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Design, synthesis, cytotoxic evaluation and tubulin inhibitory activity of 4-aryl-5-(3,4,5-trimethoxyphenyl)-2-alkylthio-1*H*-imidazole derivatives

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ABSTRACT

A new series of 4-aryl-5-(3,4,5-trimethoxyphenyl)-2-alkylthio-1*H*-imidazoles were synthesized and their cytotoxic activities in vitro against four different cell lines (HT-29, MCF-7, NIH-3T3, AGS) were evaluated. Compound **6g** bearing 3,4,5-trimethoxyphenyl moiety on ring A and 4-methoxy substituent on ring B displayed potent cytotoxic activity against all cell lines. Flow cytometry analysis and microtubule polymerization assay confirmed that cytotoxic activities of this compound were related to inhibitory effect against microtubules polymerization. Molecular modeling studies revealed that compound **6g** could strongly bind to the colchicine binding site of α,β-tubulin through hydrogen bond interactions with Thrα179 and Cysβ241.

1. Introduction

Microtubules play important role in many cellular processes such as cell division, cell signaling, and intracellular transport, and they influence the cell shape as well.[1] Thus far, three different microtubule small-molecule binding sites—vinca, taxane, and colchicine sites—have been characterized.[2] The importance of vinca alkaloids and taxanes in the treatment of human cancers has already been shown;[3] furthermore, extensive research programs have focused on introducing new anticancer drugs that inhibit microtubule polymerization through colchicine binding sites.[4,5] Combretastatin A-4 (**CA-4**) **1**, which was isolated from the bark of the South African tree Combretum caffrum,[6] is a cytotoxic and antitubulin agent that binds to tubulin at the colchicine binding site.[7] The structure of this compound has a 3,4,5-trimethoxy substituted pattern, which resembles the trimethoxyaryl ring of colchicine **2**, that shows optimal cytotoxic activity.

Structure–activity relationship (SAR) studies showed that the 3,4,5-trimethoxy-substitution and *cis* configuration in the **CA-4** structure are important for cytotoxic and antitubulin activities (Fig. 1).[8,9] To retain the *cis* configuration, different diaryl-heterocyclic analogs of **CA-4** such as furan, thiophene,[10] pyrazole,[11] imidazole,[12] isoxazole,[13] thiadiazole,[14] triazole,[15] tetrazole,[16] and thiazole,[17] have been synthesized, and their activities have been evaluated. The investigation of various substitutes on the aryl rings has revealed that methoxy substitute significantly affected the potency and tubulin activity in order that diaryl-heterocycles possessing 3,4,5-trimethoxyphenyl on ring A display low nanomolar cytotoxicity and retain the potency and efficacy of **CA-4** when 4-methoxyphenyl (with or without one of the 3-hydroxy, 3-amino and 3-fluoro substitutions) is positioned on ring B. However, the activity of some promising compounds is not greatly different from reference compound **CA-4**.

In light of the structural features of the above mentioned compounds, some 4,5-diaryl-imidazole-2-thione analogues bearing a 3,4,5-trimethoxyphenyl moiety as one of the aryl rings, which are identical to the A-ring of **CA-4**, were synthesized and the effect of OCH_3 substitutions on the other aryl moiety was studied. Cell cycle analysis was conducted for the most potent compounds, and their antitubulin activity was investigated. Furthermore, docking studies were performed to evaluate the binding mode of the active compound at the colchicine binding site of tubulin proteins.

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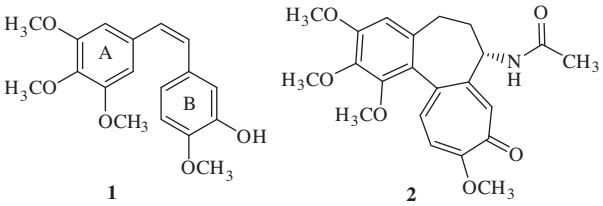


Figure 1. Structures of microtubule targeting agents, combretastatin A-4 **1** and colchicine **2**.

2. Results and discussion

2.1. Synthesis

4,5-Diaryl-1*H*-imidazole-2(3*H*)-thiones were synthesized by the reaction of different benzoins and ammonium thiocyanate.[18] The cyanide ion-catalyzed condensation of 3,4-dimethoxy or 3,4,5-trimethoxy benzaldehyde is a convenient synthetic method for the symmetrical benzoins **3a**,**b**. On the other hand, for preparing of unsymmetrical benzoins, 3,4,5-trimethoxybenzaldehyde was reacted with benzoyl chloride, potassium cyanide and aqueous potassium hydroxide in the presence of tetra-butylammonium hydrogen sulfate to obtain cyanohydrinbenzoate **4**. In the second step, the cyanohydrin was treated with different methoxy benzaldehydes to give related benzoin benzoate. Hydrolysis of resulted compound in acetonitrile and sodium hydroxide under the atmosphere of argon provided related unsymmetrical benzoins **3c**–**g**[19] (Scheme 1).

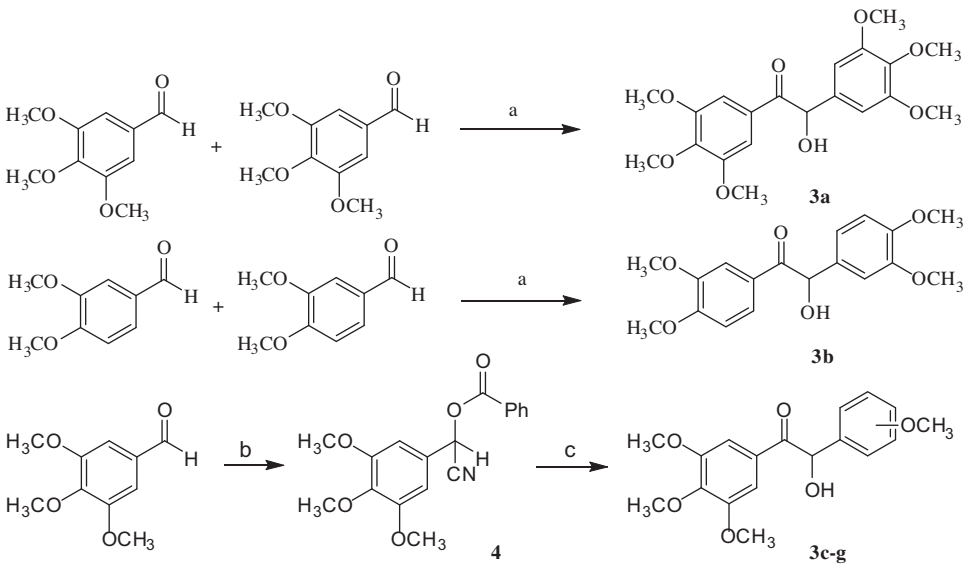
Treatment of different benzoins with a 10-fold excess of ammonium thiocyanate in *n*-butanol afforded the desired 4,5-diaryl-1*H*-imidazole-2(3*H*)-thiones **5a**–**g**. Finally, alkylation of **6a**–**g** and **7a**–**g** using alkyl iodide in basic media afforded the title compounds (Scheme 2).

As shown in Figure 2, when the ^{1}H NMR spectrum of **6g** was collected in DMSO-d_6, two sets of signals in nearly 1:1 ratio were detectable. This observation indicated a slow equilibrium conversion between two imidazole tautomers A and B on the NMR time scale. However, in $CDCl_3$ the interconversion of the two isomers is faster on the NMR time scale and consecutively the resonances are averaging to single broad signals. Khalili et al. reported similar tauto-isomerization in (4 or 5)-aryl-2-aroyl-(1*H*)-imidazole derivatives. In the latter study, variable-temperature ^{1}H NMR spectroscopy was used to investigate the barrier of H–N tauto-isomerization.[20] In another study, Laufer et al., attributed tauto-isomerization in 4,5-diaryl-2-(methylthio)-1*H*-imidazole to the sterically restricted free rotation of the bulky residues like quinolyl ring around its sigma bond with the imidazole core.[21]

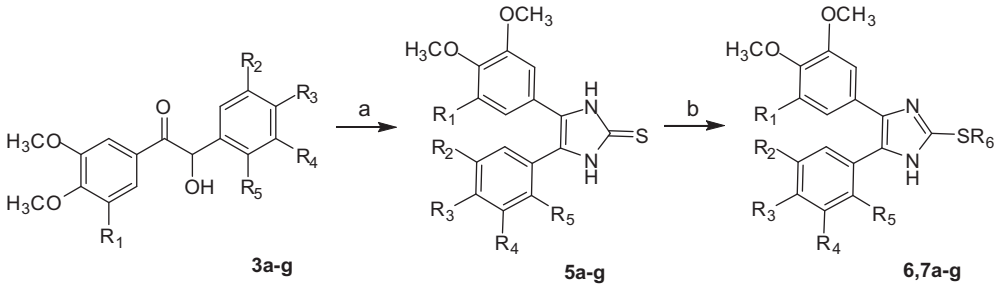
2.2. Biological evaluation

The cytotoxicity of compounds **6a**–**g** and **7a**–**g**, was evaluated against three human cancer cell lines—human colon carcinoma cells HT-29, human breast adenocarcinoma cell line MCF-7, human caucasian gastric adenocarcinoma cell line AGS—as well as fibroblast cell line NIH-3T3 by MTT assay. **CA-4** was used as the positive control. The cytotoxicity activities of the synthesized compounds are summarized in Table 1. Generally, most tested compounds showed moderate to potent cytotoxic activities. Among the synthesized compounds, the 2-alkylthio-4-(3,4,5-trimethoxyphenyl)-5-(4-methoxyphenyl)-1*H*-imidazole structure in compounds **6g** and **7g** showed the most potent activity against all tested cell lines (IC_{50} = 0.06–0.75 μM). SAR studies showed that the nature of the methoxy substituted group on the phenyl ring had a profound influence on the cytotoxicity of these compounds, for example, compounds **6a** and **7a**, both of which contain 3,4,5-trimethoxy substituent in both rings, are inactive (IC_{50} >50 μM). Moreover, removing 5-methoxy from both rings yielded to compounds **6b** and **7b**, both of which showed enhanced cytotoxic activity (IC_{50} = 15–36 μM). Clearly, the number of methoxy substitutions in both A and B rings has important effect on the cytotoxicity of this series of compounds.

In compounds **6c**–**g** and **7c**–**g**, 3,4,5-trimethoxy substitutes in phenyl (ring A) were retained and the effect of methoxy groups on the second phenyl (ring B) was evaluated. As shown in Table 1 and 4-methoxy substitutes in compounds **6g** and **7g** were favorable and showed significant cytotoxic activity. Adding extra methoxy groups at the meta- and ortho-positions (compounds **6c**, **7c** and **6d**, **7d** respectively) and replacement with methoxy group at ortho-position of ring B (compounds **6f** and **7f**), led to a decrease in potency. A comparison of the cytotoxic activities of compounds **6c**–**g** and **7c**–**g** revealed that the effects of different methoxy groups in the second phenyl (ring B) against HT-29 and



Scheme 1. Reagent and condition: (a) KCN, ethanol 50%, reflux; (b) PhCOCl, KCN, CH_2Cl_2/H_2O, rt; (c) ArylCHO, NaOH, benzene, rt.



Scheme 2. Reagents and conditions: (a) NH_4SCN, *n*-butanol, reflux; (b) alkyl iodide, CH_3OH, reflux.

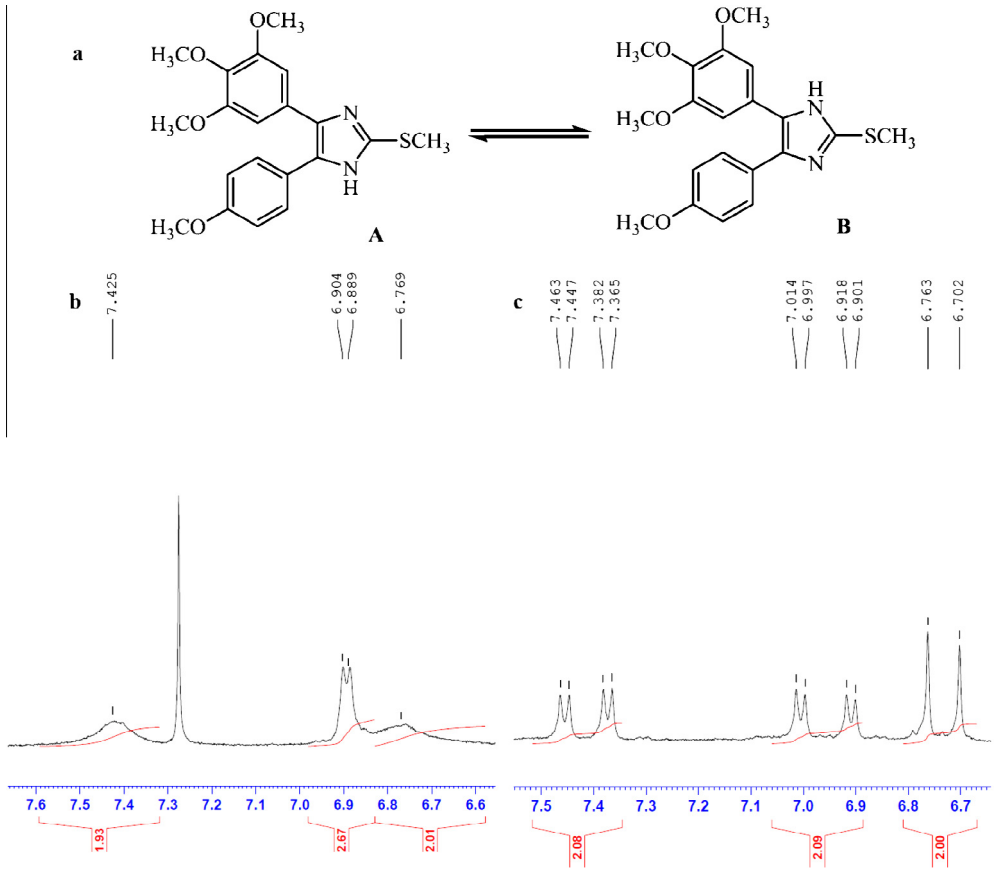


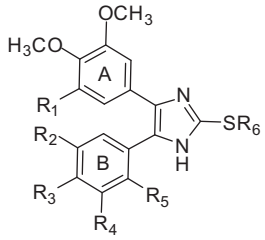
Figure 2. (a) Tautomerization in compound **6g**; (b) ^{1}H NMR spectra of **6g** in $CDCl_3$ at room temperature; (c) ^{1}H NMR spectra of **6g** in DMSO-d_6 at room temperature.

MCF-7 cell lines was para > meta, para > ortho, para > ortho > ortho, meta. In NIH-3T3 and AGS, a similar pattern with one replacement was observed in the following order para > meta, para > ortho, para > ortho, meta > ortho. From these observations, it has been concluded that only one methoxy group at the para position of ring B has beneficial effect on the cytotoxic activity, and additional methoxy groups at the ortho and/or meta positions are not favorable.

Finally, increasing the size of the thiol substituent from a methyl to an ethyl group led to a decrease in potency. However, there were two exceptions—compounds **f** and **d**—where methoxy groups were substituted at ortho and ortho, para positions of ring B, respectively. In these compounds, replacement of the 2-thiomethyl with the corresponding ethyl group led to increased cytotoxic activity. Overall, the obtained results demonstrated that the introduction of 3,4,5-trimethoxyphenyl in the 2-methylthioimidazole ring and the presence of 4-methoxy substituted in ring B led to optimal effects against all cancer cell lines. Although the activity of compound **6g** is not greatly different from reference compound **CA-4**, design and synthesis of new diaryl-heterocyclic analogs would still merit consideration in order to increase the richness of structural repertoire and optimizing the lead compound's property.

Considering the design strategy toward tubulin inhibition and the similarity of the synthesized compounds and **CA-4**, flow cytometry experiments were performed to determine the effect of compound **6g** on the cell cycle of NIH-3T3 cells, specifically, to determine whether the synthesized compound could lead to cell cycle arrest at the G_2/M phase. NIH-3T3 cells were treated with compound **6g** (0.10 μM) for 24 h, and **CA-4** (0.05 μM) was used as a positive control. The percentages of G_2/M population were 91.93% in NIH-3T3 cells that were exposed to compound **6g** as compared to 35.70% in the control group. These results demonstrated that compound **6g** could arrest cells in G_2/M phase (Fig. 3).

Table 1
In vitro cytotoxic activities of synthesized compounds **6a–g** and **7a–g**



Compounds	R_1	R_2	R_3	R_4	R_5	R_6	Cytotoxicity (IC_{50}, μM)[a]			
							HT-29	MCF-7	NIH-3T3	AGS
6a	OCH_3	OCH_3	OCH_3	OCH_3	H	CH_3	>50	nd[b]	>50	nd[b]
7a	OCH_3	OCH_3	OCH_3	OCH_3	H	CH_2CH_3	>50	nd[b]	>50	nd[b]
6b	H	H	OCH_3	OCH_3	H	CH_3	24.13 ± 1.43	17.16 ± 1.62	35.80 ± 2.56	34.84 ± 1.30
7b	H	H	OCH_3	OCH_3	H	CH_2CH_3	21.17 ± 2.06	15.05 ± 2.10	27.60 ± 3.06	33.63 ± 4.35
6c	OCH_3	H	OCH_3	OCH_3	H	CH_3	4.82 ± 0.79	4.04 ± 0.23	6.01 ± 0.86	10.57 ± 0.20
7c	OCH_3	H	OCH_3	OCH_3	H	CH_2CH_3	21.04 ± 3.11	13.19 ± 1.76	14.12 ± 0.78	37.19 ± 0.69
6d	OCH_3	H	OCH_3	H	OCH_3	CH_3	18.57 ± 0.84	4.15 ± 0.35	11.91 ± 2.84	23.22 ± 1.09
7d	OCH_3	H	OCH_3	H	OCH_3	CH_2CH_3	6.56 ± 1.12	3.55 ± 0.26	7.25 ± 1.98	18.61 ± 3.61
6e	OCH_3	H	H	OCH_3	OCH_3	CH_3	27.51 ± 1.81	18.09 ± 1.97	30.18 ± 1.68	35.31 ± 1.63
7e	OCH_3	H	H	OCH_3	OCH_3	CH_2CH_3	36.70 ± 3.50	22.90 ± 3.11	27.17 ± 1.51	38.98 ± 4.69
6f	OCH_3	H	H	H	OCH_3	CH_3	21.73 ± 1.14	12.21 ± 0.89	38.73 ± 1.87	48.43 ± 4.48
7f	OCH_3	H	H	H	OCH_3	CH_2CH_3	18.57 ± 0.96	10.99 ± 0.74	36.23 ± 0.67	40.70 ± 5.66
6g	OCH_3	H	OCH_3	H	H	CH_3	0.25 ± 0.04	0.52 ± 0.04	0.06 ± 0.01	0.06 ± 0.00
7g	OCH_3	H	OCH_3	H	H	CH_2CH_3	0.36 ± 0.05	0.75 ± 0.08	0.26 ± 0.06	0.07 ± 0.01
CA-4							0.18 ± 0.02	0.40 ± 0.03	0.02 ± 0.01	0.04 ± 0.02

[a] IC_{50}, compound concentration required to inhibit cell proliferation by 50%.
[b] nd: Not determined.

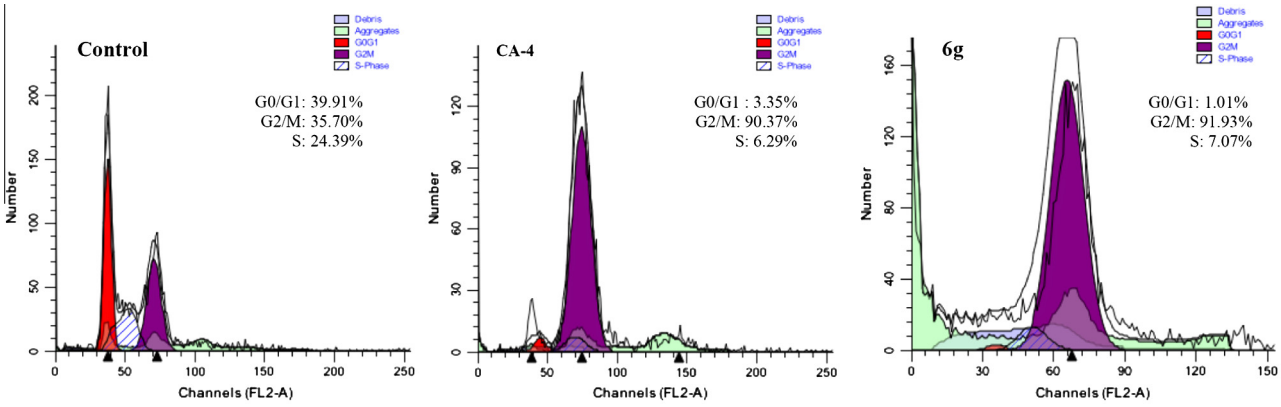


Figure 3. Effect of compound **6g** (0.10 μM) and **CA-4** (0.05 μM) on the cell cycle of NIH-3T3 cells.

To confirm whether the cytotoxic activities of the designed compounds were related to tubulin interaction, the inhibitory effect of promising compounds **6g**, **7g**, **6c** and compound **7c** on the polymerization of purified tubulin was evaluated. All compounds were evaluated at final concentration of 5 μM. **CA-4** was used as a positive control (2 μM). Figure 4 shows the rational relationship between the tubulin inhibition and the corresponding cytotoxic activities. The order of inhibition of tubulin polymerization was **CA-4** > **6g** > **7g** > **6c** > **7c**. This result suggested that tubulin is a potential target for synthesized compounds.

To investigate the binding interaction of the synthesized compounds to the colchicine binding site of α,β-tubulin, a docking study was performed on compound **6g**. The results showed that compound **6g** occupied the colchicine binding site with and orientation similar to that of crystallized colchicine fitted in a binding cavity. The trimethoxyphenyl in ring A was positioned in the hydrophobic pocket between Alaβ250, Leuβ242, Valβ238, and

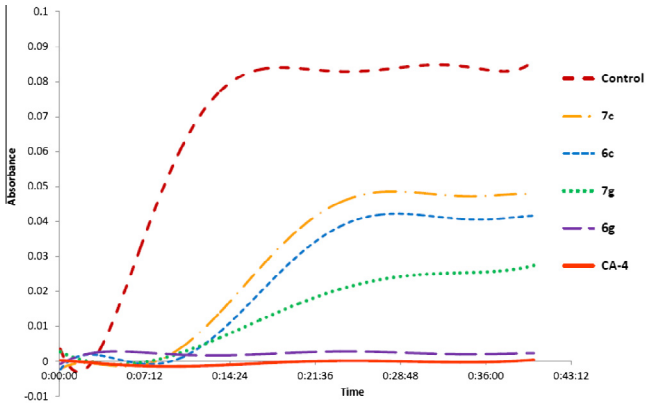


Figure 4. Effect of compounds **7c**, **6c**, **7g**, **6g** (5 μM) and **CA-4** (2 μM) on tubulin polymerization.

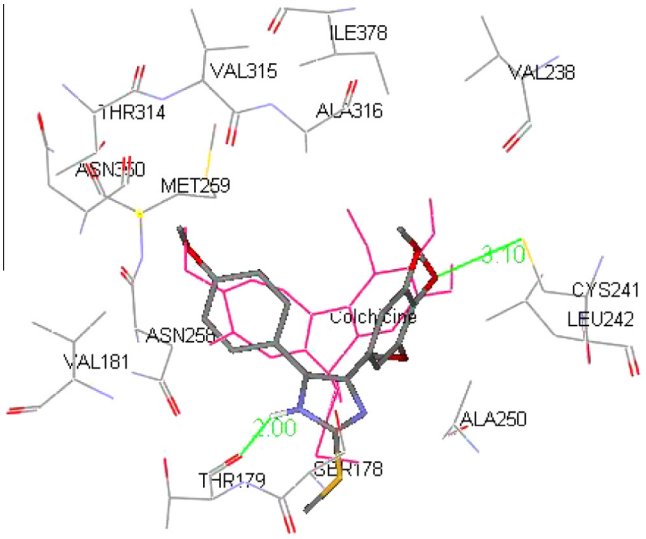


Figure 5. Superimposition of the assumed conformation of compound **6g** on the top of the X-ray structure of DAMA–colchicine (shown in pink).

Alaβ316, and the oxygen atom of the 4-methoxy substituent formed a hydrogen bond with Cysβ241 in β subunit which was always found in the interaction of colchicines and tubulin protein in crystal form. In addition, the 4-methoxy group on ring B can interact with the hydrophobic pocket Alaα180, Valα181, Thrβ314, Valβ315, Asnβ258, and Metβ259 in which only one methoxy group at the para-position was favorable to cytotoxic activity. Furthermore, the hydrogen atom of NH in the imidazole-2-thiomethyl ring can form a hydrogen bond with the CO of Thrα179. These results postulated that compound **6g** would strongly bind to the colchicine binding site between the α and the β subunits and show cytotoxic activity (Fig. 5).

3. Conclusions

In conclusion, the synthesis and biological evaluation of a series of 4-aryl-5-(3,4,5-trimethoxyphenyl)-2-alkylthio-1*H*-imidazoles was discussed. Most of these synthesized compounds showed moderate to potent cytotoxic activity against four cell lines. The SAR study demonstrated that 3,4,5-trimethoxyphenyl moiety on ring A and 4-methoxy substituent on ring B had beneficial effect on the cytotoxicity activity. The flow cytometry analysis and microtubule polymerization assay confirmed that the synthesized compound led to cell cycle arrest at the G_2/M phase by inhibitory effect against microtubules. Finally, molecular modeling studies revealed that compound **6g** could strongly bind to the colchicine binding site of α,β-tubulin through hydrogen bond interactions with Thrα179 and Cysβ241.

4. Experimental section

4.1. Chemistry

^{1}H NMR spectra were recorded on a 500 MHz Bruker spectrometer using $CDCl_3$ or DMSO-d_6 as solvent. ^{13}C NMR spectra were recorded on a 125 MHz Bruker spectrometer using $CDCl_3$ or DMSO-d_6 as solvent. Chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as internal standard. Infrared spectra were acquired on a Nicolet Magna 550-FT spectrometer. IR spectra of solids were recorded in KBr and the absorption band was given in wave numbers ν in cm^{-1}. Elemental microanalyses were within ±0.4 of the theoretical values for C, H and N. Combretastatin A-4 was prepared according to a literature procedure and used as a positive control.[22]

4.1.1. 2-(Methylthio)-4,5-bis(3,4,5-trimethoxyphenyl)-1*H*-imidazole (6a)

Yield, 76%; mp 90–92 °C; IR (KBr, cm^{-1}): ν 3260, 1582, 1506, 1408, 1239, 1120, 1002, 840; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.71 (s, 3H, SCH_3), 3.76 (s, 12H, 3,5-OCH_3), 3.85 (s, 6H, 4-OCH_3), 6.76 (br s, 4H, $H_{2,6}$-phenyl). Anal. Calcd for $C_{22}H_{26}N_2O_6S$: C, 59.18; H, 5.87; N, 6.27. Found: C, 59.34; H, 5.62; N, 6.41.

4.1.2. 2-(Ethylthio)-4,5-bis(3,4,5-trimethoxyphenyl)-1*H*-imidazole (7a)

Yield, 74%; mp 80–82 °C ; IR (KBr, cm^{-1}): ν 3272, 1588, 1511, 1419, 1229, 1122, 1004, 835; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.40 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.16 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.76 (s, 12H, 3,5-OCH_3), 3.85 (s, 6H, 4-OCH_3), 6.76 (br s, 4H, $H_{2,6}$-phenyl). Anal. Calcd for $C_{23}H_{28}N_2O_6S$: C, 59.98; H, 6.13; N, 6.08. Found: C, 59.74; H, 6.43; N, 6.37.

4.1.3. 4,5-Bis(3,4-dimethoxyphenyl)-2-(methylthio)-1*H*-imidazole (6b)

Yield, 79%; mp 89–91 °C ; IR (KBr, cm^{-1}): ν 3236, 1506, 1465, 1255, 1132, 1029, 860, 763 ; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.67 (s, 3H, SCH_3), 3.76 (s, 6H, 3-OCH_3), 3.89 (s, 6H, 4-OCH_3), 6.83 (d, J = 8.5 Hz, 2H, H_5-phenyl), 7.05 (d, J = 8.5 Hz, 2H, H_6-phenyl), 7.06 (s, 2H, H_2-phenyl); ^{13}C NMR ($CDCl_3$): δ 17.31, 55.72, 55.85, 111.06, 120.25, 124.98, 132.55, 140.61, 148.40, 148.74; MS, m/z (%) 386 (M^+, 100), 371 (16), 353 (15), 313 (16). Anal. Calcd for $C_{20}H_{22}N_2O_4S$: C, 62.16; H, 5.74; N, 7.25. Found: C, 62.32; H, 5.87; N, 7.38.

4.1.4. 4,5-Bis(3,4-dimethoxyphenyl)-2-(ethylthio)-1*H*-imidazole (7b)

Yield, 76%; mp 77–80 °C; IR (KBr, cm^{-1}): ν 3226, 1511, 1470, 1250, 1137, 1024, 860, 758; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.39 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.13 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.76 (s, 6H, 3-OCH_3), 3.89 (s, 6H, 4-OCH_3), 6.83 (d, J = 8.5 Hz, 2H, H_5-phenyl), 7.05 (d, J = 8.5 Hz, 2H, H_6-phenyl), 7.06 (s, 2H, H_2-phenyl).). Anal. Calcd for $C_{21}H_{24}N_2O_4S$: C, 62.98; H, 6.04; N, 6.99. Found: C, 62.72; H, 6.27; N, 6.75.

4.1.5. 5-(3,4-Dimethoxyphenyl)-2-(methylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (6c)

Yield, 67%; mp 99–101 °C; IR (KBr, cm^{-1}): ν 3286, 1582, 1521, 1408, 1250, 1127, 1024, 840, 768; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.67 (s, 3H, SCH_3), 3.73 (s, 6H, 3,5-OCH_3-4-aryl), 3.77 (s, 3H, 3-OCH_3-5-aryl), 3.85 (s, 3H, 4-OCH_3-4-aryl), 3.89 (s, 3H, 4-OCH_3-5-aryl), 6.76 (br s, 2H, $H_{2,6}$-4-phenyl), 6.84 (d, J = 8.5 Hz, 1H, H_5-5-phenyl), 7.01–7.10 (m, 2H, $H_{2,6}$-5-phenyl). Anal. Calcd for $C_{21}H_{24}N_2O_5S$: C, 60.56; H, 5.81; N, 6.73. Found: C, 60.72; H, 5.68; N, 6.89.

4.1.6. 5-(3,4-Dimethoxyphenyl)-2-(ethylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (7c)

Yield, 64%; mp 75–77 °C; IR (KBr, cm^{-1}): ν 3267, 1582, 1506, 1413, 1255, 1132, 1029, 840, 762; ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.38 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.12 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.71 (s, 6H, 3,5-OCH_3-4-aryl), 3.75 (br s, 3H, 3-OCH_3-5-aryl), 3.84 (br s, 3H, 4-OCH_3-4-aryl), 3.88 (br s, 3H, 4-OCH_3-5-aryl), 6.76 (br s, 2H, $H_{2,6}$-4-phenyl), 6.83 (d, J = 8.5 Hz, 1H, H_5-5-phenyl), 7.05 (m, 2H , $H_{2,6}$-5-phenyl). ^{13}C NMR ($CDCl_3$): δ 15.29, 29.52, 60.92, 104.86, 111.08, 120.54, 124.39, 127.84, 132.42, 137.35, 139.36, 148.68, 148.81, 153.14; MS, m/z (%) 430 (M^+, 100), 415 (34), 400 (38), 367 (9), 343 (14), 313 (16). Anal. Calcd for $C_{22}H_{26}N_2O_5S$ C, 61.38; H, 6.09; N, 6.51. Found: C, 61.56; H, 6.24; N, 6.76.

4.1.7. 5-(2,4-Dimethoxyphenyl)-2-(methylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (6d)

Yield, 65%; mp 82–84 °C; IR (KBr, cm^{-1}): ν 3246, 1613, 1465, 1413, 1214, 1127, 1024, 835; ^1H NMR (500 MHz, $CDCl_3$): δ 2.68 (s, 3H, SCH_3), 3.73 (s, 6H, 3,5-OCH_3-4-aryl), 3.83 (s, 3H, 4-OCH_3-5-aryl), 3.85 (s, 3H, 4-OCH_3-4-aryl), 3.86 (s, 3H, 2-OCH_3-5-aryl), 6.44 (dd, J = 8.5 Hz, J = 3 Hz, 1H, H_5-5-phenyl), 6.55 (d, J = 3 Hz, 1H, H_3-5-phenyl), 6.83 (s, 2H, $H_{2,6}$-4-phenyl), 7.28 (d, 1H, J = 8.5 Hz, H_6-5-phenyl). Anal. Calcd for $C_{21}H_{24}N_2O_5S$: C, 60.56; H, 5.81; N, 6.73. Found: C, 60.84; H, 5.69; N, 6.60.

4.1.8. 5-(2,4-Dimethoxyphenyl)-2-(ethylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (7d)

Yield, 60%; mp 74–76 °C; IR (KBr, cm^{-1}): ν 3272, 1613, 1501, 1413, 1214, 1116, 830; ^1H NMR (500 MHz, $CDCl_3$): δ 1.39 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.10 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.75 (s, 6H, 3,5-OCH_3-4-aryl), 3.83 (s, 3H, 4-OCH_3-5-aryl), 3.85 (s, 3H, 4-OCH_3-4-aryl), 3.88 (s, 3H, 2-OCH_3-5-aryl), 6.45 (d, J = 8.5 Hz, 1H, H_5-5-phenyl), 6.56 (s, 1H, H_3-5-phenyl), 6.86 (s, 2H, $H_{2,6}$-4-phenyl), 7.28 (d, J = 8.5 Hz, 1H, H_6-5-phenyl); ^{13}C NMR ($CDCl_3$): δ 15.30, 29.54, 55.46, 55.59, 55.90, 60.87, 98.83, 104.62, 104.78, 111.98, 129.71, 130.19, 131.69, 136.82, 138.37, 152.92, 157.40, 160.83; MS, m/z (%) 430 (M^+, 100), 415 (39), 397 (14), 328 (16), 211 (21), 195 (19), 165(37). Anal. Calcd for $C_{22}H_{26}N_2O_5S$ C, 61.38; H, 6.09; N, 6.51. Found: C, 61.64; H, 6.23; N, 6.78.

4.1.9. 5-(2,3-Dimethoxyphenyl)-2-(methylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (6e)

Yield, 63%; mp 77–79 °C; IR (KBr, cm^{-1}): ν 3262, 1588, 1465, 1398, 1270, 1116, 999, 830, 830, 748; ^1H NMR (500 MHz, $CDCl_3$): δ 2.69 (s, 3H, SCH_3), 3.76 (s, 6H, 3,5-OCH_3-4-aryl), 3.79 (s, 3H, 3-OCH_3-5-aryl), 3.86 (s, 3H, 4-OCH_3-4-aryl), 3.92 (s, 3H, 2-OCH_3-5-aryl), 6.85 (s, 2H, $H_{2,6}$-4-phenyl), 6.86–6.89 (m, 1H, H_4-5-phenyl), 6.93–7.02 (m, 2H, $H_{5,6}$-5-phenyl); ^{13}C NMR ($CDCl_3$): δ 17.02, 55.88, 56.00, 60.76, 60.90, 105.00, 111.83, 112.13, 122.06, 122.37, 124.26, 129.33, 137.67, 141.10, 146.10, 153.03; MS, m/z (%) 416 (M^+, 100), 401 (51), 386 (20), 312 (15), 267 (17), 91 (16). Anal. Calcd for $C_{21}H_{24}N_2O_5S$: C, 60.56; H, 5.81; N, 6.73. Found: C, 60.82; H, 5.63; N, 6.58.

4.1.10. 5-(2,3-Dimethoxyphenyl)-2-(ethylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (7e)

Yield, 59%; mp 65–67 °C; IR (KBr, cm^{-1}): ν 3262, 1583, 1470, 1396, 1276, 1122, 999, 835, 748; ^1H NMR (500 MHz, $CDCl_3$): δ 1.39 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.16 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.75 (s, 6H, 3,5-OCH_3-4-aryl), 3.77 (s, 3H, 3-OCH_3-5-aryl), 3.85 (s, 3H, 4-OCH_3-4-aryl), 3.91 (s, 3H, 2-OCH_3-5-aryl), 6.87 (s, 2H, $H_{2,6}$-4-phenyl), 6.87–6.89 (m, 1H, H_4-5-phenyl), 6.93–7.02 (m, 2H, $H_{5,6}$-5-phenyl). Anal. Calcd for $C_{22}H_{26}N_2O_5S$ C, 61.38; H, 6.09; N, 6.51. Found: C, 61.69; H, 6.27; N, 6.77.

4.1.11. 5-(2-Methoxyphenyl)-2-(methylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (6f)

Yield, 67%; mp 92–94 °C; IR (KBr, cm^{-1}): ν 3264, 1588, 1511, 1413, 1244, 1127, 1004, 758; ^1H NMR (500 MHz, $CDCl_3$): δ 2.69 (s, 3H, SCH_3), 3.73 (s, 6H, 3,5-OCH_3-4-aryl), 3.84 (s, 3H, 4-OCH_3-4-aryl), 3.90 (s, 3H, 2-OCH_3-5-aryl), 6.82 (s, 2H, $H_{2,6}$-4-phenyl), 6.89 (t, 1H, J = 8 Hz, H_5-5-phenyl), 6.99 (d, 1H, J = 8 Hz, H_3-5-phenyl), 7.29 (t, J = 8 Hz, 1H, H_4-5-phenyl), 7.39 (d, 1H, J = 8 Hz, H_6-5-phenyl). Anal. Calcd for $C_{20}H_{22}N_2O_4S$: C, 62.16; H, 5.74; N, 7.25. Found: C, 62.31; H, 5.51; N, 7.62.

4.1.12. 2-(Ethylthio)-5-(2-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (7f)

Yield, 62%; mp 72–74 °C; IR (KBr, cm^{-1}): ν 3268, 1588, 1511, 1419, 1244, 1116, 1004, 758; ^1H NMR (500 MHz, $CDCl_3$): δ 1.40 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.15 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.72 (s, 6H, 3,5-OCH_3-4-aryl), 3.85 (s, 3H, 4-OCH_3-4-aryl), 3.89 (s, 3H, 2-OCH_3-5-aryl), 6.83 (s, 2H, $H_{2,6}$-4-phenyl), 6.90 (t, 1H, J = 8 Hz, H_5-5-phenyl), 7.00 (d, 1H, J = 8 Hz, H_3-5-phenyl), 7.29 (t, 1H, J = 8 Hz, H_4-5-phenyl), 7.40 (d, 1H, J = 8 Hz, H_6-5-phenyl); ^{13}C NMR ($CDCl_3$): δ 15.29, 29.52, 55.61, 55.93, 60.89, 104.82, 111.28, 118.89, 120.81, 125.51, 129.50, 129.65, 130.89, 137.11, 138.91, 152.99, 156.11; MS, m/z (%) 400 (M^+, 100), 385 (29), 367 (7), 313 (7), 298 (7). Anal. Calcd for $C_{21}H_{24}N_2O_4S$: C, 62.98; H, 6.04; N, 6.99. Found: C, 62.63; H, 6.32; N, 6.67.

4.1.13. 5-(4-Methoxyphenyl)-2-(methylthio)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (6g)

Yield, 67%; mp 82–84 °C; IR (KBr, cm^{-1}): ν 3262, 1588, 1495, 1511, 1413, 1244, 1127, 830; ^1H NMR (500 MHz, $CDCl_3$): δ 2.67 (s, 3H, SCH_3), 3.72 (s, 6H, 3,5-OCH_3-4-aryl), 3.83 (s, 3H, 4-OCH_3-5-aryl), 3.85 (s, 3H, 4-OCH_3-4-aryl), 6.76 (br s, 2H, $H_{2,6}$-4-phenyl), 6.89 (d, 2H, J = 8 Hz, $H_{3,5}$-5-phenyl), 7.38–7.53 (m, 2H, $H_{2,6}$-5-phenyl). ^1H NMR (500 MHz, DMSO-d_6): δ 2.59 (s, 3H, SCH_3), 3.61 and 3.66 (two s, 6H, OCH_3), 3.64 and 3.67 (two s, 3H, OCH_3), 3.75 and 3.78 (two s, 3H, OCH_3), 6.70 and 7.76 (two s, 2H, $H_{2,6}$-4-phenyl), 6.90 and 7.00 (two d, J = 8.5 Hz, 2H, $H_{3,5}$-5-phenyl), 7.37 and 7.45 (two d, J = 8.5 Hz, 2H, $H_{2,6}$-5-phenyl), 12.40 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 17.11, 55.32, 55.95, 60.90, 104.58, 113.99, 128.86, 129.34, 129.51, 137.14, 140.73, 153.13, 159.18; MS, m/z (%) 386 (M^+, 100), 371 (46), 353 (7), 313 (8), 135(7), 97 (12), 69 (16). Anal. Calcd for $C_{20}H_{22}N_2O_4S$: C, 62.16; H, 5.74; N, 7.25. Found: C, 62.36; H, 5.89; N, 7.53.

4.1.14. 2-(Ethylthio)-5-(4-methoxyphenyl)-4-(3,4,5-trimethoxyphenyl)-1*H*-imidazole (7g)

Yield, 63%; mp 64–66 °C; IR (KBr, cm^{-1}): ν 3246, 1588, 1495, 1509, 1403, 1255, 1116, 830; ^1H NMR (500 MHz, $CDCl_3$): δ 1.39 (t, J = 7.5 Hz, 3H, SCH_2CH_3), 3.11 (q, J = 7.5 Hz, 2H, SCH_2CH_3), 3.72 (s, 6H, 3,5-OCH_3-4-aryl), 3.83 (s, 3H, 4-OCH_3-5-aryl), 3.86 (s, 3H, 4-OCH_3-4-aryl), 6.76 (br s, 2H, $H_{2,6}$-4-phenyl), 6.90 (d, 2H, J = 8 Hz, $H_{3,5}$-5-phenyl), 7.38–7.53 (m, 2H, $H_{2,6}$-5-phenyl). Anal. Calcd for $C_{21}H_{24}N_2O_4S$: C, 62.98; H, 6.04; N, 6.99. Found: C, 62.71; H, 6.27; N, 6.73.

4.2. Cytotoxicity assay

The cytotoxic activity was measured using the MTT assay in four different cell lines (HT-29, MCF-7, NIH-3T3 and AGS).[23] Cells from different cell lines were seeded in 96-well plates at the density of 8000–10,000 viable cells per well and incubated for 24 h. Attached cells were treated with various concentrations of compounds **6**, **7a**–**g** (0.001–100 μM) and incubated for another 48 h. In the following, 20 μL of MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL) were added to each well and incubated for additional 4 h. Finally, 100 μL dimethyl sulfoxide was added and absorbance of each well was measured by plate reader (Anthous 2020; Austria) at a test wavelength of 550 nm against a standard reference solution at 690 nm. Two independent experiments in triplicate were performed for determination of sensitivity to each compound. The IC_{50} values were determined by a nonlinear regression analysis and expressed in mean ± SD.

4.3. Cell cycle analysis

For flow cytometric analysis of DNA content, 10^6 NIH-3T3 cells were treated with (0.10 μM) compound **6g** for 24 h. After centrifugation, the cell pellet was fixed in 75% ethanol at kept in 4 °C for 0.5 h. The cell pellet was resuspended in 500 μL of PBS containing

0.1% (v/v) Triton X-100, 10 μg/mL propidium iodide (PI, Sigma, St. Louis, MO), and 100 μg/mL RNase A and incubated in 37 °C for 0.5 h. Finally, the fluorescence cell was measured by FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA).

4.4. Microtubule polymerization assay

Microtubule protein (MTP) was prepared and purified as described previously.[24] Breifly, test compounds in final concentration 5 μM were preincubated with purified tubulin (12 μM) and PEM buffer (100 mM PIPES, pH 6.9, 1 mM $MgSO_4$, and 1 mM EGTA) for 15 min and then cooled to 0 °C. **CA-4** was used as a positive control in final concentration 2 μM. After adding the final 1 mM concentration of GTP, the assembly was initiated by warming the solution from 0 to 37 °C and polymerization process was monitored by observing the variations in absorbance at 350 nm.

4.5. Molecular modeling

Structure of compound **6g** was designed in chem3D professional Cambridge software and output file was minimized under MM2 method (RMS gradient = 0.05 kcal/mol).[25] Molecular docking was performed by AutoDockTools 4.0 version1.5.4 using a Lamarckian genetic algorithm.[26] The high resolution crystal structure of tubulin complex with DAMA–colchicine was retrieved from RCSB Protein Data Bank (PDB entry: 1SA0).[27] The docking grid size with 40, 40 and 40 points in *X*, *Y* and *Z* directions was built and the maps were center located (118.921, 89.188, 5.32) in the catalytic site of protein. The docking parameter files were generated by Genetic Algorithm and Local Search Parameters (GALS). Moreover, the number of generations and maximum number of energy evaluations was set to 150 and 2,500,000, respectively. Docking results were clustered with a root mean square deviation tolerance (RMSD) of 0.5 Å and evaluated by Accelrys DS Visualizer 2.0.1.[28]

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