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Synthesis, cytotoxic evaluation and molecular docking study of 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazoles as tubulin polymerization inhibitors

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ABSTRACT

A series of *cis*-restricted 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazole analogues of combretastatin A-4 were synthesized and investigated for inhibition of cell proliferation against three cancer cell lines, HT-29, MCF-7, and AGS, and a normal mouse fibroblastic cell line, NIH-3T3, using an MTT assay. The biological study showed that 2-(methylthio) substituted compounds showed little cytotoxic activity against the four cell lines. In contrast, the presence of the 2-(benzylthio) group on the thiazole ring resulted in a significant improvement in cytotoxic activity relative to the 2-(methylthio) substituted derivatives. Furthermore, the inhibition of tubulin polymerization by some potent compounds was evaluated. All the compounds studied were moderate tubulin polymerization inhibitors. The flow cytometry analysis confirmed that the synthesized compounds led to cell cycle arrest at the G_2/M phase. Docking simulation was performed to insert these compounds into the crystal structure of tubulin at the colchicine binding site to determine a probable binding model.

1. Introduction

Microtubules are attractive molecular targets for anticancer therapeutics because microtubule polymerization dynamics can greatly affect critical processes, such as cell signaling and mitosis.[1] At the same time, polymerization dynamics can be affected by natural products and synthetic small molecules.[2] There are three major binding sites on tubulin, the taxus, the vinca, and the colchicine binding site.[3] Taxanes are examples of compounds that bind to the taxus binding site and stabilize the microtubule, whereas the majority of compounds that bind to the vinca and colchicine binding sites destabilize the microtubule and promote depolymerization by inhibition of tubulin.[4] The first colchicine site inhibitor, colchicine (Fig. 1), has limited therapeutic application because of its high cytotoxicity.[3] Combretastatin A-4 (**CA-4**) (Fig. 1), isolated from the South African willow tree *Combretum caffrum*, strongly inhibits the polymerization of tubulin by binding to the colchicine site.[5] Although **CA-4** expresses high levels of in vitro activity, it does not show efficacy in vivo because of its low aqueous solubility and the isomerization of its *cis*-double bond into a more thermally stable, but inactive, *trans*-isomer.[6] As **CA-4** has a simple structure, with only two aromatic rings linked by a double bond in the *cis* configuration, a wide number of **CA-4** analogues have been developed and synthesized to date. Among these analogues, compounds in which the olefinic bond is replaced with five-membered rings are promising targets. Imidazole,[7] triazole,[8] thiazole,[9] and pyrazoline[10] are example of five-membered heterocyclic bridges.

In continuation of our research on tubulin inhibitors,[11,12] a series of 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazole (Fig. 1) analogues of **CA-4** were synthesized, and their cytotoxicity and tubulin polymerization inhibitory activity were evaluated. The binding mode of these compounds to tubulin was also studied by molecular docking.

2. Results and discussion

2.1. Chemistry

2-Alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazoles were synthesized by the reaction of different α-bromoketones with methyl

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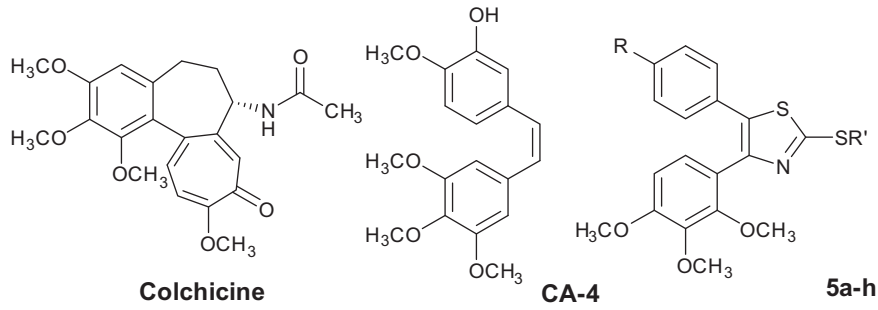


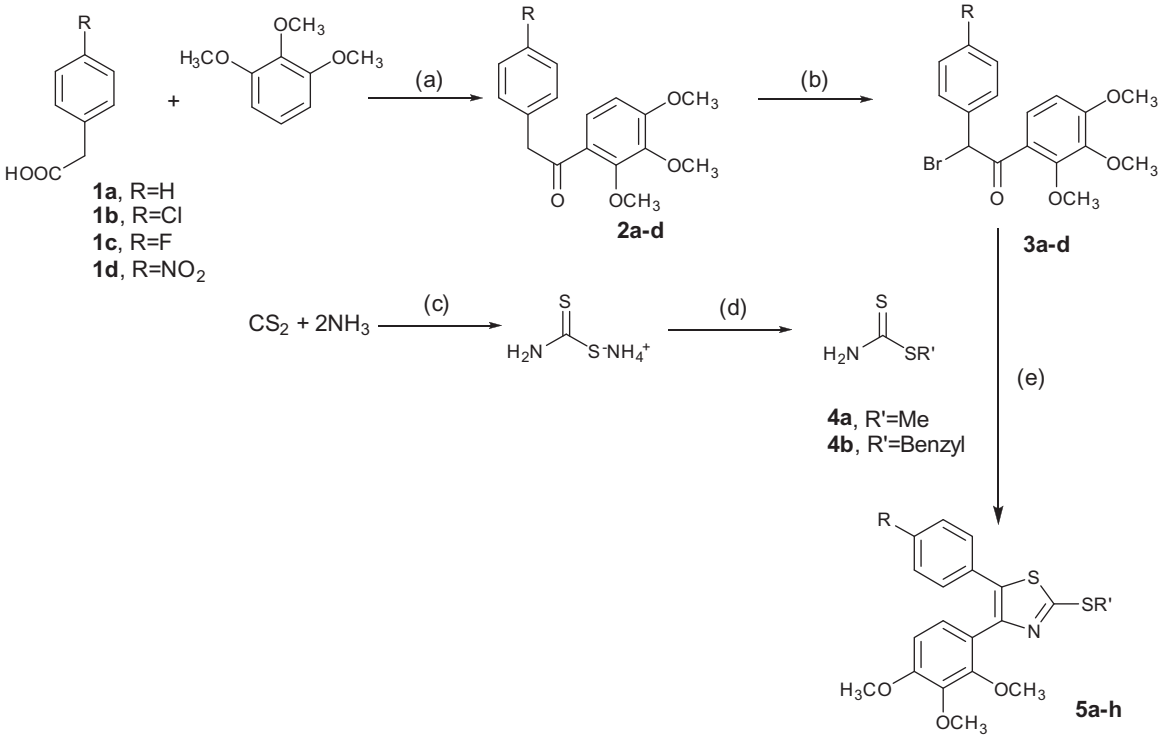
Figure 1. Structure of colchicine, combretastatin A-4, and 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazoles (**5a**–**h**).

and benzyl carbamodithioate **4a**–**b**. In the first step, α-bromoketones **3a**–**d** were prepared from bromination of 1-(2,3,4-trimethoxyphenyl)-2-(4-substitutedphenyl)-1-ethanones **2a**–**d** in glacial acetic acid.[13] These ethanones **2a**–**d** were synthesized by reaction of 1,2,3-trimethoxybenzene and appropriate phenyl acetic acid **1a**–**d** according to the literature.[14] From two expected regio isomers, 2-aryl-1-(2,3,4-trimethoxyphenyl)ethanone and 2-aryl-1-(3,4,5-trimethoxyphenyl)ethanone one of the former was obtained. The structure of **2a**–**d** were confirmed by NMR spectroscopy. In another experiment, ammonium dithiocarbamate was prepared by passing ammonia through a solution of carbon disulfide in tetrahydrofuran.[15] In the next step, methyl and benzyl dithiocarbamate were obtained from reaction of methyl and benzyl iodide with ammonium dithiocarbamate. Finally, treatment of different α-bromoketones with twofold excess of **4a**–**b** in methanol afforded the desired 4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazole-2-thio substituted compounds **5a**–**h** (Scheme 1).

The CA-4 was synthesized as positive control according to the literature.[16] All of synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR and CHN analysis.

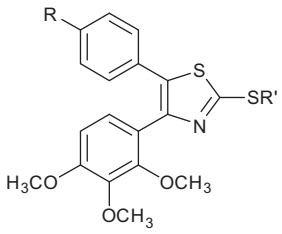
2.2. Biological study

To test the anticancer activity of the synthesized compounds, the antiproliferative activity of all the derivatives against three cancer cell lines, human colon adenocarcinoma (HT-29), human breast adenocarcinoma (MCF-7), and human stomach adenocarcinoma (AGS), as well as a mouse embryonic fibroblast cell line, NIH-3T3, was evaluated by an MTT colorimetric assay.[17,18] **CA-4** was used as a positive control. The cultured cells were treated with several concentrations of test compounds for 48 h. The ability of these analogues to inhibit cell growth is summarized in Table 1. The results revealed that most of the tested compounds showed moderate to potent cytotoxic activities. Structure-activity relationship (SAR) studies showed that the nature of the benzylthio group on the thiazole ring has a profound influence on the cytotoxicity of the compounds. For example, compounds **5d** and **5f**, both of which contain the 2-(benzylthio) group on thiazole rings, were active (IC_{50} <20 μM) in all three cancer cell lines. In contrast, the replacement of the 2-(benzylthio) group on the thiazole ring with a 2-(methylthio) moiety, namely compounds **5c** and **5e**, led to a



Scheme 1. Reagents and conditions: (a) H_3PO_4, $(CF_3CO)_2O$, 25 °C, 1 min; (b) Br_2, CH_3COOH, 25 °C, 2 h; (c) THF, 25 °C; (d) methanol, benzyl or methyl iodide, 25 °C; (e) methanol, reflux, 24 h.

Table 1
Structure and cytotoxicity of analogues of CA-4



Compounds	R	R′	Cytotoxicity[a] (IC_{50}, μM)			
			HT-29	MCF-7	AGS	NIH-3T3
5a	H	CH_3	77.9 ± 2.3	N.D[b]	27.2 ± 1.2	22.1 ± 1.4
5b	H	CH_2Ph	46.0 ± 1.7	27.3 ± 0.6	38.5 ± 1.5	56.5 ± 1.8
5c	Cl	CH_3	>100	N.D[b]	>100	95.7 ± 2.2
5d	Cl	CH_2Ph	8.9 ± 0.7	12.6 ± 0.9	15.8 ± 1.2	20.5 ± 0.8
5e	F	CH_3	80.3 ± 2.7	N.D[b]	97.8 ± 2.6	42.1 ± 0.9
5f	F	CH_2Ph	18.5 ± 0.9	18.5 ± 1.1	18.1 ± 0.4	41.5 ± 1.0
5g	NO_2	CH_3	45.3 ± 2.0	N.D[b]	>100	>100
5h	NO_2	CH_2Ph	33.8 ± 1.5	11 ± 1.3	13.8 ± 0.9	16.3 ± 1.1
CA-4			1.58 ± 0.5	0.67 ± 0.2	0.064 ± 0.04	0.097 ± 0.08

[a] Values are the mean ± SD. All the experiments were performed at least two times.
[b] N.D, not determined.

reduction in the cytotoxic activity (IC_{50} >80 μM). The compound **5d** with *para* chloro substituted on the phenyl ring and the 2-(benzylthio) group had the highest cytotoxic activity against the HT-29 cell line (IC_{50} = 8.9 μM). This compound also showed good activity against the MCF-7 and AGS cell lines. Moreover, the replacement of chloro substituted with fluoro and nitro led to less active compounds. In contrast, **5b** with the 5-phenyl group and the 2-methylthio substitution on the thiazole ring had little cytotoxic activity against all the cell lines. The results suggest that removing the *para* substitution from the 5-phenyl ring led to a decrease in the potency. Generally, most of the tested compounds showed no cytotoxic activity or only moderate cytotoxic activity against the NIH-3T3 cell line.

Finally, the introduction of the 2-(benzylthio) group on the thiazole ring seemed to play an important role in the cytotoxic activity. Furthermore, the presence of the *para* substitution in the 5-phenyl ring in the 2-(benzylthio) derivatives led to optimal effects against all the cancer cell lines. The same results were observed when **5a** and **5b** and **5g** and **5h** were compared. However, the cytotoxic effect of the synthesized compounds **5a**–**d** was less than that of the reference CA-4 in all the tested cell lines.

As the inhibition of tubulin polymerization has been implicated in G_2/M phase cell cycle arrest in various cancer cell lines,[19] the effect of compound **5d** on the cell cycle progression of the NIH-3T3 cells was determined by flow cytometry analysis (Fig. 2). The NIH-3T3 cells were treated with compound **5d** (25 and 50 μM) for 24 h, and **CA-4** (0.05 μM) was used as a positive control. The findings indicated that **5d** results in weak accumulation of cells in the G_2/M phase (37.64% compared to 35.70% for untreated cells) in 25 μM and in more evident accumulation (57.41%) at a higher concentration of 50 μM. These results demonstrated that the compound **5d** could arrest cells in the G_2/M phase.

After determination of the cytotoxic activity of the synthesized compounds, the abilities of some derivatives in inhibiting the polymerization of tubulin were studied. For this purpose, the effect of three potent compounds **5d**, **5f**, and **5h** on the polymerization of purified tubulin was evaluated at a final concentration of 10 μM. **CA-4** was also evaluated by this assay as a positive control. Figure 3 shows that all three compounds decreased the polymerization activity of the microtubule relative to the control and indicates the rational relationship between the inhibition of tubulin and the corresponding cytotoxic activities. The order of inhibition of tubulin polymerization was **CA-4** > **5d** > **5f** > **5h**. The compound with the 2-(benzylthio) group on the thiazole ring and the *para* chloro substituted 5-phenyl ring was the most potent inhibitor of tubulin polymerization. From these results, it seems that tubulin is a possible target for the synthesized compounds.

3. Molecular modeling

Molecular modeling studies were performed to investigate the binding ability of the synthesized compounds to the colchicine binding site of α, β-tubulin (PDB: 1SA0). Docking studies revealed that the 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazole compound, as well as CA-4, occupied the colchicine binding site of α, β-tubulin mostly buried in the β subunit (Fig. 4). In these compounds, the oxygen atom of the 4-methoxy substituent formed a hydrogen bond with Cysβ241 in the β subunit, similar to that seen in the X-ray complex of DAMA-colchicine with tubulin.

The 5-phenyl ring moiety lay in the hydrophobic pocket between Valα181, Metβ259, and Lys352. Furthermore, the thiazole ring interacted with Asnβ258 in compounds with the 2-(benzylthio) group, but replacements of this group with a 2-(thiomethyl moiety) increased the distance between these two groups. For example, the observed distance between thiazole and Asn258 was 3.05 Å for **5d**, whereas this value was 4.85 Å for **5c**. The increase in the distance led to a lack of interaction between the thiazole ring and Asn258 in **5c**. It is possible that this difference in the distance between the two molecules is one of the factors leading to the decreased antiproliferative activity observed for **5c** and **5d**. The docking scores varied from −7.45 to −6.38 kcal/mol for compounds with the 2-(benzylthio) group and were 1 kcal/mol lower than the average 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-methyl-thiazole score values.

4. Conclusions

In conclusion, the synthesis and biological evaluation of a series of 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazoles were discussed. Most of the synthesized compounds showed moderate to potent cytotoxic activity against the four cell lines. It seems that

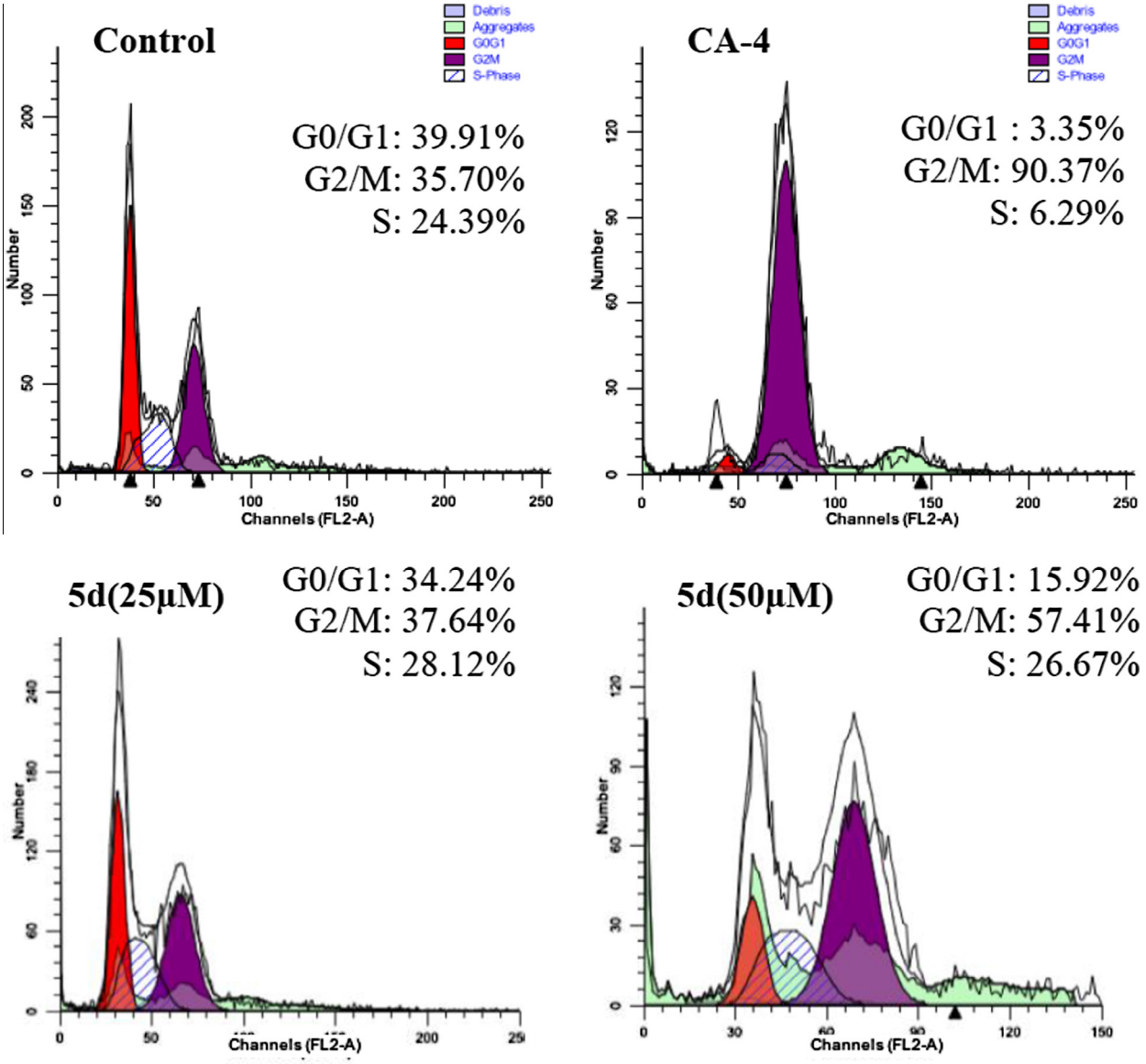


Figure 2. Effect of compound **5d** (25 and 50 μM) and CA-4 (0.05 μM) on the cell cycle of the NIH-3T3 cells.

the introduction of the 2-(benzylthio) group on the thiazole ring played an important role in the cytotoxic activity and that the *para* substitution in the 5-phenyl ring in the 2-(benzylthio) derivatives led to optimal effects against all the cancer cell lines. The flow cytometry analysis and microtubule polymerization assay confirmed that the synthesized compounds led to cell cycle arrest at the G_2/M phase by inhibitory effects against the microtubules. The molecular modeling studies revealed that these compounds could strongly bind to the colchicine binding site of α, β-tubulin through hydrogen bond interactions with Cysβ241 and that the thiazole ring also interacted with Asnβ258 in compounds with the 2-(benzylthio) group. The ability of some of the compounds to inhibit tubulin polymerization shows that tubulin is a good target for diarylthiazole derivatives.

5. Experimental section

5.1. General

Melting points were taken on a Kofler hot-stage apparatus (Reichert, Vienna, Austria) and are uncorrected. The ^{1}H NMR spectra were record on Bruker FT-500 MHz spectrometer. The instrument was set as 125 MHz for acquiring ^{13}C NMR spectra. Coupling constant (*J*) values are presented in hertz (Hz) and spin multiples are given as s (singlet), d (double), t (triple) and m (multiple). Elemental analyses were carried out with a Perkin Elmer Model 240-C apparatus. Elemental analyses were within ± 0.4% of theoretical values for C, H and N. Ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA), guanosine-5′-triphosphate type II-S (GTP), adenosine-5′-triphosphate (ATP), phenylmethylsulphonyl fluoride (PMSF), glycerol, $MgSO_4$ were purchased from Sigma (Deisenhofen, Germany). Piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), dimethyl sulfoxide (DMSO) and all starting material for synthesis were purchased from Merck (Darmstadt, Germany). MTT was purchased from Carl Roth (Karlsruhe, Germany). $MgSO_4$ (1 M) was added to both GTP and ATP 100 mM stock solutions, as a ratio of 1:10 (v/v). All cell line obtained from Pasteur Institute (Tehran, Iran). The cells were cultured in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA), penicillin (100 U/ml), and (100 μg/ml) streptomycin (Roche, Germany) at 37 °C in a humidified incubator with 5% CO_2. All of the test compounds, used in this study, were

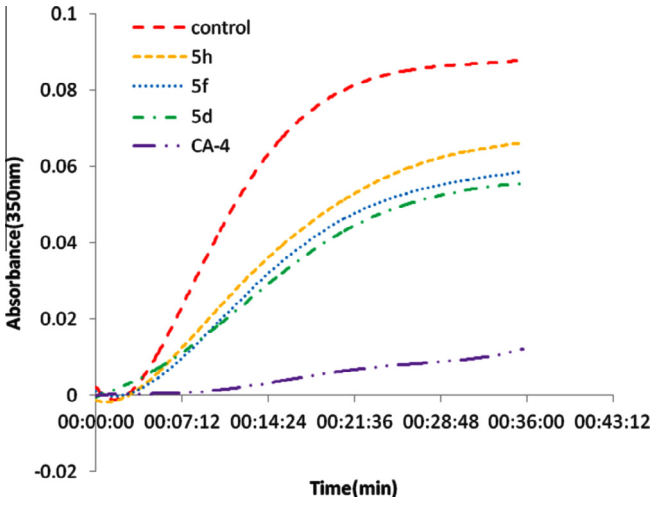


Figure 3. Effect of compounds **5d**, **5f**, **5h**, and **CA-4** on the polymerization of tubulin. The results represent the mean of duplicate experiments for each compound.

dissolved in DMSO (dimethyl sulfoxide) as 1 mM stock. For cell culture tests, DMSO was used at the final concentrations <0.1% (v/v). DMSO alone in the mentioned concentration did not show any cytotoxic effect.

5.2. Chemistry

5.2.1. General procedure for the preparation of 5(a–h)

To a solution of ammonium dithiocarbamate (1 mmol) in methanol, methyl iodide (1.5 mmol) (or benzyl iodide) was added. The reaction mixture was stirred at room temperature for 1 h. Then solvent was evaporated under reduced pressure. Water (10 mL) was added to the residue and extracted with dichloromethane. The organic phase was separated, dried by sodium sulfate and evaporated under vacuum. The residue (1.5 mmol) was added to a solution of α-bromo-1,2-(*p*-substituted)diaryl-1-ethanone (1 mmol) in methanol. The mixture was refluxed overnight. Then the reaction was cooled to room temperature and the precipitated product was filtered and recrystallized from ethanol to yield 2-alkylthio-4-(2,3,4-trimethoxyphenyl)-5-aryl-thiazole derivatives.

5.2.1.1. 4-(2,3,4-Trimethoxyphenyl)-5-(phenyl)-2-(methylthio)-thiazole (5a). Yield, 65%; mp 107–110 °C; ^{1}H NMR ($CDCl_3$) δ: 2.75 (s, 3H, SCH_3), 3.57 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3). 3.88 (s, 3H, OCH_3), 6.67 (d, J = 8.5 Hz, 1H, H_3-Ar_4), 7.05 (d, J = 8.5 Hz, 1H, H_2-Ar_4), 7.21–7.23 (m, 5H, Ar_5); ^{13}C NMR ($CDCl_3$) δ: 16.68 (SCH_3), 55.99 (OCH_3), 60.76 (OCH_3), 60.85 (OCH_3), 107.15 (C_5-Ar_4), 121.83 (C_1-Ar_4), 126.01 (C_6-Ar_4), 127.62 (C_4-Ar_5), 128.31 ($C_{3,5}$-Ar_5), 128.52 ($C_{2,6}$-Ar_5), 132.14 (C_1-Ar_5), 133.57 (C_3-Ar_4), 142.32 (C_5-thiazole), 146.87 (C_4-Ar_4), 152.01 (C_2-Ar_4), 154.11 (C_4-thiazole), 163.33 (C_2-thiazole). MS, *m*/*z* (%) 373 (M^+, 100), 267 (73), 207 (28), 171 (14), 121 (17), 79 (13). Anal. Calcd for $C_{19}H_{19}NO_3S_2$: C, 61.10; H, 5.13; N, 3.75. Found: C, 61.22; H, 5.33; N, 3.54.

5.2.1.2. 4-(2,3,4-Trimethoxyphenyl)-5-(phenyl)-2-(benzylthio)-thiazole (5b). Yield, 52%; mp 91–94 °C; ^{1}H NMR ($CDCl_3$) δ: 3.54 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.49 (s, 2H, SCH_2), 6.70 (d, J = 8.5 Hz, 1H, H_3-Ar_4), 7.05 (d, J = 8.5 Hz, 1H, H_2-Ar_4), 7.21–7.23 (m, 5H, Ar_5), 7.27–7.32 (m, 3H, benzyl), 7.41–7.48 (m, 2H, benzyl); ^{13}C NMR ($CDCl_3$) δ: 38.93 (CH_2), 56.01 (OCH_3), 60.79 (OCH_3), 60.88 (OCH_3), 107.19 (C_5-Ar_4), 121.36 (C_1-Ar_4), 126.07 (C_6-Ar_4), 127.69 (C_4-Benzyl), 127.80 (C_4-Ar_5), 128.34 ($C_{3,5}$-Ar_5), 128.54 ($C_{2,6}$-Ar_5), 128.66 ($C_{2,6}$-Benzyl), 129.16 ($C_{3,5}$-Benzyl), 131.90 (C_1-Ar_5), 134.48 (C_3-Ar_4), 136.36 (C_1-Benzyl), 142.20 (C_5-thiazole), 146.66 (C_4-Ar_4), 152.01 (C_2-Ar_4), 154.21 (C_4-thiazole), 161.26 (C_2-thiazole). MS, *m*/*z* (%) 449 (M^+, 11), 281 (23), 252 (15), 207 (100), 96 (17), 73 (19). Anal. Calcd for $C_{25}H_{23}NO_3S_2$: C, 66.79; H, 5.16; N, 3.12. Found: C, 66.85; H, 5.29; N, 3.02.

5.2.1.3. 4-(2,3,4-Trimethoxyphenyl)-5-(4-cholorophenyl)-2-(methylthio)-thiazole (5c). Yield, 59%; mp 150–151 °C. ^{1}H NMR ($CDCl_3$) δ: 2.74 (s, 3H, SCH_3), 3.61 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), 6.68 (d, J = 8.5 Hz, 1H, H_3-Ar_4), 7.03 (d, J = 8.5 Hz, 1H, H_2-Ar_4), 7.15 (d, J = 8.5 Hz, 2H, $H_{3,5}$-Ar_5), 7.21 (d, J = 8.5 Hz, 2H, $H_{2,6}$-Ar_5); ^{13}C NMR ($CDCl_3$) δ: 16.62 (SCH_3), 56.0 (OCH_3), 60.91 (OCH_3), 60.96 (OCH_3), 107.31 (C_5-Ar_4), 121.54 (C_1-Ar_4), 125.89 (C_6-Ar_4), 128.71 ($C_{3,5}$-Ar_5), 129.45 ($C_{2,6}$-Ar_5), 130.72 (C_1-Ar_5), 132.12 (C_3-Ar_4), 133.45 (C_4-Ar_5), 142.52 (C_5-thiazole), 147.33 (C_4-Ar_4), 151.97 (C_2-Ar_4), 154.64 (C_4-thiazole), 163.65 (C_2-thiazole). MS, *m*/*z* (%) 409 (M^++2, 33), 407 (M^+, 100), 301 (25), 281 (37), 266 (61), 207 (87), 73 (37). Anal. Calcd for $C_{19}H_{18}ClNO_3S_2$: C, 55.94; H, 4.45; N, 3.43. Found: C, 56.07; H, 4.56; N, 3.35.

5.2.1.4. 4-(2,3,4-Trimethoxyphenyl)-5-(4-cholorophenyl)-2-(benzylthio)-thiazole (5d). Yield, 67%; mp 75–77 °C; ^{1}H NMR ($CDCl_3$) δ: 3.58 (s, 3H, OCH_3), 3.83 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.49 (s, 2H, SCH_2), 6.69 (d, J = 8.5 Hz, 1H, H_3-Ar_4), 7.05 (d, J = 8.5 Hz, 1H, H_2-Ar_4), 7.13 (d, J = 8.5 Hz, 2H, $H_{3,5}$-Ar_5),7.21 (d, J = 8.5 Hz, 2H, $H_{2,6}$-Ar_5), 7.29-7.35 (m, 3H, benzyl), 7.43–7.46 (m,

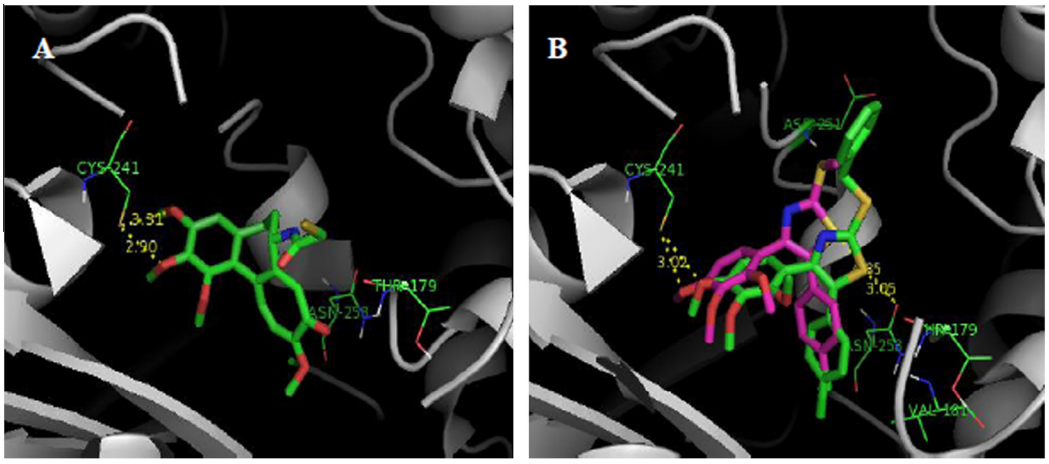


Figure 4. Binding modes of DAMA-colchicine (A) and the comparison between the binding modes of **5c** (magenta in B) and **5d** (green in B).

2H, benzyl); ^{13}C NMR ($CDCl_3$) δ: 38.67 (CH_2), 56.01 (OCH_3), 60.95 (OCH_3), 107.30 (C_5-Ar_4), 121.49 (C_1-Ar_4), 125.92 (C_6-Ar_4), 127.67 (C_4-benzyl), 128.65 ($C_{2,6}$-benzyl), 128.71 ($C_{3,5}$-Ar_5), 129.12 ($C_{3,5}$-benzyl), 129.48 ($C_{2,6}$-Ar_5), 130.64 (C_1-Ar_5), 133.01 (C_4-Ar_5), 133.55 (C_3-Ar_4), 136.43 (C_1-benzyl), 142.20 (C_5-thiazole), 147.36 (C_4-Ar_4), 151.95 (C_2-Ar_4), 154.23 (C_4-thiazole), 161.27 (C_2-thiazole). MS, *m*/*z* (%) 485 (M^++2, 3), 483 (M^+, 10), 281 (35), 207 (100), 147 (15), 73 (22). Anal. Calcd for $C_{25}H_{22}ClNO_3S_2$: C, 62.03; H, 4.58; N, 2.89. Found: C, 62.25; H, 4.61; N, 2.77.

5.2.1.5. 4-(2,3,4-Trimethoxyphenyl)-5-(4-flourophenyl)-2-(methylthio)-thiazole (5e). Yield, 43%; mp 98–101 °C; ^{1}H NMR ($CDCl_3$) δ: 2.78 (s, 3H, SCH_3), 3.61 (s, 3H, OCH_3), 3.81 (s, 3H, OCH_3). 3.91 (s, 3H, OCH_3), 6.68 (d, *J* = 8.5 Hz, 1H, H_3-Ar_4), 6.95 (t, *J* = 8.5 Hz, 2H, $H_{3,5}$-Ar_5), 7.05 (d, *J* = 8.5 Hz, 1H, H_2-Ar_4), 7.19 (dd, *J* = 8.5 Hz, *J* = 5.5 Hz, 2H, $H_{2,6}$-Ar_5); ^{13}C NMR ($CDCl_3$) δ: 16.64 (SCH_3), 56.0 (OCH_3), 60.76 (OCH_3), 60.91 (OCH_3), 107.26 (C_5-Ar_4), 115.54 (d, J_{CF} = 21.25 Hz,$C_{3,5}$-Ar_5), 121.58 (C_1-Ar_4), 126.03 (C_6-Ar_4), 128.31 (C_1-Ar_5), 130.01 (d, J_{CF} = 8.75 Hz, $C_{2,6}$-Ar_5), 133.93 (C_3-Ar_4), 142.22 (C_5-thiazole), 147.03 (C_4-Ar_4), 152.16 (C_2-Ar_4), 154.15 (C_4-thiazole), 160.18 (C_2-thiazole), 161.67 (d, J_{CF} = 242.0 -Hz,C_4-Ar_5). MS, *m*/*z* (%) 391 (M^+, 100), 300 (14), 285 (76), 207 (17), 189 (15), 139 (19). Anal. Calcd for $C_{19}H_{18}FNO_3S_2$: C, 58.29; H, 4.63; N, 3.58. Found: C, 58.35; H, 4.71; N, 3.35.

5.2.1.6. 4-(2,3,4-Trimethoxyphenyl)-5-(4-flourophenyl)-2-(benzylthio)-thiazole (5f). Yield, 74%; mp 81–82 °C; ^{1}H NMR ($CDCl_3$) δ: 3.56 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3), 4.48 (s, 2H, SCH_2), 6.69 (d, *J* = 8.5 Hz, 1H, H_3-Ar_4), 6.93 (t, *J* = 8.5 Hz, 2H, $H_{3,5}$-Ar_5), 7.04 (d, *J* = 8.5 Hz, 1H, H_2-Ar_4),7.17 (dd, *J* = 8.5 Hz, *J* = 5.5 Hz, 2H, $H_{2,6}$-Ar_5), 7.29–7.37 (m, 3H, Benzyl), 7.43-7.46 (m, 2H, Benzyl); ^{13}C NMR ($CDCl_3$) δ: 38.71 (CH_2), 56.0 (OCH_3), 60.72 (OCH_3), 60.90 (OCH_3), 107.26 (C_5-Ar_4), 115.54 (d, J_{CF} = 21.25 Hz, $C_{3,5}$-Ar_5), 121.52 (C_1-Ar_4), 125.98 (C_6-Ar_4), 127.66 (C_4-benzyl), 128.19 (C_1-Ar_5), 128.64 ($C_{2,6}$-benzyl), 129.13 ($C_{3,5}$-benzyl), 130.04 (d, J_{CF} = 7.5 Hz, $C_{2,6}$-Ar_5), 133.26 (C_3-Ar_4), 136.48 (C_1-benzyl), 142.39 (C_5-thiazole), 147.06 (C_4-Ar_4), 151.97 (C_2-Ar_4), 154.16 (C_4-thiazole), 160.85 (C_2-thiazole), 162.20 (d, J_{CF} = 246.0 Hz,C_4-Ar_5). MS, *m*/*z* (%) 467 (M^+, 44), 281 (37), 253 (18), 207 (100), 91 (18), 73 (20). Anal. Calcd for $C_{25}H_{22}FNO_3S_2$: C, 64.22; H, 4.74; N, 3.00. Found: C, 64.25; H, 4.81; N, 2.98.

5.2.1.7. 4-(2,3,4-Trimethoxyphenyl)-5-(4-nitrophenyl)-2-(methylthio)-thiazole (5g). Yield, 81%; mp 161–162 °C; ^{1}H NMR ($CDCl_3$) δ: 2.77 (s, 3H, SCH_3), 3.61 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3). 3.91 (s, 3H, OCH_3), 6.72 (d, *J* = 8.5 Hz, 1H, H_3-Ar_4), 7.08 (d, *J* = 8.5 Hz, 1H, H_2-Ar_4), 7.36 (d, *J* = 8.5 Hz, 2H, $H_{3,5}$-Ar_5), 8.10 (d, *J* = 8.5 Hz, 2H, $H_{2,6}$-Ar_5); ^{13}C NMR ($CDCl_3$) δ: 16.55 (SCH_3), 56.04 (OCH_3), 61.01 (OCH_3), 61.11 (OCH_3), 107.15 (C_5-Ar_4), 121.18 (C_1-Ar_4), 123.84 ($C_{3,5}$-Ar_5), 125.76 (C_6-Ar_4), 128.44 ($C_{2,6}$-Ar_5), 130.65 (C_3-Ar_4), 139.17 (C_1-Ar_5), 142.55 (C_5-thiazole), 146.48 (C_4-Ar_4), 149.0 (C_4-Ar_5), 151.82 (C_2-Ar_4), 154.64 (C_4-thiazole), 165.83 (C_2-thiazole). MS, *m*/*z* (%) 418 (M^+, 16), 281 (38), 253 (19), 207 (100), 73 (25). Anal. Calcd for $C_{19}H_{18}N_2O_5S_2$: C, 54.53; H, 4.34; N, 6.69. Found: C, 54.75; H, 4.47; N, 6.48.

5.2.1.8. 4-(2,3,4-Trimethoxyphenyl)-5-(4-nitrophenyl)-2-(benzylthio)-thiazole (5h). Yield, 75%; mp 140–141 °C; ^{1}H NMR ($CDCl_3$) δ: 3.58 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 3.92 (s, 3H, OCH_3), 4.52 (s, 2H, SCH_2), 6.75 (d, *J* = 8.5 Hz, 1H, H_3-Ar_4), 7.08 (d, *J* = 8.5 Hz, 1H, H_2-Ar_4), 7.29–7.38 (m, 5H, benzyl), 7.45 (d, *J* = 8.5 Hz, 2H, $H_{2,6}$-Ar_5), 8.09 (d, *J* = 8.5 Hz, 2H, $H_{3,5}$-Ar_5); ^{13}C NMR ($CDCl_3$) δ: 38.49 (CH_2), 56.05 (OCH_3), 61.02 (OCH_3), 61.07 (OCH_3), 107.59 (C_5-Ar_4), 121.08 (C_1-Ar_4), 123.82 ($C_{3,5}$-Ar_5), 125.78 (C_6-Ar_4), 127.79 (C_4-benzyl), 128.50 ($C_{2,6}$-benzyl), 128.70 ($C_{2,6}$-Ar_5), 129.12 ($C_{3,5}$-Benzyl), 131.40 (C_3-Ar_4), 136.18 (C_1-benzyl), 139.11 (C_1-Ar_5), 142.61 (C_5-thiazole), 146.58 (C_4-Ar_4), 148.96 (C_4-Ar_5), 151.80 (C_2-Ar_4), 154.63 (C_4-thiazole), 163.52 (C_2-thiazole). MS, *m*/*z* (%) 494 (M^+, 11), 281 (34), 253 (21), 207 (100), 133 (12), 73 (23). Anal. Calcd for $C_{25}H_{22}N_2O_5S_2$: C, 60.71; H, 4.48; N, 5.66. Found: C, 60.34; H, 4.73; N, 5.85.

5.3. Biology

5.3.1. Growth inhibition assay

The response of three different carcinoma cell lines HT-29 (colon carcinoma cell), AGS (stomach carcinoma cell) and MCF7 (breast carcinoma cells) to synthesized compounds was evaluated by the determination of cell survival using MTT assay. NIH-3T3 (mouse fibroblast cells) also used as normal cell line. **CA-4** was applied as a positive control. Cells from different cell lines were cultured in 96-well plates at the density of 8000–10,000 viable cells per well and incubated for 48 h to allow cell attachment. Then cell were treated with various concentrations of each compound (0.001–100 μM). Cells were then incubated for another 24 or 48 h (depends to cell cycle of each cell line). At the end of this period the response of cells to compounds were evaluated by determining cell survival using 3-(4,5-dimethylthiazoyl-2-yl)2,5-diphenyl tetrazolium bromide. For this purpose Cells were washed in PBS, and 20 μL of MTT reagent (5 mg/ml) in phosphate buffered serum (PBS) was added to each well and the cells were incubated for another 4 h at 37 °C. Finally the supernatants were then aspirated and dimethyl sulfoxide (100 μL) was added to each well.[18] The absorbance was determined by plate reader (Anthous 2020; Austria) for each well at a test wavelength of 550 nm against a standard reference solution at 690 nm. Each experiment was performed in three replicates. The concentration required for 50% inhibition of cell viability (IC_{50}) were determined by a nonlinear regression analysis and expressed in mean ± SD.

5.3.2. Cell cycle analysis

For flow cytometric analysis of DNA content, 10^6 NIH-3T3 cells were treated with (25 and 50 μM) compound **5d** 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).

5.3.3. In vitro tubulin polymerization assay

Microtubule protein (MTP) was isolated from fresh sheep brain[4] and then MAP-free tubulin was purified from the microtubule protein by DEAE chromatography.[20] The protein content was estimated by Bradford method using BSA as a standard.[21]

For investigation of tubulin polymerization test compounds in final concentration 10 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. 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. The results represent the mean of duplicate experiments.[22]

5.4. Molecular docking

The X-ray crystal structure of the DAMA-colchicine–tubulin complex (PDB code 1SA0) was used as the tubulin protein template.[23] The 3D structures of ligand molecules were built, optimized and saved in Mol2 format with the aid of the hyperchem 8.0 program. These partial charges of Mol2 files were further

modified by using the ADT package (version 1.5.4) so that the charges of the nonpolar hydrogens atoms assigned to the atom to which the hydrogen is attached. The resulting files were saved as pdbqt files. The box center was set to the center of co-crystalized ligand, DAMA colchicines ($x = 117.2187$, $y = 90.1800$, $z = 6.2898$) and a grid of 40 - 40 - 40 was used to ensure that the area probed was adequate for the ligands to explore all possible binding modes. Moreover, the number of generations and maximum number of energy evaluations was set to 150 and 2,500,000, respectively. To validate the utility of method for docking ligand into the active site, DAMA colchicine was built using the hyperchem8.0, energy minimized and docked to the active site using the above parameter and the best scored pose of DAMA colchcine compared with the crystal structure. At the end of a docking job with multiple runs, results differing by less than 0.5 Å in positional root–mean–square deviation (RMSD) were clustered together and the results of the most favorable free energy of binding were selected as the resultant complex structures.[24]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.10.030.

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