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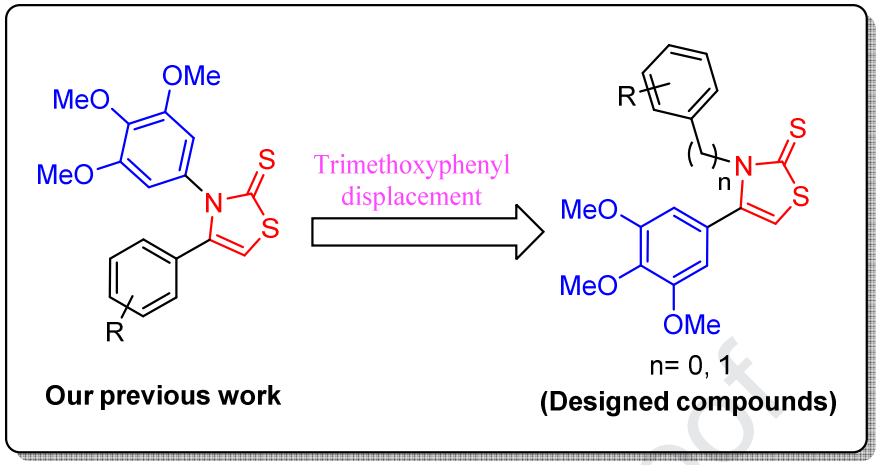
Graphical Abstract



New thiazole-2(3*H*)-thiones containing 4-(3,4,5-trimethoxyphenyl) moiety as anticancer agents

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Abstract: A new series of thiazole-2(3*H*)-thiones containing 4-(3,4,5-trimethoxyphenyl) moiety were synthesized as diaryl-heterocylic analogs of combretastatin A-4 with anticancer activity. The cytotoxicity evaluation of synthesized compounds against cancer cell lines (A549, MCF-7 and SKOV3) revealed that most of them had potent cytotoxic activity toward all tested cell lines (IC_{50}s <10 µg/mL). Among them, 3-(chlorobenzyl) derivatives **5c** and **5d** showed the best inhibitory effect on MCF-7 cells (IC_{50} values of 1.14 and 2.41 µg/mL, respectively). Furthermore, the ability of tubulin polymerization inhibition and apoptosis induction were evaluated for the promising compounds **5c** and **5d**. Results suggested that these compounds remarkably inhibit tubulin polymerization and induce apoptosis resulting in cell death. *In vitro* studies revealed that these compounds had no significant cytotoxicity against normal cells at the concentrations required for growth inhibition of cancer cells. *In vitro* biding assay and *in silico* docking study also confirmed the binding of prototype compound to the colchicine binding site of tubulin.

Keywords: Anticancer agents; Thiazole-2(3*H*)-thione; Cytotoxic activity; 3,4,5-trimethoxyphenyl; Tubulin polymerization

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1. Introduction

Nowadays, cancer after cardiovascular disease has become one of the most prominent factors of death worldwide. Deaths due to cancer are globally about 17% of total human deaths [1]. The lack of efficient and safe drugs is a driving force for continuous efforts to discovery of new anticancer agents [2].

Microtubule/tubulin is a promising target for design and development of new anticancer agents. Microtubules are major components of the cytoskeleton and are formed by tubulin polymerization during interphase which have pivotal role in division of cells [3,4]. Hereupon, diverse molecules inhibiting tubulin assembly and suppressing microtubule formation have been synthesized and reported as anti-mitotic agents [5,6]. One of the most potent anti-mitotic agents which soon gained the attention of medicinal chemists and cancer biologists is combretastatin A-4 (CA-4, Fig. 1), a trimethoxyphenyl (TMP) containing stilbenoid that first was found in *Combretum caffrum* [7]. CA-4 belongs to a group of tubulin binding agents which inhibits intensely tubulin polymerization and disrupts microtubule dynamics by binding to the colchicine binding site (CBS) in beta tubulin. Other well-known colchicine binding drugs containing TMP moiety are AVE8062, and BCN-105P (Fig. 1) [8,9].

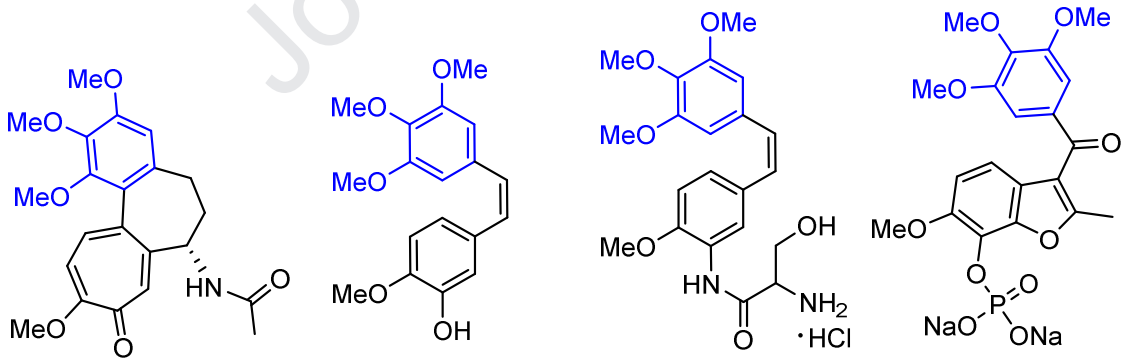


Figure 1. Structures of some potent anti-mitotic agents targeting CBS of tubulin.

SAR studies on CA-4 demonstrated that both TMP moiety and *cis* configuration in alkene bridge which connects two aromatic rings are essential structural features and required for the activity of this compound [10]. Since olefinic double bond in CA-4 is instable and undergoes geometrical isomerization, considerable efforts have been made to modify the structure of CA-4, maximizing its stability and bioavailability. Replacement of the olefinic bridge with diverse heterocyclic rings such as thiophene, tetrazole, 1,2,3-triazole, 1,2,3-thiadiazole, 1,3-oxazole, and oxazolone was the main modifications [11,12]. In particular, Nam et al. have reported two series of combretoxazolones including 3,4-diaryloxazolones and 4,5-diaryloxazolones as potent antitumor agents. The representative compound **A** (Fig. 2) with IC_{50} values of 1.1-4.3 nM showed potent cytotoxic activity against B16, MCF-7, HCT116, A549, and PC-3 cell lines. This compound had effective antitumor activity at the dose of 30 mg/kg/day in BDF1 mice bearing B16 murine melanoma cells [13].

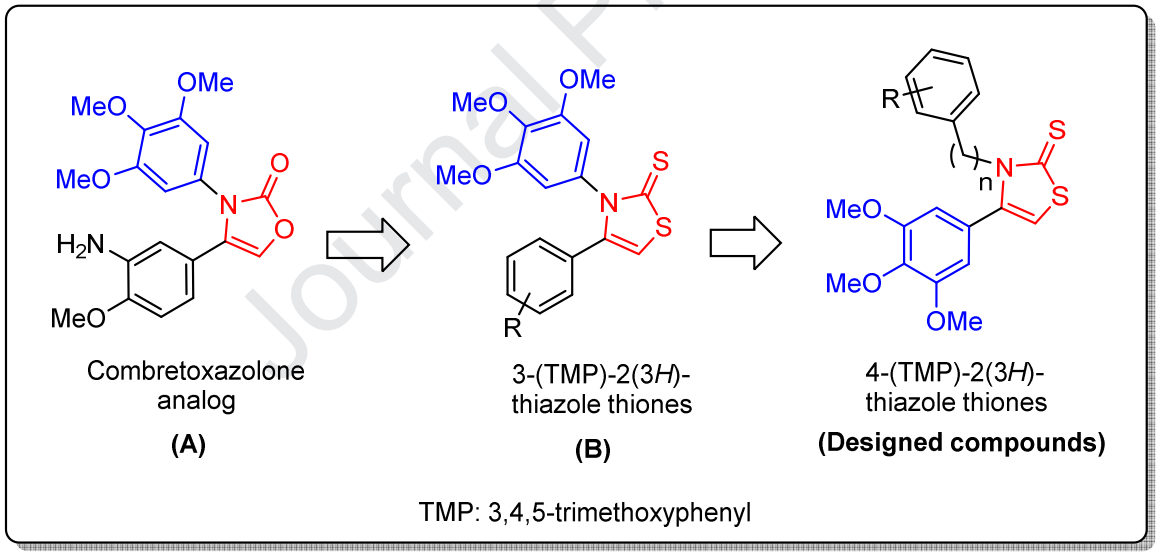


Figure 2. Structures of lead compounds (**A** and **B**) and newly designed compounds.

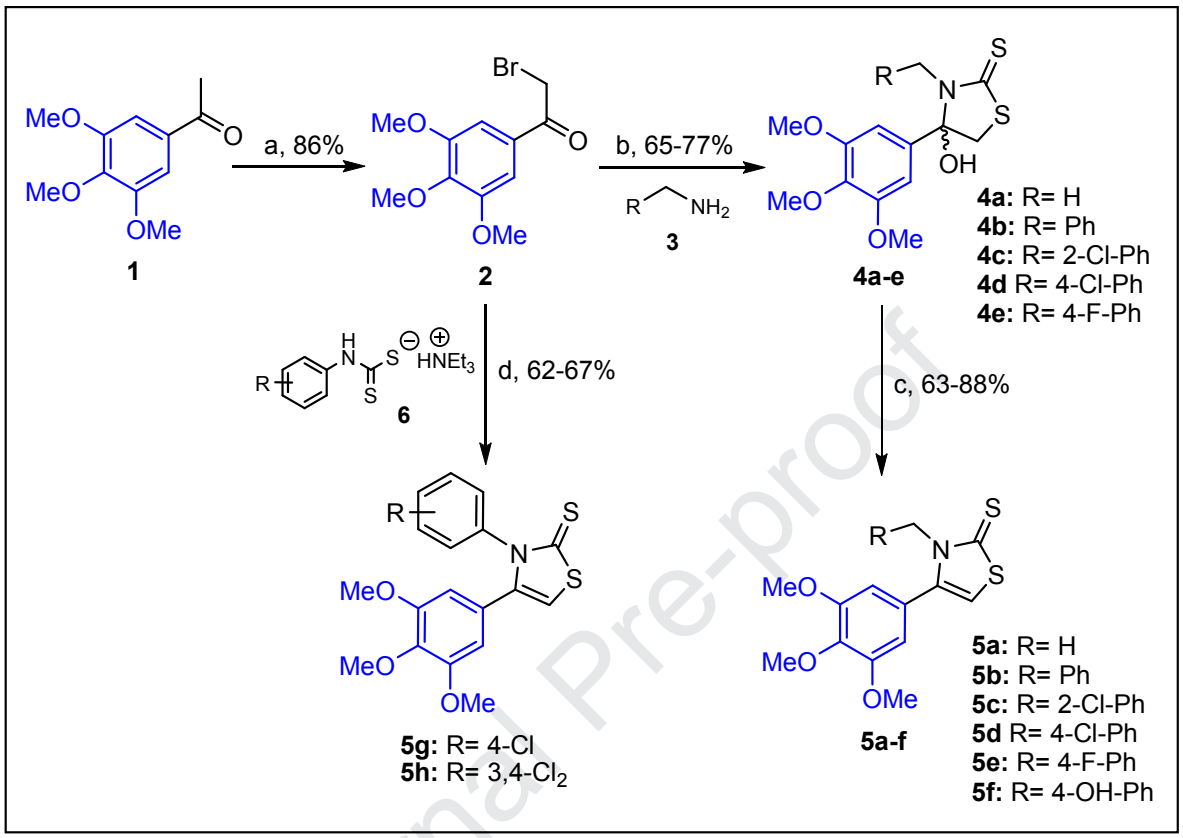
In continuation of our works on tubulin-targeting anticancer agents [14-17] we have recently, designed and synthesized several 3-(TMP)-2(3*H*)-thiazole thiones **B** as new CA-4-modified analogs. These compounds are combretoxazolone analogs in which both oxygen atoms of central

heterocyclic ring have been replaced with sulfur atoms [18]. This isosteric replacement could affect the size and electronic character of central ring, resulting in possible alteration of binding properties and increase of lipophilicity of the new designed molecules namely thiazole thiones **B**. The cytotoxicity assay of compounds **B** toward T47D, MCF-7 and MDA-MB-231 cancer cell lines revealed that 4-methyl derivative (R= 4-Me) with IC_{50} values of 11.8-19.7 µg/mL had the highest activity against all tested cell lines. In conductance with our previous publications, 4-(TMP)-thiazole-2(3*H*)-thione derivatives (Fig. 2) as new series of anticancer agents targeting tubulin were designed and in these new series of compounds, the TMP moiety has been displaced from position 3 to position 4 of thiazole-2(3*H*)-thione core structure.

2. Results and discussion

2.1. Chemistry

The synthetic route followed for the preparation of 4-(TMP)thiazole-2(3*H*)-thiones **5a-h** was illustrated in Scheme 1. Firstly, treatment of 3,4,5-trimethoxyacetophenone (**1**) with cupper (II) bromide in refluxing chloroform-ethyl acetate afforded α-bromo-3,4,5-trimethoxyacetophenone (**2**) [19]. In the next step, the *in situ* generated cabodithioate form the reaction of different primary amines (**3**) with CS_2 in the presence of potassium acetate, was condensed with compound **2** to give 4-hydroxythiazolidine-2-thione intermediates **4a-e** [20]. Subsequently, these alcoholic compounds were refluxed with HCl 0.5% in methanol to yield pure target compounds **5a-f** [21]. On the other hand, the appropriate *N*-aryldithiocarbamate salt (**6**) prepared according to the literature method [21], was treated with α-bromo- compound **2** in absolute ethanol at room temperature. After addition of hydroxylamine hydrochloride (as catalyst), the reaction mixture was heated under microwave irradiation (T_1= 150 ºC, t_1= 15 min, t_2= 30 min). In these cases, the obtained products were characterized to be corresponding 4-(TMP)thiazole-2(3*H*)-thiones **5g,h**.



Scheme 1. Synthesis of thiazolidine-2-thiones **4a-e** and thiazole-2(3*H*)-thiones **5a-h**. *Reagents and conditions*: (a) $CuBr_2$, $CHCl_3$-EtOAc, reflux; (b) CS_2, CH_3COOK, MeOH, rt; (c) HCl 0.5%, MeOH, reflux; (d) absolute ethanol, rt, and then $HONH_2$.HCl, MW.

2.2. Biology

2.2.1. Cell viability assay

The cytotoxicity of all newly synthesized compounds **4a-e** and **5a-h** towards human cancer cell lines A549 (lung), MCF-7 (breast) and SKOV3 (ovarian) was examined by employing the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [22]. Etoposide was served as a positive control. The IC_{50} values of the tested compounds are reported in Table 1.

Table 1. Cytotoxicity (IC_{50} values, µg/mL) of compounds **4a-e** and **5a-h** against human cancer cell lines (*Note*: the related IC_{50} values in µM are shown into parentheses below the values in µg/mL).

Compound	A549	MCF-7	SKOV3
4a	3.21 ± 0.41 (10.2)	8.23 ± 0.12 (26.1)	4.23 ± 0.14 (13.4)
4b	5.02 ± 0.12 (12.8)	5.22 ± 0.17 (13.4)	5.38 ± 0.16 (13.8)
4c	6.19 ± 0.23 (14.6)	5.22 ± 0.10 (12.3)	5.13 ± 0.18 (12.1)
4d	4.91 ± 0.14 (11.6)	7.97 ± 0.09 (18.8)	7.68 ± 0.28 (18.1)
4e	8.74 ± 0.22 (21.4)	4.92 ± 0.17 (12.0)	5.05 ± 0.22 (12.4)
5a	5.20 ± 0.24 (17.5)	8.22 ± 0.15 (27.7)	6.94 ± 0.13 (23.4)
5b	4.52 ± 0.12 (12.1)	5.18 ± 0.22 (13.9)	6.52 ± 0.24 (17.5)
5c	5.62 ± 0.23 (13.8)	1.14 ± 0.18 (2.8)	5.50 ± 0.18 (13.5)
5d	2.72 ± 0.15 (6.7)	2.41 ± 0.11 (5.9)	7.37 ± 0.08 (18.1)
5e	6.18 ± 0.16 (15.8)	3.91 ± 0.10 (10.0)	7.29 ± 0.16 (18.6)
5f	6.07 ± 0.22 (15.6)	8.58 ± 0.24 (22.0)	7.27 ± 0.18 (18.7)
5g	6.04 ± 0.23 (15.4)	6.63 ± 0.08 (16.9)	6.95 ± 0.11 (17.7)
5h	5.16 ± 0.10 (12.1)	4.11 ± 0.21 (9.6)	7.62 ± 0.25 (17.9)
Etoposide	3.52 ± 0.23 (6.0)	3.37 ± 0.24 (5.7)	5.41 ± 0.06 (9.2)

The values are mean of three experiments.

Based on the observed results, the majority of compounds exhibited good cytotoxic activity, displaying IC_{50} values in the range of 1.14-8.74 µg/mL. The reference drug etoposide showed

IC_{50} values between 3.37 and 5.41 µg/mL against tested cell lines. The highest inhibitions were obtained with 3-(chlorobenzyl)thiazole thione derivatives **5c** and **5d** against MCF-7 cells, displaying IC_{50} values of 1.41 and 2.41 µg/mL. The activity of **5c** was better than that of etoposide against MCF-7 cell line. Also, the 3-(4-chlorobenzyl)thiazole thione **5d** was found to be the most potent compound against A549 cell line (IC_{50} = 2.72 µg/mL). Nevertheless, the 3-methyl derivative from 4-hydroxythiazolidine-2-thione series (compound **4a**) was next potent compound. Moreover, thiazolidine-2-thiones **4a-c**, **4e** along with thiazole-2(3*H*)-thione **5c** were found to be the most active derivatives against SKOV3 cells.

A survey on the IC_{50} values of benzyl analogs **4b-e** and **5b-e** against A549 and MCF-7 cells revealed that the activity of thiazole-2(3*H*)-thiones is slightly better than that of their corresponding intermediates 4-hydroxythiazolidine-2-thiones. In the case of SKOV3 cell line, the differences between thiazole-2(3*H*)-thiones and 4-hydroxythiazolidine-2-thiones were not significant, with the exception of 4-fluorophenyl derivatives **4e** (IC_{50} = 5.05 µg/mL) and **5e** (IC_{50} = 7.29 µg/mL). Furthermore, by comparing 3-(4-chlorobenzyl)thiazole thione **5d** and 3-(4-chlorophenyl)thiazole thione **5g**, it can be concluded that the insertion of methylene between 4-chlorophenyl moiety and thiazole-2(3*H*)-thione ring improves the potency towards A549 and MCF-7 cancer cells.

As described in Introduction, firstly we have designed and synthesized 3,4-diarylthiazole-2(3*H*)-thiones bearing a TMP moiety at 3-position [18]. Modification of substituents on 4-phenyl ring resulted in 4-methyl derivative with promising activity against breast cancer cells (IC_{50}s < 20 µg/mL), which should be improved using different strategy. Thus, we displaced the pharmacophoric portion TMP from 3-position to 4-position of the central core thiazole-2(3*H*)-thione to generate 4-(TMP)-thiazole-2(3*H*)-thiones. The obtained compounds in these series showed significantly lower IC_{50} values especially against MCF-7 cells. For example, 4-(4-chlorophenyl)- analog (**7**, Fig. 3) from 3-(TMP)thiazole thione series was inactive on MCF-7, its reverse analog **5g** had IC_{50} value of 6.63 µg/mL.

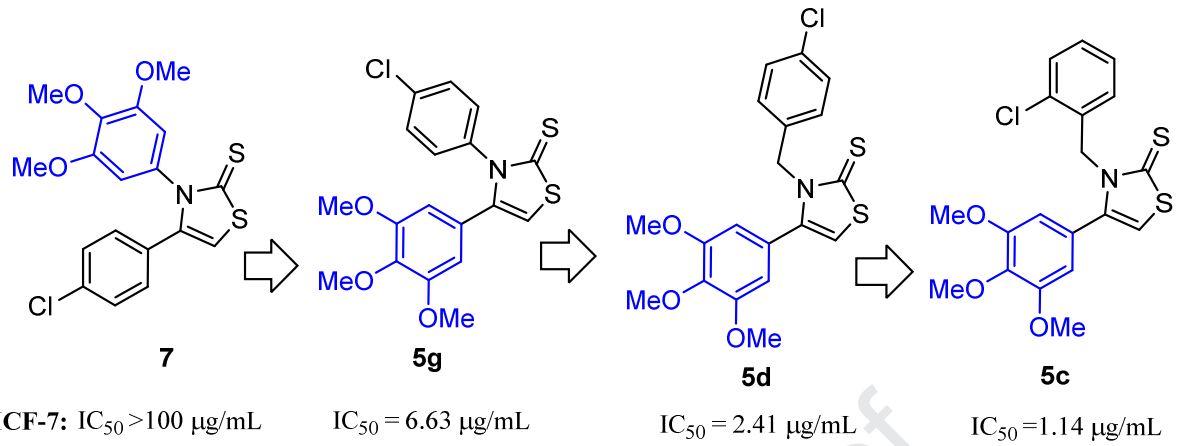


Figure 3. Structure-activity relationships of thiazole-2(3*H*)-thiones

Introduction of a methylene linker between 4-chlorophenyl and 4-(TMP)thiazole thione in compound **5g** resulted in compound **5d** with improved potency (IC_{50}= 2.41 µg/mL). Further moving of chloro group from *para* to *ortho* position of benzyl moiety produced compound **5c** with highest activity against MCF-7 (Fig. 3). So, it proved that regional isomerization of 3-TMP derivatives to 4-TMP and replacement of another aryl ring with benzyl moiety could significantly increase the cytotoxic activity.

*2.2.2. Effect of compounds **5c** and **5d** on normal cells L929 and red blood cells*

Although all compounds showed pronounced cytotoxicity against cancer cell lines, but the important issue that should be considered was selectivity of compounds. Therefore, the representative compounds **5c** and **5d** were tested against normal cells L929 (mouse fibroblast cell line) *in vitro*. As seen in Fig. 4, the viability of normal cells was not affected significantly at low concentrations by these compounds. Even at high concentrations of 200 µg/mL, the percent of viability was more than 50%. Accordingly, the IC_{50} values of compounds **5c** and **5d** are determined to be > 200 µg/mL. Since the IC_{50} values of compounds **5c** and **5d** against cancer cell lines were in the range of 1.14-7.37 µg/mL, thus they have acceptable selectivity against cancer cells over normal cells. As investigated below, the possible mechanism of designed compounds is inhibition of tubulin polymerization which has pivotal role in cell division. Thus, the increased

rate of cell division in cancer cells and significant divergent of metabolic pathways in cancer cells make them more sensitive to anti-mitotic agents over the normal cells.

Some of the chemotherapeutic agents have potential toxicity due to hemolysis which results in anemia [23]. Thus we studied the hemolytic activity of representative compounds **5c** and **5d** in erythrocytes. Based on the results (Fig. 5), the hemolysis rate of the tested compounds was less than 25% at the concentration of 10 µg/mL. Notably, compound **5d** showed no significant hemolytic activity at the concentrations of 1 and 5 µg/mL, compared to control. It should be noted that the rates of hemolysis in negative control group (CT) and positive control (Triton X, TX) were 6.2 and 100%, respectively.

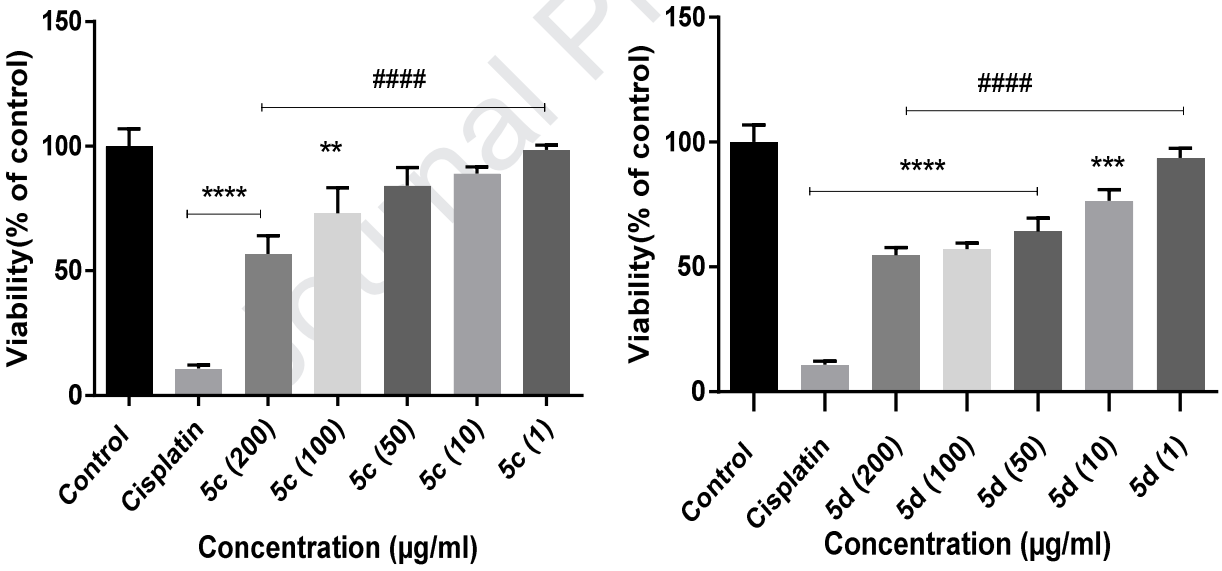


Figure 4. Cytotoxic activity of compounds **5c** and **5d** toward normal cells L929 at the concentrations of 1, 10, 50 100 and 200 µg/mL in comparison with cisplatin (50 µg/mL). Each experiment was repeated three times and the values were calculated as Mean ± SD. Significantly different from control (***P* <0.01; ****P* <0.001 *****P* < 0.0001).

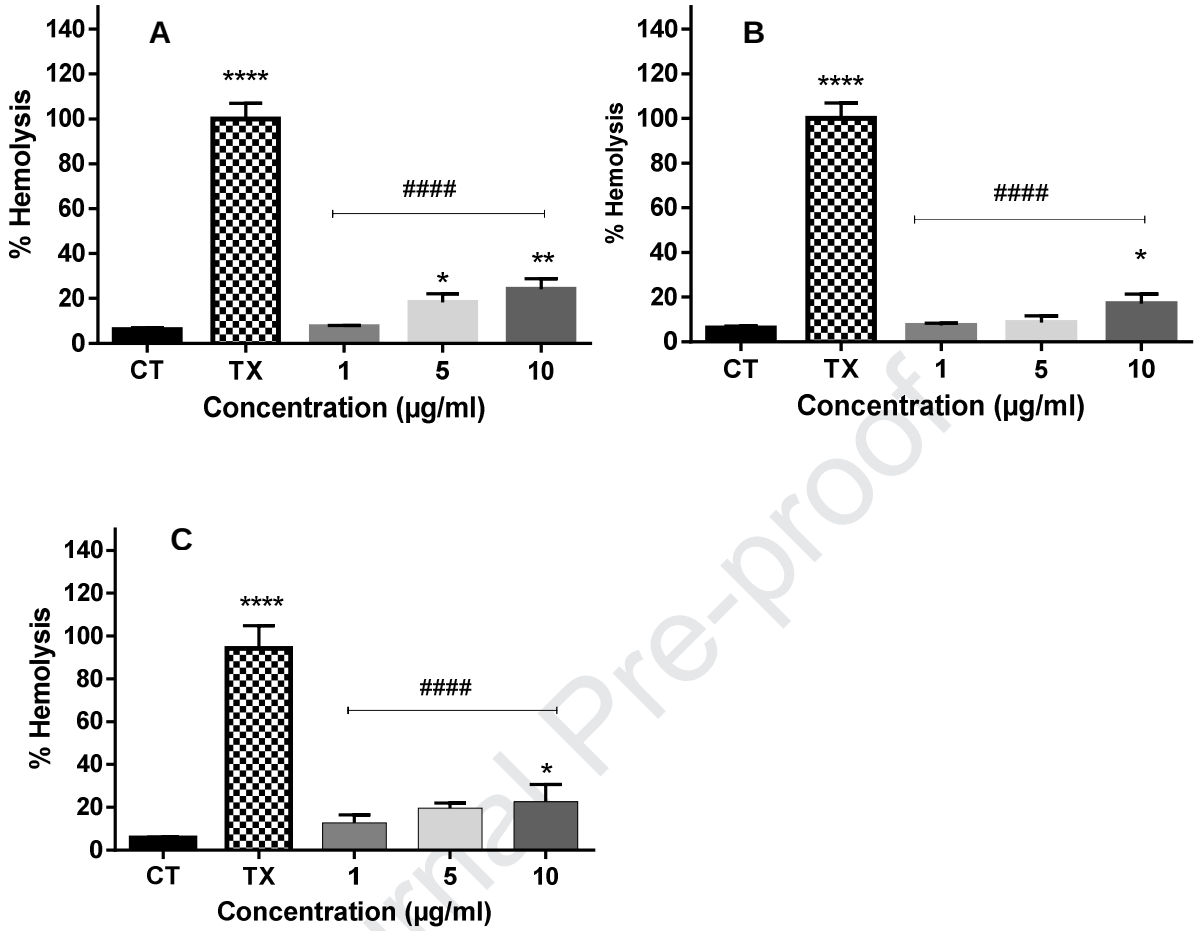


Figure 5. The rates of hemolysis induced by compounds **5c** (A), **5d** (B) and etoposide (C) in human erythrocytes at the concentrations of 1, 5 and 10 µg/mL. The negative control (CT) consisted of erythrocytes treated with DMSO and the positive control consisted of erythrocytes treated with Triton X-100 (TX, 2% v/v). Each experiment was repeated three times and the values were calculated as Mean ± SD. Significantly different from CT (*$P < 0.05$; ****$P < 0.0001$). Significantly different from TX ($^{####}P < 0.0001$).

*2.2.3. Investigation of the genotoxicity of compounds **5c** and **5d** by comet assay*

The comet assay is a simple and sensitive method based on single cell gel electrophoresis that enables the detection of DNA damage in individual eukaryotic cells. In this method, the electrophoresis of nucleic acids from a single cells immobilized in agarose layer, results in migration of the DNA fragments in an electric field related to their mass, making a tail. The

percentage of fragmented DNA in the comet tail as well as the tail length and tail moment is considered as parameters for evaluating potential genotoxicity of compounds [24,25]. Accordingly, the potential genotoxic effect of compounds **5c** and **5d** was explored by the alkaline comet assay in normal cells L929. The compounds **5c** and **5d** were tested at the concentration of 10 µg/mL and compared to etoposide (0.5 µg/mL) as positive control. As parameters scored, we used tail length, % DNA in tail and tail moment, and the results were presented in Fig. 6. Based on the results, etoposide (as reference drug) at lower concentration of 0.5 µg/mL showed significant levels of DNA damage in L929 cells. We observed that compound **5c** at 10 µg/mL concentration didn't induce a significant increase in DNA damage as compared to the negative control group. On the other hand, compound **5d** increased significantly ($P < 0.01$) the level of DNA fragmentation in L929 cells as compared to the control group. However, the level of DNA damage produced by compound **5d** (10 µg/mL) was significantly less than that caused by etoposide (0.5 µg/mL), indicating the lower genotoxicity of **5d**.

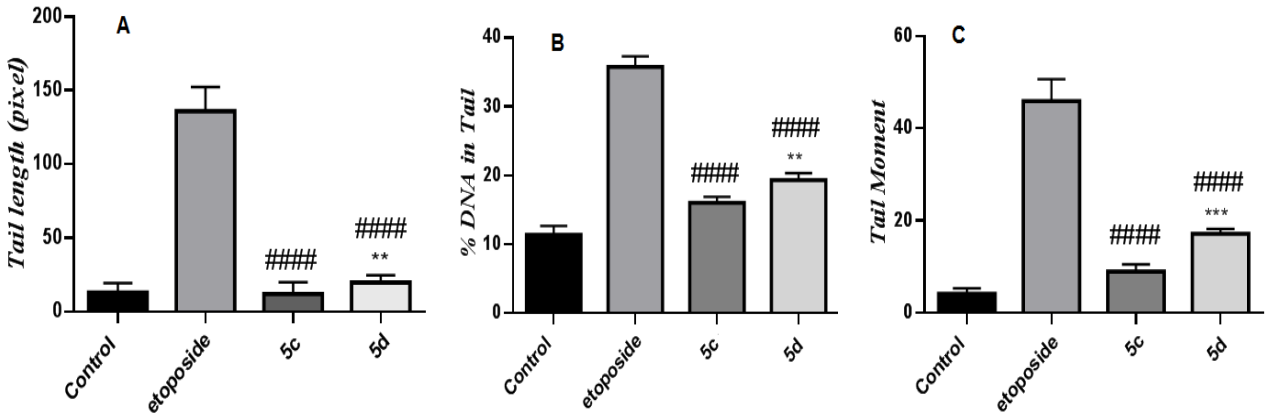


Figure 6. Quantitative representation of the DNA damage evaluated by the comet assay in L929 cells as tail parameters: (A) Tail length; (B) % DNA in tail and (C) tail moment. The cells were exposed to the compounds **5c** and **5d** (10 µg/mL) and compared to etoposide (0.5 µg/mL) and negative control (untreated). Each graph has been represented as Mean ± SEM. Significantly different from control (**$P < 0.01$; ***$P < 0.001$). Significantly different from etoposide ($^{\#\#\#\#}P < 0.0001$).

*2.2.4. Effect of compounds **5c** and **5d** on tubulin polymerization*

To evaluate whether the novel synthesized 4-(TMP)-thiazole-2(3*H*)-thiones could target the microtubule system also to elucidate the inhibitory effect of them on tubulin polymerization *in vitro*, compounds **5c** and **5d** which showed better cytotoxic activities were chosen for further evaluations. Colchicine was included as the reference drug and the polymerization was measured according to the literature method [26, 27]. The obtained results indicated that these compounds could inhibit tubulin polymerization at a concentration-dependent manner (Fig. 7). The IC_{50} values of compounds **5c** and **5d** were 5.14 and 9.97 μg/mL, respectively.

*2.2.5. Colchicine-tubulin binding inhibition by compound **5c***

Previous studies revealed that the TMP-derived tubulin inhibitors such as colchicine and combretastatin A-4 can bind to the CBS in tubulin, resulting in microtubule depolymerization [9]. In our study, the *in vitro* bioassay indicated that the prototype compounds **5c** and **5d** as diaryl-heterocylic analogs of combretastatin A4 are also capable to inhibit tubulin polymerization. In order to investigate the binding of ligand **5c** to tubulin at the CBS, we used the fluorescence assay of the colchicine-tubulin complex as reported previously [28-30]. In the fluorescence assay, if a ligand has binding affinity to the CBS, colchicine molecule is displaced by the ligand and the fluorescence intensity is reduced. Accordingly, we determined the changes in the fluorescence of the colchicine-tubulin complex after exposure to compound **5c**. Fig. 8 shows that the addition of compound **5c** to the different mixtures of tubulin (0.25 μM) and colchicine (0.1, 0.25, 0.5 or 1 μM) significantly reduces the relative fluorescence intensity (F/F0). These findings indicate that compound **5c** has specific affinity to the colchicine pocket and prevents the binding of colchicine to tubulin.

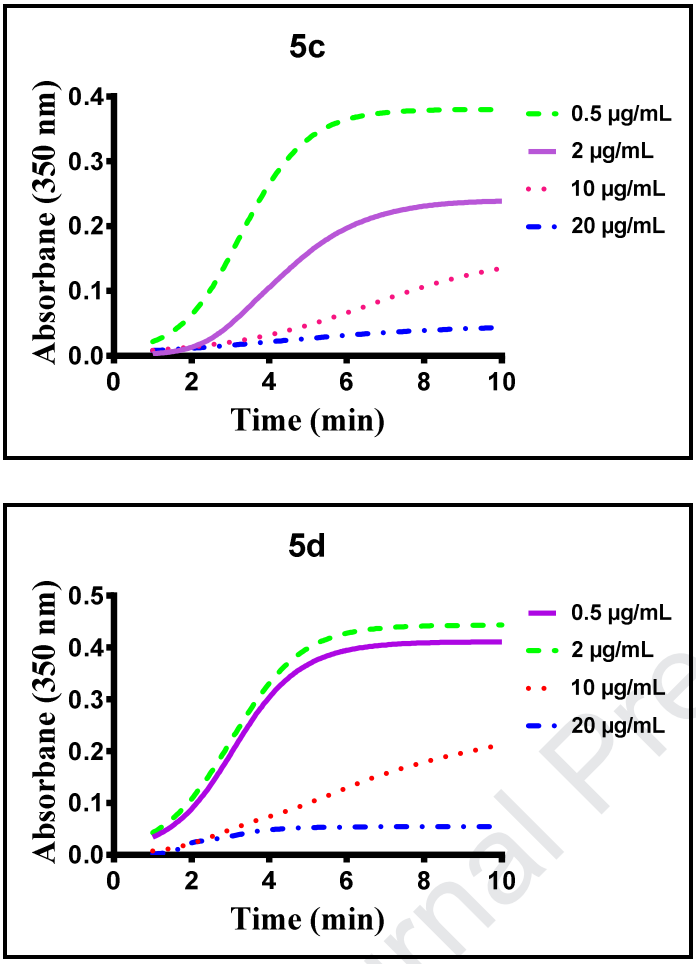


Figure 7. Tubulin polymerization inhibitory activity of compounds **5c** and **5d**.

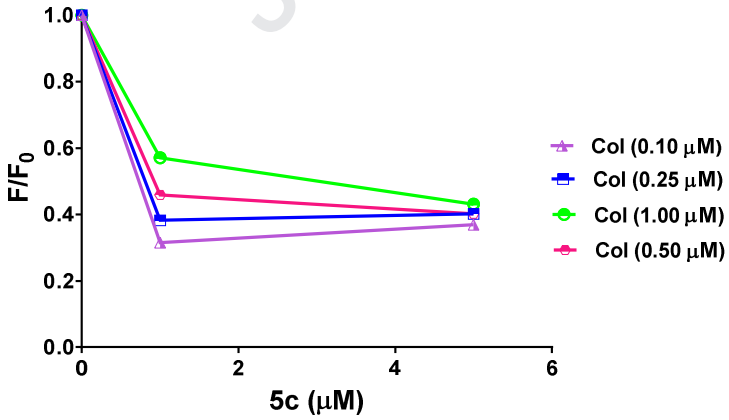


Figure 8. Inhibition of the binding of colchicine to tubulin by ligand **5c**. The concentrations of colchicine (Col) are presented in the figure.

2.2.6. Cell cycle analysis

The effect of compounds **5c** and **5d** on cell division in MCF-7 cell line was investigated by determining the distribution of the cells in different phases of the cell cycle using flow cytometric technique. The results show that compounds **5c** and **5d** significantly increased the number of cells in the sub-G1 (Fig. 9). The percentage of cells in the sub-G1 after treatment with **5c** and **5d** increased from 0.66 to 14.74 and 17.28%, respectively. As the cells were treated with **5c** and **5d**, the percentage of cell in G1 phase reached from 44.45 to 78.02 and 54.57 respectively. Therefore, a significant increase in G1 phase cell with decrease percentage of cells in G2/M phase, revealed that this compounds can arrest cell cycle in G1 and S phase.

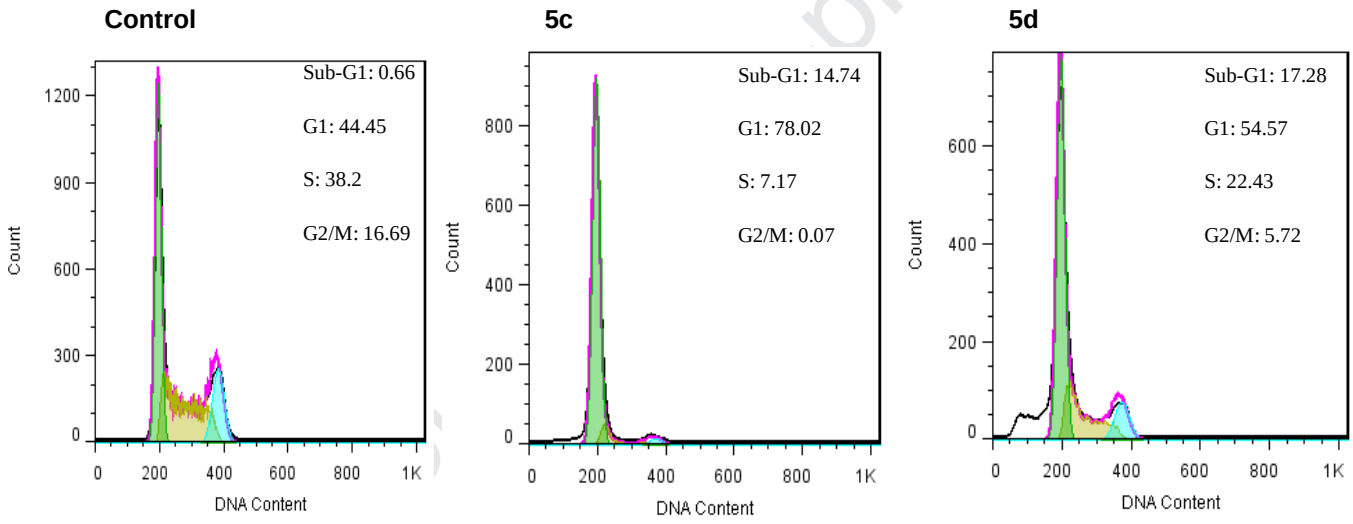


Figure 9. Effects of compounds **5c** and **5d** on cell cycle in MCF-7 cells. The cells were treated with compounds **5c** and **5d** (12µg/mL) for 48 h. The control group was untreated cells.

2.2.7. Annexin-V binding assay

Flow cytometry assay using propidium iodide (PI) and protein Annexin-V in SKOV3 cells was carried out to evaluate the potential of **5c** and **5d** to induce apoptosis in cancer cells [31]. The SKOV3 cells were treated with different concentrations (2 and 4 µg/mL) of compounds **5c** and **5d** for 48 h. Then the cells were stained with the Annexin-V-FITC apoptosis detection kit. As shown in Fig. 10, quadrant plots have been divided into four regions labeled Q1–Q4

(Q1 = necrotic cells, Q2 = late-apoptotic cells, Q3 = viable cells, and Q4 = early-apoptotic cells). The total percent of apoptotic cells are obtained by summation of Q2 and Q4. According to these plots, compounds **5c** and **5d** relatively induce apoptosis in SKOV3 cells in a concentration-dependent manner. Results demonstrated the rise in the percentage of apoptosis from 18.54 and 19.75 in 2 µg/mL to 27.41 and 21.69 in 4 µg/mL, respectively.

2.3. Molecular modeling for the tubulin-binding

Molecular docking studies were accomplished to further confirm the capability of the promising compounds **5c** and **5d** for binding to the CBS of tubulin. AutoDock software (version 4.2) was used for this purpose. The coordinates of the target co-crystallized with colchicine were extracted from the Protein Data Bank (PDB ID: 4O2B). Colchicine was firstly re-docked into the pocket of the tubulin to validate the docking protocol.

Docking poses shown in Fig. 11a and Fig. 11c indicate that the TMP scaffold in both **5c** and **5d** was interred in the hydrophobic pocket of the CBSin the β-chain and surrounded by the residues of amino acids such as βAsn249, βAsn258, βAla250, βLys254, βleu255, and βLys352, similar to that of colchicine. 2D representation of compound **5c** displayed two hydrogen bonding interactions between the methoxy group and amino acid residues βAsn258 and βAla317. Besides this, binding mode of **5c** also established a pi-sigma interaction between the thiazole thione ring and βLeu248 and two pi-alkyl interactions between 2-chlorophenyl moiety and amino acid residues βLys352 and βAla316 in addition to some pi-sulfur and sulfur-x interactions with the residues of βAla354 and βMet259, respectively. Compound **5d**, showed one hydrogen bonding interaction between the methoxy group and βAsn258. Furthermore, as can be seen in Fig. 11d, several secondary interactions like van der Waal and polar interactions with the residues of βLeu255, βVal315, βThr314, βAla317, βLys245 and βThr353 were involved. Further van der Waal interaction between βLeu248 and central thiazole thione ring was a pi-sigma interaction. The *p*-chlorophenyl group of **5d** was perfectly cinctured by several alkyl and pi-alkyl interactions with the hydrophobic residues of βAla354, βMet259, βLys352 and βAla316.

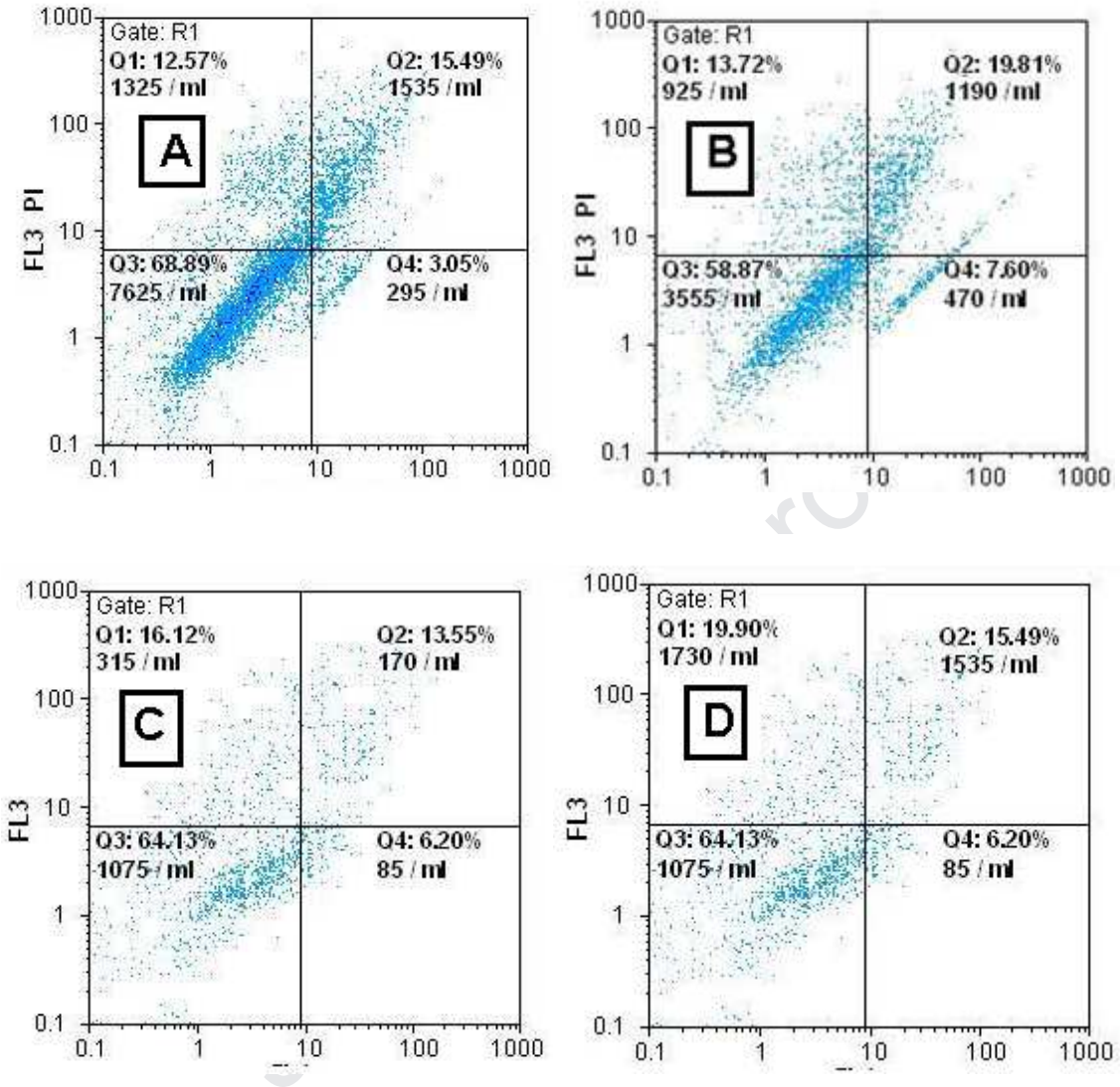


Figure 10. Flow cytometry of **5c-** or **5d**-treated SKOV3 cells at the doses of 2 and 4 µg/mL after 48 h; A: **5c** (2 µg/mL), B: **5c** (4 µg/mL), C: **5d** (2 µg/mL), D: **5d** (4 µg/mL).

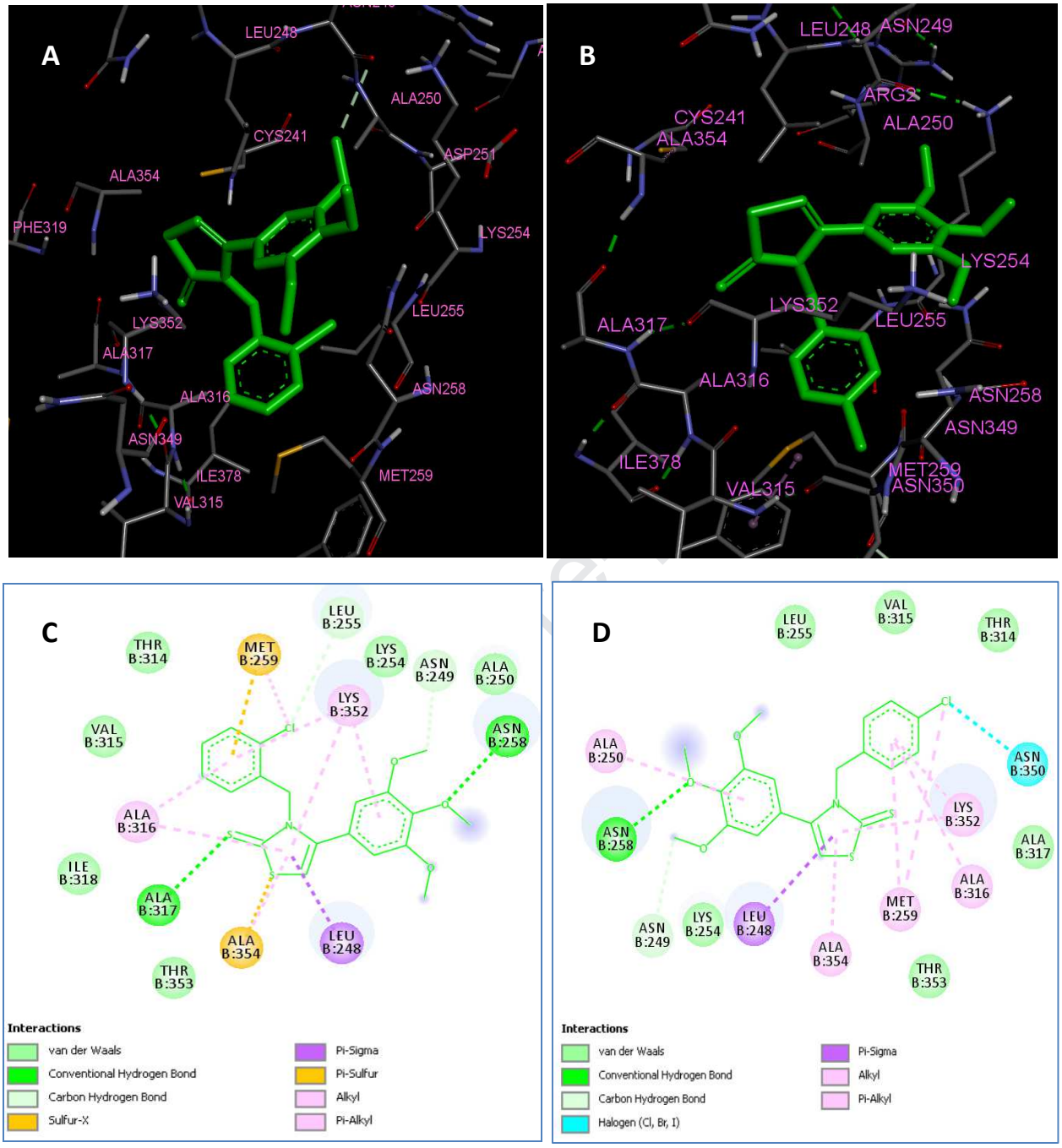


Figure 11. 3D and 2D presentations for the binding modes of compounds **5c** (A, C), and **5d** (B, D) with tubulin (PDB ID: 4O2B).

3. Conclusion

In the field of anticancer drug design and discovery, combretastatin-modified analogs have been considered as attractive target molecules to find new anti-tubulin agents. The hit molecules namely 3-(TMP)-2(3*H*)-thiazole thiones were a starting point for finding anti-tubulin agents derived from combretastatin with 2(3*H*)-thiazole thione core structure. In this work, we improved the anticancer activity of primary hits by displacing TMP moiety on the 2(3*H*)-thiazole thione to introduce 4-(TMP)-2(3*H*)-thiazole thiones as new lead compounds. The cytotoxicity assay against cancer cell lines (A549, MCF-7 and SKOV3) revealed that all compounds had remarkable cytotoxic activity (IC_{50} values <10 μg/mL). Among them, the 3-(2-chlorobenzyl)- and 3-(4-chlorobenzyl)- analogs (compounds **5c** and **5d**, respectively) with IC_{50} values of 1.14 and 2.41 μg/mL, exhibited the highest inhibitory activity against MCF-7 breast cancer cell line. The *in vitro* tubulin polymerization assay proved that compounds **5c** and **5d** could interfere with microtubule assembly with an IC_{50} <10 μg/mL. The *in vitro* colchicine-tubulin binding assay and molecular modeling study indicated that these compounds bind at the CBS of the tubulin. The representative compounds **5c** and **5d** had no significant effect on the viability of tested normal cells (L929) at the range of concentrations required for the inhibition of cancer cell proliferation, indicating the selective action and good safety profile of the designed compounds.

4. Experimental

4.1. Chemistry

All reagents and solvents were obtained from commercial suppliers and were used without further purification. The triethylammonium phenylcarbamodithioate salts **6** were prepared according to the literature method [21].

*4.1.1. Synthesis of compound **2***

To a warm and well-stirred suspension of powdered cupper (II) bromide (10 mmol, 2.23 g) in $CHCl_3$-EtOAc (20 mL, 1:1), a solution of 1-(3,4,5-trimethoxyphenyl)ethan-1-one (**1**, 10 mmol,

2.10 g) in $CHCl_3$ (10 mL) was added, and the reaction mixture was refluxed for 4 h. After completion of the reaction, the reaction mixture was filtered to remove produced cupper (I) bromide and the solution was evaporated using rotary evaporator under vacuum. Then the oily product was crystallized from hexane-ether to afford pure compound **2**. Yield: 86%; m.p.: 65-67 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.93 (s, 6H, 2×OMe), 3.94 (s, 3H, OMe), 4.41 (s, 2H, CH_2), 7.24 (s, 2H, H-2 and H-6 Ar). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 190.32, 153.25, 143.42, 128.99, 106.65, 61.11, 56.39, 30.62. EI-MS (m/z, %): 290 (M+2, 18), 288 (M^+, 17), 196 (15), 195 (100), 181 (10), 137 (8).

*4.1.2. General procedure for the preparation of compounds **4a-e***

A mixture of methylamine (40% solution in water, 2 mmol) or benzylamine derivatives (**3**, 2 mmol) and potassium acetate (2 mmol, 196.3 mg) in methanol (3 mL) was stirred in an ice bath following by addition of carbon disulfide (2 mmol, 152 mg, 0.12 mL). After 2h, compound **2** (1 mmol, 289 mg) was added portion wise during 30 min. After consumption of compound **2** (monitored by TLC), water (10 mL) was added and the mixture was kept in refrigerator overnight to complete precipitation. The solid product was filtrated, washed with water and dried to give alcoholic intermediates **4a-e**.

*4-Hydroxy-3-methyl-4-(3,4,5-trimethoxyphenyl)thiazolidine-2-thione (**4a**)*

Yield: 72%; m.p.: 110-112 °C; IR (KBr, cm^{-1}): 3352, 2833, 1656, 1595, 1505, 1437, 1332, 1129, 1066, 869, 678. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.05 (s, 3H, NMe), 3.52 (d, 1H, *J*= 12.4 Hz, H_a), 3.73 (d, 1H, *J*= 12.4 Hz, H_b), 3.89 (s, 3H, OMe), 3.90 (s, 6H, 2×OMe), 6.70 (s, 2H, H-2 and H-6). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 195.32, 153.35, 137.97, 136.99, 103.23, 100.00, 60.46, 56.36, 42.93, 32.86. EI-MS (m/z, %): 315 (M^+, 6), 297 (30), 282 (16), 242 (38), 210 (6), 195 (100), 181 (9), 167 (7), 152 (14), 137 (9), 122 (6), 104 (14), 73 (42).

*3-Benzyl-4-hydroxy-4-(3,4,5-trimethoxyphenyl)thiazolidine-2-thione (**4b**)*

Yield: 77%; m.p.: 150-152 °C; IR (KBr, cm^{-1}): 3405, 3086, 2829, 1594, 1400, 1318, 1239, 1126, 997, 841, 702, 465. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.34 (s, 1H, OH), 3.42 (d, 1H, *J*= 12.4 Hz,

H_a), 3.73 (s, 6H, 2×OMe), 3.77 (d, 1H, *J*= 12.4 Hz, H_b), 3.87 (s, 3H, OMe), 4.84 (d, 1H, *J*= 14.8 Hz, CH_2 benzyl), 4.91 (d, 1H, *J*= 14.8 Hz, CH_2 benzyl), 6.57 (s, 2H, H-2 and H-6), 7.14-7.19 (m, 2H, H-2′ and H-6′), 7.20-7.25 (m, 3H, H-3′, H-4′ and H-5′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 197.14, 152.76, 137.91, 137.51, 136.05, 128.58, 128.06, 126.99, 104.21, 100.22, 60.35, 56.11, 49.07, 42.77. EI-MS (m/z, %): 391 (M^+< 1), 373 (100), 358 (13), 340 (66), 282 (16), 267 (6), 251 (78), 236 (16), 224 (30), 209 (20), 195 (18), 181 (10), 150 (9), 121 (6), 91 (93), 65 (13).

*3-(2-Chlorobenzyl)-4-hydroxy-4-(3,4,5-trimethoxyphenyl)thiazolidine-2-thione (**4c**)*

Yield: 65%; m.p.: 162-164 °C; IR (KBr, cm^{-1}): 2965, 2926, 1593, 1509, 1461, 1443, 1343, 1126, 974, 889, 748. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.44 (d, 1H, *J*= 12.4 Hz, H_a), 3.74 (s, 3H, OMe), 3.79 (d, 1H, *J*= 12.8 Hz, H_b), 3.96 (s, 6H, 2×OMe), 4.87 (s, 2H, CH_2 benzyl), 6.61 (s, 2H, H-2 and H-6), 7.04 (d, 1H, *J*= 6.8 Hz, H-6′), 7.12-7.32 (m, 2H, H-4′ and H-5′), 7.35 (d, 1H, *J*= 6.4, H-3′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 197.80, 152.72, 137.88, 135.67, 133.82, 131.44, 129.26, 129.11, 128.68, 126.95, 104.11, 100.08, 60.30, 56.17, 46.49, 42.71. EI-MS (m/z, %): 425 (M^+< 1), 372 (4), 289 (11), 242 (14), 195 (79), 184 (21), 152 (7), 125 (100), 109 (5), 89 (19), 63 (8).

*3-(4-Chlorobenzyl)-4-hydroxy-4-(3,4,5-trimethoxyphenyl)thiazolidine-2-thione (**4d**)*

Yield: 69%; m.p.: 158-160 °C; IR (KBr, cm^{-1}): 3254, 3014, 2941, 1597, 1509, 1444, 1416, 1399, 1233, 958, 665. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.45 (d, 1H, *J*= 12.4 Hz, H_a), 3.52 (br s, 1H, OH), 3.75 (s, 6H, 2×OMe), 3.81 (d, 1H, *J*= 12.4 Hz, H_b), 3.88 (s, 3H, OMe), 4.77 (d, 1H, *J*= 14.8 Hz, CH_2 benzyl), 4.90 (d, 1H, *J*= 14.8 Hz, CH_2 benzyl), 6.57 (s, 2H, H-2 and H-6), 7.12 (d, 2H, *J*= 8.8 Hz, H-2′ and H-6′), 7.18 (d, 2H, *J*= 8.8 Hz, H-3′ and H-5′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 197.13, 152.79, 137.93, 136.52, 135.99, 131.63, 129.92, 128.01, 104.21, 99.98, 60.33, 56.08, 48.29, 42.75. EI-MS (m/z, %): 425 (M^+< 1), 242 (21), 195 (100), 183 (15), 167 (3), 152 (6), 137 (4), 125 (80), 109 (4), 89 (15), 77 (4), 63 (6).

*3-(4-Fluorobenzyl)-4-hydroxy-4-(3,4,5-trimethoxyphenyl)thiazolidine-2-thione (**4e**)*

Yield: 69%; m.p.: 146-148 °C; IR (KBr, cm^{-1}): 340, 2967, 1601, 1509, 1439, 1318, 1167, 1125, 851. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.44 (br s, 1H, OH), 3.45 (d, 1H, *J*= 12.4 Hz, H_a), 3.76 (s,

6H, 2×OMe), 3.80 (d, 1H, *J*= 12.8 Hz, H_b), 3.88 (s, 3H, OMe), 4.72 (s, 1H, CH_2 benzyl), 4.84 (d, 1H, *J*= 2.8 Hz, CH_2 benzyl), 6.59 (s, 2H, H-2 and H-6), 6.90 (t, 2H, *J*= 8.4 Hz, H-3′ and H-5′), 7.17 (dd, 2H, *J*= 8.4 and 5.6 Hz, H-2′ and H-6′). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 198.06, 162.09 (d, $J_{C,F}$= 245.4 Hz), 153.17, 138.50, 133.73, 132.88 (d, $J_{C,F}$= 3.5 Hz), 130.40 (d, $J_{C,F}$= 7.9 Hz), 115.05 (d, $J_{C,F}$= 21.3 Hz), 103.61, 100.06, 60.92, 56.09, 48.64, 44.04. EI-MS (m/z, %): 409 (M^+< 1), 391 (5), 358 (3), 292 (32), 242 (17), 210 (4), 195 (100), 183 (11), 167 (5), 152 (8), 124 (67), 109 (74), 83 (11), 66 (3).

*4.1.3. General procedure for the preparation of compounds **5a-f***

A solution of corresponding alcoholic compound **4** in methanol (3 mL) was treated with 0.5% HCl in water (10 mL). The mixture was refluxed for 2-4 h to completing the reaction (checked by TLC). Then the mixture was concentrated under reduced pressure, cooled to room temperature and kept in refrigerator (4 °C) overnight. The precipitated solid was separated by filtration, and washed with water to give pure final compounds **5a-f**.

*3-Methyl-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5a**)*

Yield: 88%; m.p.: 116-120 °C; IR (KBr, cm^{-1}): 2932, 2833, 1584, 1500, 1307, 1241, 1127, 1182, 978, 835, 670. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.61 (s, 3H, Me), 3.91 (s, 6H, 2×OMe), 3.93 (s, 3H, OMe), 6.51 (s, 1H, H-5 Thiazoline), 6.55 (s, 2H, H-2 and H-6). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 188.16, 153.62, 144.71, 139.42, 126.02, 108.23, 106.49, 60.98, 56.34, 36.28. EI-MS (m/z, %): 297 (M^+, 6), 256 (56), 224 (7), 192 (36), 160 (49), 128 (53), 96 (27), 64 (100).

*3-Benzyl-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5b**)*

Yield: 73%; m.p.: 132-134 °C; IR (KBr, cm^{-1}): 2934, 2835, 1580, 1500, 1409, 1364, 1236, 1192, 1124, 1030, 986, 831, 694. ^{1}H NMR (500 MHz, $CDCl_3$) δ: 3.58 (s, 6H, 2×OMe), 3.86 (s, 3H, OMe), 5.38 (s, 2H, CH_2 benzyl), 6.25 (s, 2H, H-2 and H-6), 6.51 (s, 1H, H-5 Thiazoline), 7.02 (d, 2H, *J*= 7.0 Hz, H-2′ and H-6′), 7.20-7.32 (m, 3H, H-3′, H-4′and H-5′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 188.25, 153.11, 144.70, 138.85, 136.29, 128.92, 127.63, 126.83, 125.94, 110.32, 107.41, 60.52, 56.15, 50.97. EI-MS (m/z, %): 373 (M^+, 100), 358 (83), 340 (38), 282 (10), 251

(49), 236 (10), 224 (19), 209 (14), 195 (5), 181 (6), 166 (4), 150 (6), 135 (3), 121 (4), 106 (3), 91 (59), 65 (8).

*3-(2-Chlorobenzyl)-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5c**)*

Yield: 63%; m.p.: 147-149 °C; IR (KBr, cm^{-1}): 3336, 2965, 1678, 156, 1504, 1442, 1329, 1242, 1123, 1117, 751. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.60 (s, 6H, 2×OMe), 3.84 (s, 3H, OMe), 5.45 (s, 2H, CH_2 benzyl), 6.27 (s, 2H, H-2 and H-6), 6.60 (s, 1H, H-5 Thiazoline), 7.04 (d, 1H, *J*= 6.8 Hz, H-6′), 7.19-7.33 (m, 2H, H-4′ and H-5′), 7.35 (dd, 1H, *J*= 7.6 and 1.2 Hz, H-3′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 188.12, 162.06, 153.13, 148.22, 144.50, 133.20, 132.71, 131.21, 130.98, 130.11, 127.95, 125.33, 122.25, 107.13, 60.46, 56.13, 49.03. EI-MS (m/z, %): 407 (M^+, 59), 392 (7), 372 (97), 356 (5), 339 (6), 312 (7), 297 (23), 282 (27), 266 (7), 251 (100), 236 (47), 208 (24), 193 (17), 178 (17), 163 (7), 142 (8), 125 (94), 107 (14), 89 (22), 63 (12).

*3-(4-Chlorobenzyl)-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5d**)*

Yield: 71%; m.p.: 138-140 °C; IR (KBr, cm^{-1}): 3096, 2931, 1584, 1496, 1338, 1239, 1187, 1126, 986, 557. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.67 (s, 6H, 2×OMe), 3.90 (s, 3H, OMe), 5.38 (s, 2H, CH_2 benzyl), 6.26 (s, 2H, H-2 and H-6), 6.53 (s, 1H, H-5 Thiazoline), 6.98 (d, 2H, *J*= 8.4 Hz, H-2′ and H-6′), 7.27 (d, 2H, *J*= 8.4 Hz, H-3′ and H-5′). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 189.11, 153.23, 144.60, 139.34, 134.33, 133.49, 128.72, 128.43, 125.47, 108.74, 106.74, 60.96, 56.00, 50.60. EI-MS (m/z, %): 407 (M^+, 100), 392 (8), 374 (31), 339 (9), 282 (16), 267 (3), 251 (61), 236 (20), 224 (22), 209 (15), 193 (2), 181 (8), 150 (7), 125 (54), 89 (10), 63 (4).

*3-(4-Fluorobenzyl)-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5e**)*

Yield: 78%; m.p.: 128-130 °C; IR (KBr, cm^{-1}): 3101, 2932, 1606, 1585, 1509, 1497, 1363, 1223, 1162, 1128, 768. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.67 (s, 6H, 2×OMe), 3.90 (s, 3H, OMe), 5.38 (s, 2H, CH_2 benzyl), 6.28 (s, 2H, H-2 and H-6), 6.53 (s, 1H, H-5 Thiazoline), 6.94-7.12 (m, 4H, F-Ph). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 188.22, 161.73 (d, $J_{C,F}$= 242.6 Hz), 153.17, 144.54, 138.93, 132.40, 129.17, 125.95, 115.67 (d, $J_{C,F}$= 21.2 Hz), 107.49, 60.59, 56.26, 50.24, 47.65. EI-MS (m/z, %): 391 (M^+, 67), 376 (5), 358 (27), 282 (10), 266 (3), 251 (44), 224 (15), 209 (11), 195 (3), 181 (6), 166 (3), 150 (5), 124 (4), 109 (100), 83 (10), 63 (3).

*3-(4-Hydroxybenzyl)-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5f**)*

Yield: 73%; m.p.: 113-115 °C; IR (KBr, cm^{-1}): 3336, 2931, 1597, 1531, 1446, 1410, 1239, 1121, 883, 668. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.67 (s, 6H, 2×OMe), 3.89 (s, 3H, OMe), 5.34 (s, 2H, CH_2 benzyl), 6.29 (s, 2H, H-2 and H-6), 6.51 (s, 1H, H-5 Thiazoline), 6.73 (d, 2H, *J*= 8.4 Hz, H-3′ and H-5′), 6.89 (d, 2H, *J*= 8.4 Hz, H-2′ and H-6′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 196.68, 156.53, 152.75, 137.90, 136.16, 129.68, 127.84, 114.78, 104.25, 102.21, 60.41, 56.10, 48.61, 42.80. EI-MS (m/z, %): 389 (M^+< 1), 256 (3), 242 (11), 210 (17), 195 (75), 181 (2), 165 (6), 152 (6), 137 (6), 122 (4), 107 (100), 91 (2), 77 (19), 64 (4), 51 (5).

*4.1.4. Synthesis of 3-(4-chlorophenyl)-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5g**)*

A suspension of triethylammonium (4-chlorophenyl)carbamodithioate (1 mmol, 305 mg) and compound **2** (1 mmol, 289 mg) in absolute ethanol (5 mL) was stirred at room temperature for 3 h. After complete consumption of the starting materials, hydroxylamine hydrochloride (3 mmol, 207 mg) was added and the reaction was continued under microwave irradiation for 30 min. Then, water (10 mL) was added and the mixture was kept in refrigerator overnight. The precipitate was separated by filtration, washed with water and recrystallized from ethanol to give pure compound **5g**. Yield: 62%; m.p.: 123-125 °C; IR (KBr, cm^{-1}): 3003, 2932, 1606, 1580, 1483, 1321, 1122, 1083, 987, 710, 466. ^{1}H NMR (250 MHz, $CDCl_3$) δ: 3.66 (s, 6H, 2×OMe), 3.82 (s, 3H, OMe), 6.12 (s, 1H, H-5 Thiazoline), 6.27 (s, 2H, H-2 and H-6), 7.07 (d, 2H, *J*= 7.2 Hz, H-2′, and H-6′), 7.40 (d, 2H, *J*= 7.2 Hz, H-3′ and H-5′). ^{13}C NMR (100 MHz, DMSO-d_6) δ: 189.58, 149.80, 148.42, 144.34, 138.36, 132.26, 131.45, 130.14, 122.71, 113.25, 111.67, 109.88, 55.83. EI-MS (m/z, %): 395 (M+2, 13), 393 (M^+, 37), 377 (31), 362 (13), 304 (40), 256 (18), 211 (8), 193 (17), 169 (17), 149 (13), 127 (31), 111 (40), 85 (66), 69 (82), 57 (100).

*4.1.5. Synthesis of 3-(3,4-dichlorophenyl)-4-(3,4,5-trimethoxyphenyl)thiazole-2(3H)-thione (**5h**)*

A suspension of triethylammonium (3,4-dichlorophenyl)carbamodithioate (1 mmol, 339 mg) and compound **2** (1 mmol, 289 mg) in absolute ethanol (5 mL) was stirred at rt for 3 h. After

complete consumption of the starting materials, the reaction mixture was refluxed for 48 h. Then, water (10 mL) was added and the mixture was kept in refrigerator overnight. The precipitate was separated by filtration, washed with water and recrystallized from ethanol to give pure compound **5h**. Yield: 67%; m.p.: 180-182 °C; IR (KBr, cm^{-1}): 2968, 2836, 1656, 1582, 1505, 1414, 1320, 1243, 1128, 1033, 992, 755. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 3.71 (s, 6H, 2×OMe), 3.84 (s, 3H, OMe), 6.29 (s, 2H, H-2 and H-6), 6.65 (s, 1H, H-5 Thiazoline), 7.07 (d, 1H, *J*= 8.4 Hz, H-6′), 7.42 (s, 1H, H-2′), 7.50 (d, 1H, *J*= 8.4 Hz, H-5′). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 193.28, 153.16, 143.22, 138.90, 134.56, 132.56, 131.01, 130.50, 129.21, 126.80, 123.65, 106.31, 61.02, 56.34. EI-MS (m/z, %): 427 (M^+< 1), 281 (8), 207 (70), 195 (57), 181 (8), 161 (17), 146 (10), 135 (29), 119 (16), 105 (83), 91 (24), 77 (100), 64 (80), 51 (55).

4.2. Biological tests

4.2.1. Cell-based assays

4.2.1.1. Drug/sample solution preparation for cell-based assays

The stock solution of compounds and standard drugs was prepared in DMSO. In the cell viability assay the stock solution was diluted to the desired concentrations in culture medium to reach the final concentrations of 100, 50, 10, 5 and 1 µg/mL. The final concentration of DMSO in culture medium was less than 1%, which had no effect on cell viability. In the hemolysis assay, an aliquot (10 µL) of stock solution was added to diluted erythrocytes (190 µL), resulting final concentrations of 1, 5 and 10 µg/mL. The selected concentrations for other tests were mentioned in each corresponding section.

4.2.1.2. Cytotoxicity assay

Three human cancer cell lines (A549, MCF-7 and SKOV3) and normal cells L929 were purchased from Pasture Institute, Tehran (Iran). The cytotoxic activities of thiazolidine-2-thiones **4a-e** and thiazole-2(3*H*)-thiones **5a-h** were evaluated in comparison with reference drug

etoposide by using MTT assay [22]. The detailed procedure can be followed in Supplementary Material.

4.2.1.3. Hemolysis assay

The *in vitro* hemolytic activity of compound **5c** and **5d** was determined against human red blood cells according to the reported method as described in Supplementary Material [32].

4.2.1.4. Cell cycle analysis

MCF-7 cells were cultured in 12-well plates with optimum density (25 $\times 10^4$ cells/well). After 24 h, cells were treated with 12µg/mL of compounds **5c** and **5d** and incubated for 48 h. After harvesting, cells were washed with cold PBS twice, suspended in 70% ethanol (for cell fixation) at least for 3 h. Afterward, 5 µL of RNase A (10 mg/mL) was added and incubated at room temperature for 1 h. Then, 10 µL of propidium iodide (PI, 50 µg/mL) was added. Finally, percentages of cells in different phases of the cell cycle were determined using a FACS Calibur flow cytometer. The cytographs were analyzed by Flowjo software.

4.2.1.5. Annexin V-FITC analysis for apoptosis

The apoptotic effect of compounds **5c** and **5d** was evaluated by Annexin-V FITC/PI assay [31]. SKOV3 cells were cultured in 6-well plates (1×10^6 cells/well) and incubated for 24 h. Then the medium was removed and a complete medium containing **5c** or **5d** at 2 and 4 µg/mL concentrations was added. After 48 h, the cells from the supernatant were harvested by trypsinization, washed with PBS at 3000 rpm and stained with 5 mL of Annexin-V/FITC (Sigma Chemical Co) in PBS at rt for 15 min and then with PI solution (Keygen Biotech, China) for another additional 10 min. Flow cytometric analyses were implemented using a FACScan (Becton Dickinson) appointed with a single 488 nm argon laser. The EXPO32 ADC analysis software was used for calculating the percentage of apoptotic cells.

4.2.1.6. Alkaline comet assay

The possible effect of promising compounds **5c** and **5d** on DNA damage in normal cells L929 was investigated by alkaline single cell gel electrophoresis method with slightly modification [24,25]. L929 Cells were seeded in 24 well plate (250000 cell/well). The cells were exposed with **5c** and **5d** at the concentration of 10 µg/mL for 1 h. Untreated cells and cell treated with etoposide (0.5 µg/mL) were considered as negative and positive controls, respectively. Afterwards, 10 µL of cell suspensions were mixed with 100 µL of low-melting-point agarose (LMA). The mixture was expanded onto microscope slides which were pre-coated with 1.0% normal-melting point agarose (NMA). After solidification of the agarose (4 °C, 10 min), slides were lysed in cold lysis buffer including 2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10% DMSO and 1% TritonX-100 (4 °C, 1 h). Then, slides were submerged in electrophoretic buffer including 1 mM Na_2-EDTA, 300 mM NaOH, pH>13 for 40 min to open DNA prior to electrophoresis. The electrophoresis was performed at 25 V and a current of 300 mA for 20 min. Then, the slides were washed with cold neutralization buffer solution three times for 15 min. Subsequently, the slides were stained with 30 µL ethidium bromide and observed with a fluorescence microscope. The most reliable factors, tail moment, tail length and tail DNA percentage were measured using Comet Score software project (CASP) to assess DNA damage.

4.2.2. Tubulin extraction and tubulin polymerization assay

Ethics approval for the use of sheep brain for tubulin extraction was granted by the Ethics Committee of the Shahid Beheshti University of Medical Sciences. Sheep fresh brains were obtained from a local slaughterhouse and rapidly transported to the laboratory on ice in 0.9% saline solution for further activities. Tubulin protein was extracted from the sheep brain by three cycles of temperature-dependent polymerization/depolymerization as described in Supplementary Material based on reported method [33]. Tubulin was re-suspended in a buffer and incubated in the presence and absence of compounds **5c** and **5d** at the final concentrations of 0.5, 2, 10 and 20 µg/mL for 15 min on ice. For induction of tubulin polymerization, GTP (final concentration 1 mM) was added at 37 °C and tubulin assembly was monitored turbidimertically

at 350 nm [26]. The concentration that inhibits tubulin polymerization by 50% (IC_{50}) was determined using area under the curve (AUC). The AUC of the untreated controls were considered as 100% polymerization. The IC_{50} value for each compound was computed using GraphPad Prism Software.

4.2.3. Fluorescence assay of colchicine-tubulin complex

The tubulin inhibitor **5c** was subjected to binding assay using fluorescence technique according to the reported method [34,35]. The purified tubulin (0.25 μM) was incubated at 37°C with the different concentrations of colchicine (0.1, 0.25, 0.5 or 1 μM) for 1 h. The tubulin-colchicine complexes were treated with compound **5c** at the final concentration of 1 and 5 μM. Fluorescence emission spectra for each experiment (n=3) were recorded at 430 nm following excitation at 360 nm using Cytation 3, multi-mode imaging and plate Reader (BioTek, USA). Fluorescent data was corrected by subtracting the background fluorescence intensity for compound **5c** at each concentration. "F_0" and "F" are the fluorescence intensity (a.u) of the [tubulin+colchicine] and [tubulin+colchicine+ligand **5c**] mixtures, respectively.

4.3. Molecular docking studies

The binding mode of promising compounds **5c** and **5d** to the CBS of tubulin was investigated using AutoDock software (version 4.2). The 3D structure of compounds was constructed and optimized by ChemBioDraw Ultra16.0 and ChemBio3D Ultra 16.0 softwares. The β-subunit of the target (chain D) was chosen and the rest was deleted. The final receptor pdbqt file was prepared following AutoDock protocol [36]. A grid map with 40, 40, and 40 grid points (X, Y, Z dimensions) and Centroid: X = 17.0192, Y = 65.9939, Z =43.3901 was selected. The pose with the best scoring was selected and visualized for analyzing the involved interactions. The 3D, 2D and pose of studied compounds **5c** and **5d** were taken using discovery studio 2016.

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Research Highlights

- Some new thiazole-2(3*H*)-thiones containing 4-(3,4,5-trimethoxyphenyl) were synthesized.
- Most compounds had potent antiproliferative activity (IC_{50}s <10 µg/mL).
- The best results were obtained by 3-(chlorobenzyl) analogs **5c** and **5d** on MCF-7 cells.
- These compounds remarkably inhibit tubulin polymerization and induce cell death by apoptosis.
- **5c** and **5d** had acceptable safety profile as tested in normal cells and red blood cells.
- Results also confirmed the binding of **5c** to the colchicine binding site of tubulin.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.