Original article

Synthesis and biological evaluation of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones as combretastatin analogs

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A B S T R A C T

A series of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones 5 were designed as new heterocyclic analogs of combretastatin A-4 (CA-4). Indeed, the olefinic core structure of CA-4 has been replaced by 2(3H)-thiazole thione. The general synthetic strategy to prepare compounds 5 was based on the cyclocondensation reaction between triethylammonium N-(trimethoxyphenyl)dithiocarbamate and appropriate phenacyl halide. The cytotoxic activity evaluation of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones 5 against human cancer cell lines T47D, MCF-7 and MDA-MB-231 demonstrated that 4-methyl analog 5f showed the highest activity against all cell lines. Compound 5f had no significant toxicity towards non-tumoral cells MRC-5 and its cytotoxicity was apparently selective for cancer cells. The results of bioassays showed that the representative compound 5f depolymerized tubulin, inhibited cell proliferation, and induced apoptosis in cancer cells.

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1. Introduction

Currently, cancer is one of the most serious problem threats human health in the world. Chemotherapy has still been an important fundament for cancer treatment. Drugs that perturb microtubule/tubulin dynamics are used widely in cancer chemotherapy. Several binding sites including taxane, vinca alkaloids and colchicine binding sites have been identified on tubulin. Anti-mitotic agents with the capability of binding at the colchicine site of tubulin have received much attention and some of them such as combretastatin A-4 (CA-4) and its water-soluble prodrug CA-4P are undergoing clinical trials as antitumor drugs [2,3].

CA-4 is the most biologically significant member of cis-stilbenes isolated from the bark of African willow tree Combretum caffrum [4]. This compound was found to be a potent antiproliferative agent against a broad spectrum of cancer cell lines including multi drug-resistant cells [5,6]. Structure–activity relationship studies on CA-4 derivatives have established that pharmacophore structure for binding to tubulin is the cis-orientation of the two ethenyl-bridged aromatic rings which one of them bearing 3,4,5-trimethoxy substituents [7–9]. The cis-stilbene structure of CA-4 is unstable and during storage and administration, the cis-configuration is isomerized to trans-form results in decreasing of both antitubulin activity and cytotoxicity [10,11]. Moreover, the double bond reduction of CA-4 leads compound with moderately reduced activity [8,9,12]. Accordingly, considerable efforts have been focused on the cis-restriction of pharmacophoric backbone (Fig. 1), particularly by the replacement of the double bond with heterocyclic rings [13,14]. For example, a number of five-membered heterocyclic analogs of CA-4 such as imidazoles, pyrazoles, thiazoles, triazoles, oxazolones and furanones, have been reported as cis-restricted biologically active congeners of CA-4 [13–15].
With the aim of developing new heterocyclic analogs of CA-4, we designed a series of 3-(trimethoxyphenyl)-2(3H)-thiazole thione derivatives in which the olefinic core structure of CA-4 has been replaced by 2(3H)-thiazole thione (Fig. 1). Thus, we report here the convenient synthesis and biological activity of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones and their related compounds.

2. Chemistry

The synthesis of target compounds 5a–j was carried out according to Scheme 1. Firstly, 3,4,5-trimethoxyaniline (1a) was treated with carbon disulfide in triethylamine, at room temperature to obtain N-(trimethoxyphenyl) dithiocarbamate salt 2. Compound 2 was reacted with appropriate phenacyle halide to produce a gummy adduct, which was dehydrated by refluxing in 0.5% HCl to give 4-aryl-3-(3,4,5-trimethoxyphenyl)-2(3H)-thiazole thione 5. Structurally, the intermediate adduct would be either acyclic S-phenacyle dithiocarbamate 3 or cyclic 4-hydroxythiazolidine-2-thione 4. In the case of the reaction of dithiocarbamate 2 and 4-methoxyphenacyle bromide, the intermediate was isolated and characterized as acyclic adduct 3a. Moreover, the reaction 4-bromophenacyle bromide or 2,4-dichlorophenacyle chloride with the dithiocarbamate salt 2 resulted in corresponding cyclic adducts which were unambiguously purified and characterized as 4-hydroxythiazolidine-2-thione 4a.b. It should be noted that compounds 4a and 4b which have a chiral center at the C-4 of their thiazoline-2-thione scaffold are racemates. Due to the problem of isolation and purification the rather labile intermediates 3 and 4, the rest of final compounds 5 were obtained from refluxing of crude mixture of 3 and 4 in 0.5% hydrochloric acid.

For preparation of 3-(4-methoxyphenyl) and 3-(3,4-dimethoxyphenyl) analogs of 5 (compounds 6a,b), we have attempted to synthesize corresponding dithiocarbamates by following the general procedure which was used for compound 2. Due to the low solubility of 4-methoxyaniline and 3,4-dimethoxyaniline in triethylamine, these attempts were failed. Luckily, compounds 6a,b were prepared by using an one-pot sequential reaction (Scheme 2). Thus, treatment of 4-methoxyaniline or 3,4-dimethoxyaniline with carbon disulfide in DMF in the presence of K2PO4 afforded corresponding dithiocarbamate which was subsequently reacted with phenacyle bromide. The crude product was refluxed in 0.5% HCl to give pure compound 6.

As described above, the intermediate compounds 3a, 4a and 4b were isolated and unambiguously characterized by spectral data. The IR spectrum of compound 3a showed a strong band at 1681 cm⁻¹ due to the carbonyl group. The NH absorption of compound 3a was appeared at 3313 cm⁻¹. In the ¹H NMR of compound 3a, a signal was observed at 3.79 ppm that belongs to the protons of CH₂. The chemical shift value of aromatic H-2 and H-6 signal (8.05 ppm) was in accord to the protons ortho to the carbonyl group. The ¹³C NMR data showed that the resonance related to the carbonyl carbon of compound 3a was occurred at 197.96 ppm. Cyclization of compound 3a to 5j resulted in disappearance of carbonyl signal from this region of ¹³C NMR spectrum. These data confirmed the acyclic form of intermediate 3a.

In the ¹H NMR spectra of compound 4a, two characteristic doublets at 3.62 and 3.80 ppm with geminal coupling constants ~12.3 Hz indicated the diastereotopic nature of C-5 methylene protons because of neighboring with C-4 stereocenter in thiazolidine ring.

Furthermore, the IR, ¹H NMR, ¹³C NMR and MS spectral data were used for structural characterization of target compounds 5. Representatively, the ¹H NMR spectrum of compound 5b showed six protons as a singlet at 3.56 ppm, which was related to the 3- and 5-methoxy groups of trimethoxyphenyl moiety. The protons of 4-methoxy group were appeared at 3.69 ppm as a singlet peak. A singlet at 6.57 ppm attributed to the H-5 of 2(3H)-thiazole thione. The H-2 and H-6 aromatic protons of trimethoxyphenyl moiety were appeared at 6.26 ppm upfield respect to the other aromatic protons. The protons of 4-bromophenyl displayed two doublets at 6.86 and 7.27 ppm bearing coupling constant of 8.35 Hz. In the decoupled ¹³C NMR of compound 5b, 13 signals was detected which confirmed correct carbon skeleton of the compound. The mass spectral data of compound 5b provided further evidence of its correct structure. The molecular ion peak was observed at 437 and 439 as expected for m/z values of M⁺ and [M + 2].

3. Biology

3.1. Cytotoxicity assay

The in vitro cytotoxic activity of synthesized compounds against three human cancer cell lines, including MCF-7, MDA-MB-231 and T47D was determined by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay [16]. MTT assay is based on reduction of the tetrazolium salt to blue colored formazan by mitochondrial dehydrogenases in viable cells. The relative cell viability was expressed as the mean percentage of viable cells comparing to control cells, and the inhibition percentages of compounds were assessed by the following formula: 

\[ \frac{[\text{Abs control cells} - \text{Abs treated cells}]}{\text{Abs control cells}} \times 100 \]

The IC₅₀ values were calculated from the concentration–response curves by regression analysis. The IC₅₀ values of the compounds against different cancer cell lines are shown in Table 1.

Fig. 1. (A) Structure of combretastatin A-4 (CA-4) as unstable cis-stilbenes undergoing to trans-isomerism; (B) cyclic combretastatin analogs locked as cis-oriented stilbenoids; (C) 3-(trimethoxyphenyl)-2(3H)-thiazole thiones as new heterocyclic analogs of CA-4.
3.2. Tubulin polymerization assay

To further characterize the effects of selected compound 5f on microtubule polymerization, we examined microtubules assembly incubated with different concentration of compound 5f using UV–Visible spectrometer. The absorbances (λ 350 nm) were recorded for a period of 30 min and the results were compared to the DMSO-treated control group to evaluate the relative degree of change in optical density. The percent of inhibition was calculated as follow: % inhibition = (1 – A350 treated control group/A350 control) × 100.

3.3. Acridine orange/ethidium bromide staining test

The potential of compounds 4b and 5f to induce apoptosis in T47D and MCF-7 cells was determined morphologically by acridine orange/ethidium bromide double staining test. Using fluorescence microscopy, cells can be distinguished as live cells (uniformly stained green) and apoptotic cells that are stained orange because of cell membrane destruction and the intercalation of ethidium bromide between the nucleotide bases of DNA [17].

3.4. Flow cytometry analyses of the apoptotic cells with Annexin V-PE and 7-aminoactinomycin D (7-AAD) double staining

The MCF-7 cells were treated with IC50 concentrations of the selected compounds 4b, 5f and etoposide as reference drug. Then, the apoptosis induction was evaluated by Annexin-V binding and 7-AAD uptake test. Annexin V-PE was used to quantitatively determine the percentage of cells within a population that are undergoing apoptosis [18]. Using this technique, cells that are viable are Annexin V-PE and 7-AAD negative; cells that are in early apoptosis are Annexin V-PE positive and 7-AAD negative; and cells that are in late apoptosis or necrosis are both Annexin V-PE and 7-AAD positive. The double stained cells were analyzed by flow cytometry.

4. Results and discussion

The IC50 values of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones 5a–j in comparison with etoposide as standard drug are presented in Table 1. Among the compounds 5a–j, 4-methyl analog 5f showed the highest activity against all cell lines with IC50 values 11.8–19.7 μg/mL. Moreover, compounds 5c, 5e and 5h exhibited significant growth inhibitory activity against all tested cell lines (IC50 values < 50 μg/mL).

The comparison of IC50 values of 5f and 5h with those of unsubstituted analog 5a revealed that the introduction of methyl or hydroxyl group on 4-phenyl ring increases the cytotoxic activity. The compounds 5b–e containing halogen substituent showed lower activities against T47D cells compared to unsubstituted counterpart 5a. Thus, halogen substitution could not improve the inhibitory activity against T47D cell line. While, the introduction of 4-fluoro- or 2,4-dichloro- substituents improved the cytotoxic activity toward MCF-7 and MDA-MB-231 (compounds 5c and 5e vs. 5a). The O-methylation of 4-hydroxy derivative (compound 5j compared to 5h) reduced the cytotoxic potential against all cell lines. The observed activity with 4-hydroxy derivative 5h and 3,4-dihydroxy analog 5i demonstrated that the insertion of second hydroxy group could not improve the cytotoxic activity.

To investigate the impact of 3,4,5-trimethoxyphenyl functionality on cytotoxic activity, we synthesized and evaluated the mono- methoxy and dimethoxy congeners of compound 5a (compounds 6a and 6b, respectively). Interestingly, these compounds showed comparable or superior activity respect to the corresponding 3,4,5-trimethoxyphenyl derivative 5a. These findings revealed that the intrinsic cytotoxic activity of compounds significantly depends on 3,4-diaryl-2(3H)-thiazole thione backbone.
<table>
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<tr>
<th>Compound</th>
<th>Structure</th>
<th>T47D</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
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<td>3a</td>
<td><img src="image" alt="Structure 3a" /></td>
<td>29.9 ± 5.2</td>
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<td><img src="image" alt="Structure 4a" /></td>
<td>16.3 ± 3.3</td>
<td>16.5 ± 1.0</td>
<td>15.1 ± 2.7</td>
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<td>4b</td>
<td><img src="image" alt="Structure 4b" /></td>
<td>16.9 ± 4.0</td>
<td>9.6 ± 2.5</td>
<td>14.5 ± 2.1</td>
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<tr>
<td>5a</td>
<td><img src="image" alt="Structure 5a" /></td>
<td>31.6 ± 3.8</td>
<td>&gt;100</td>
<td>52.0 ± 14.1</td>
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<tr>
<td>5b</td>
<td><img src="image" alt="Structure 5b" /></td>
<td>&gt;100</td>
<td>&gt;100</td>
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<td><img src="image" alt="Structure 5c" /></td>
<td>38.9 ± 6.7</td>
<td>38.5 ± 2.1</td>
<td>19.5 ± 1.9</td>
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<td>43.4 ± 8.8</td>
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(continued on next page)
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<th>Compound</th>
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<td><img src="image" alt="Structure" /></td>
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<td>5i</td>
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<td>&gt;100</td>
<td>53.8 ± 8.4</td>
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<td>36.8 ± 5.6</td>
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<td>6b</td>
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<td>33.1 ± 8.2</td>
<td>17.6 ± 1.5</td>
<td>19.1 ± 1.4</td>
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<tr>
<td>Etoposide</td>
<td><img src="image" alt="Structure" /></td>
<td>8.2 ± 1.2</td>
<td>0.4 ± 3.2</td>
<td>7.2 ± 1.6</td>
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</tbody>
</table>
Furthermore, the cytotoxic activity of acyclic and alcoholic inter-
mediates (compounds 3a and 4a,b, respectively) were also 
evaluated against test cell lines (Table 1). Surprisingly, by 
comparing the IC50 values of acyclic analog 3a with those of h
terocyclic congener 5j, it was demonstrated that acyclic form had 
more cytotoxicity than compound 5j. It was worthy of note that the 
alkaloid derivatives 4a and 4b showed more potent activity 
respect to the corresponding dehydrated analogs 5b and 5e, 
respectively. Alcoholic compound 4b was as potent as standard 
drug etoposide against MCF-7 cells. Its activity against the latter cell 
line was superior than all tested compounds.

The cytotoxic activity of promising compounds 4b and 5f were 
also evaluated on non-tumoral cell line MRC-5. Results of MTT 
assay showed that compounds 4b and 5f have less cytotoxic activity 
against non-tumoral cells. The IC50 values of compounds 4b and 5f 
on MRC-5 cells were 47.3 ± 4.2 µg/mL and >100 µg/mL, respect-
ively. According to the results, the cytotoxicity of these compounds 
was apparently selective for cancer cell lines T47D, MCF-7 and 
MDA-MB-231. Particularly, compound 5f had no significant toxicity 
towards non-tumoral cell line MRC-5 (Selectivity index > 5).

To verify whether the cytotoxic activity of the designed com-
pounds was correlated to tubulin inhibition, the inhibitory effect of 
the most active compound 5f on the polymerization of purified 
tubulin was evaluated. The effect of compound 5f on the microtu-
bules polymerization was shown in Fig. 2. The results showed that 
compound 5f evidently inhibited the assembly of purified sheep 
brain MTP in a concentration dependent manner (Fig. 2).

It is well documented that exposure to microtubule-targeting 
agents leads to malformed mitotic spindles, mitotic arrest, and 
apoptosis. Thus, we next examined the effects of selected com-
pounds 4b and 5f on the apoptosis of T47D and MCF-7 cells by 
acridine orange/ethidium bromide double staining technique. T47D 
and MCF-7 cells were treated with and without IC50 concentration 
of compounds 4b and 5f for 24 h and stained with a mixture of 
adrenaline orange and ethidium bromide. Analysis of the acridine 
orange/ethidium bromide staining results revealed that the test 
compounds 4b and 5f induced apoptosis in T47D and MCF-7 cell 
lines. As shown in Fig. 3, the non-apoptotic control cells were 
stained green and the apoptotic cells had orange particles in their 
nuclei due to nuclear DNA fragmentation. The appearance of 
chromatin condensation and nuclear fragmentation are evident in 
this figure.

To further confirm of apoptosis induction by compounds 4b and 
5f, the treated MCF-7 cells were subjected to Annexin V/7-AAD 
double staining followed by flow cytometry analyses (Figs. 4 and 
5). Annexin V/7-AAD flow cytometric analyses revealed that cells 
undergo apoptosis after treatment with IC50 concentrations of 
compounds 4b and 5f. As shown in Fig. 5, compounds 4b and 5f 
induced 51.67% and 66.47% apoptosis in the cancer cells, respec-
tively. Therefore, it is evident that these compounds have strong 
anti-proliferative activity.

5. Conclusion

In summary, we designed a series of 3-(trimethoxyphenyl)- 
2-(3H)-thiazole thiones 5 as new heterocyclic analogs of CA-4, in 
which the olefinic core structure of CA-4 replaced by 2-(3H)-thiazole 
thione. The general synthetic strategy employed to prepare the 3-
(trimethoxyphenyl)-2(3H)-thiazole thiones 5 was based on the 
cyclocondensation reaction between N-(trimethoxyphenyl)dithio-
carbamate and appropriate phenacyl halide. In some cases, the 
intermediate adducts including acyclic S-phenacyl dithiocarbamate 
3 or cyclic 4-hydroxythiazolidine-2-thione 4 were purified and 
characterized as individual compounds. The cytotoxic activity 
evaluation of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones 5 
against human cancer cell lines, including T47D, MCF-7 and 
MDA-MB-231 demonstrated that 4-methyl analog 5f showed the 
highest activity against all cell lines. The cytotoxicity of compound 
5f was apparently selective for tested cancer cell lines and com-
pound 5f had no significant toxicity towards non-tumoral cell line 
MRC-5. Besides 2(3H)-thiazole thiones 5, 4-hydroxy-3-(3,4,5-
trimethoxyphenyl)-4-(2,4-dichlorophenyl)thiazolidine-2-thione 
(4b) which was separated and characterized as a stable interme-
diate showed better profile of antiproliferative activity against 
tested cell lines. The representative compound 5f depolymerized 
tubulin, inhibited cell proliferation, and induced apoptosis in can-
cer cells. These compounds can be considered as more stable an-
logs of combretastatin A-4 for further biological evaluations.

6. Experimental protocols

6.1. Chemistry

6.1.1. General methods

Triethylammonium N-(3,4,5-trimethoxyphenyl)dithiocarba-
mate was prepared according to the literature method [19]. 
Required phenacyl halides including 2-bromo-4’-hydroxy-
acetophenone and 2-bromo-4’-chloroacetophenone were syn-
thesizied by bromination of corresponding acetophenones using 
CuBr2 in CHCl3–EtOAc [20]. Other phenacyl halides were
commercially available materials from Merck or Fluka companies. The progress of reactions was checked by thin-layer chromatography (TLC) using silica gel 60 F254 plastic sheets (Merck). The ultra-violet light (254 nm) was used for TLC visualization. Yields are based on isolated product and were not optimized. Melting points were determined in open glass capillaries using Bibby Stuart Scientific SMP3 apparatus (Stuart Scientific, Stone, UK) and are uncorrected. The IR spectra were obtained on a PerkinElmer FT-IR spectrophotometer using KBr disks. The NMR spectra were recorded using a Bruker 500 spectrometer and chemical shifts are expressed as δ (ppm) with tetramethylsilane (TMS) as internal standard. The mass spectra were obtained using a HP 5937 Mass Selective Detector (Agilent technologies).

6.1.2. General procedure for the synthesis of intermediates S-(Trimethoxyphenyl) dithiocarbamate 3 or 4-hydroxythiazolidine-2-thione 4

N-(Trimethoxyphenyl) dithiocarbamate salt 2 (1.5 mmol) was dissolved in acetone (10 mL) and the solution was stirred in an ice bath. An appropriate phenacyl halide (1.5 mmol) was added portionwise over a 30 min period. After completion of the reaction (consumption of phenacyl halide, 0.5–2 h), the minimum volume of water to cause solution was added and the cold solution was stirred for 15 min longer. After evaporation of acetone under reduced pressure, a viscous oily residue was separated. Water (5 mL) was added to the oily residue and the aqueous phase was decanted. The crude product was crystallized from methanol or diethyl ether.

6.1.3. General procedure for the synthesis of 3-(trimethoxyphenyl)-2(3H)-thiazole thiones 5

N-(Trimethoxyphenyl) dithiocarbamate salt 2 (1.5 mmol) was dissolved in acetone (10 mL) and the solution was stirred in an ice bath. An appropriate phenacyl halide (1.5 mmol) was added portionwise over a 30 min period. After completion of the reaction (consumption of phenacyl halide, 0.5–2 h), the minimum volume of water to cause solution was added and the cold solution was stirred for 15 min longer. After evaporation of acetone under reduced pressure, a viscous oily residue was separated. Water (5 mL) was added to the oily residue and the aqueous phase was decanted. The oily crude product was mixed with 0.5% HCl (20 mL) and methanol (4 mL), and the mixture was refluxed for 45 min. After cooling up to room temperature, the reaction mixture was left in refrigerator overnight. The precipitated solid was collected by filtration and washed with water. The precipitated solid was collected by filtration and washed with water. The obtained crude product was mixed with 0.5% HCl (20 mL) and methanol (4 mL), and the mixture was refluxed for 45 min. After cooling up to room temperature, the reaction mixture was left in refrigerator overnight. The precipitated solid was collected by filtration and washed with water. The product was recrystallized from methanol or diethyl ether to give compound 5.

6.1.4. General procedure for the synthesis of 2-(4-methoxyphenyl)-and 3-(3,4,5-trimethoxyphenyl)-2(3H)-thiazole thiones 6

A mixture of 4-methoxyaniline or 3,4-dimethoxyaniline (1 mmol) and K2PO4 (215 mg, 1 mmol) in DMF (7.5 mL) was cooled in ice bath, and then CS2 (380 mg, 5 mmol) was added. After stirring for 20 min, 2-bromoacetophenone (199 mg, 1 mmol) was added and the mixture was allowed to warm to room temperature gradually. Water (20 mL) was added to the mixture and left in refrigerator overnight. The precipitated solid was collected by filtration and washed with water. The obtained crude product was mixed with 0.5% HCl (20 mL) and methanol (4 mL), and the mixture was refluxed for 45 min. After cooling up to room temperature, the reaction mixture was left in refrigerator overnight. The precipitated solid was collected by filtration and washed with water. The product was recrystallized from methanol to give compound 6.

6.1.5. Physicochemical and spectral data of synthesized compounds

6.1.5.1. 4-Methoxyphenyl N-(3,4,5-trimethoxyphenyl)dithiocarba- 
mate (3a). Yield 60%; mp 142–143 °C; IR (KBr, cm⁻¹) 3313, 3076, 2938, 2838, 1681, 1599, 1508, 1454, 1312, 1261, 1177, 1015, 987, 835. 1H NMR (500 MHz, CDCl3) δ: 3.79 (s, 2H, CH2), 3.85 (s, 6H, 3-OCH3 and 5-OCH3), 3.87 (s, 3H, 4-CONH), 4.75 (br s, 1H, NH), 6.35 (s, 2H, H-2 and H-6), 7.00 (d, 2H, J = 8.2 Hz, H-3’ and H-5’), 8.05 (d, 2H, J = 8.8 Hz, H-2’ and H-6’). 13C NMR (125 MHz, CDCl3) δ: 56.02, 56.49, 56.68, 61.36, 101.19, 107.19, 114.06, 114.51, 127.83, 131.59, 153.29, 153.71, 160.45, 164.74, 197.96. MS (m/z, %): 406 (M⁺, 5), 389 (100), 343 (20), 288 (16), 164 (18), 135 (43). Anal. Calcd for C23H21NO5S2: C, 56.02; H, 5.08; N, 3.44. Found: C, 56.23; H, 5.08; N, 3.50.

6.1.5.2. (±)-4-Hydroxy-3-(3,4,5-trimethoxyphenyl)-4-(4-bromophenyl)thiazolidine-2-thione (4a). Yield 92%; mp 134–135 °C; IR (KBr, cm⁻¹) 3265, 2933, 1593, 1504, 1403, 1198, 1072, 990, 833, 820, 781, 631, 567. 1H NMR (500 MHz, CDCl3) δ: 3.57 (s, 6H, 3-OCH3 and 5-OCH3), 3.62 (d, 1H, J = 12.25 Hz, H-5a Thia-
zolidine), 3.68 (s, 3H, 4-CONH), 3.80 (d, 1H, J = 12.28 Hz, H-5b Thiazolidine), 6.23 (s, 2H, H-2 and H-6), 7.33 (s, 4H, BrPh), 7.53 (s,
Fig. 4. Histogram of flow cytometric analysis of MCF-7 cells treated with compounds 4b and 5f. Cells were stained with Annexin V/7-AAD and quantitated by flow cytometry. MCF-7 cells were treated with DMSO 1% (negative control) or with IC50 concentrations of etoposide (positive control) and compounds 4b and 5f. Fluorescence intensity of Annexin V-PE was shifted to the right.
6.1.5.4. 3-(3,4,5-Trimethoxyphenyl)-4-(4-bromophenyl)thiazole-2(3H)-thione (4b). Yield 47%; mp 151–152 °C; IR (KBr, cm⁻¹) %: 3408 (42), 395 (35), 243 (38), 194 (64), 183 (100), 155 (34), 89 (35), 76 (26), 50 (37). Anal. Calcd for C₁₈H₁₇BrNO₄S₂: C, 47.37; H, 3.98; N, 3.77. 1H NMR (500 MHz, CDCl₃) δ: 44.99, 56.37, 56.53, 61.04, 100.77, 107.32, 123.16, 128.38, 130.44, 131.58, 132.37, 133.85, 137.89, 140.51, 153.00, 197.77. MS (m/z, %): 455 (M⁺, 5), 423 (45), 408 (42), 395 (35), 243 (38), 194 (64), 183 (100), 155 (34), 89 (35), 76 (26), 50 (37). Anal. Calcd for C₁₈H₁₇BrNO₄S₂: C, 47.33; H, 4.22; N, 3.05.

6.1.5.5. 3-(3,4,5-Trimethoxyphenyl)-4-(4-chlorophenyl)thiazole-2(3H)-thione (5a). Yield 66%; mp 184–185 °C; IR (KBr, cm⁻¹) %: 3954, 2827, 1600, 1505, 1450, 1228, 1126. ¹H NMR (500 MHz, CDCl₃) δ: 3.71 (s, 6H, 3-OCH₃ and 5-OCH₃), 3.85 (s, 3H, 4-OCH₃), 4.42 (s, 2H, H-2 and H-6), 6.64 (s, 1H, Thiazole-H), 7.11 (d, 2H, J = 7.0 Hz, H-2' and H-6'), 7.25 (t, 2H, J = 7.5 Hz, H-3' and H-5'), 7.31 (t, 1H, J = 7.5 Hz, H-4'). MS (m/z, %): 359 (M⁺, 100), 344 (43), 312 (18), 258 (18), 135 (35), 105 (31), 77 (37), 43 (20). Anal. Calcd for C₁₈H₁₇Cl₂NO₄S₂: C, 60.14; H, 4.77; N, 3.07. Found: C, 47.34; H, 3.90; N, 3.77.

6.1.5.6. 3-(3,4,5-Trimethoxyphenyl)-4-(4-fluorophenyl)thiazole-2(3H)-thione (5c). Yield 47%; mp 190–191 °C; IR (KBr, cm⁻¹) %: 3060, 2932, 1601, 1505, 1459, 1415, 1271, 1226, 1136, 1004, 852, 729. ¹H NMR (500 MHz, CDCl₃) δ: 3.73 (s, 6H, 3-OCH₃ and 5-OCH₃), 3.87 (s, 3H, 4-OCH₃), 4.42 (s, 2H, H-2 and H-6), 6.64 (s, 1H, Thiazole-H), 6.95–6.99 (m, 2H, H-3' and H-5'), 7.09–7.14 (m, 2H, H-2' and H-6'). ¹C NMR (125 MHz, CDCl₃/DMSO-d₆) δ: 56.73, 61.39, 106.80, 109.44, 116.14 (d, JCF = 21.75 Hz), 127.29, 130.96 (d, JCF = 8.5 Hz), 133.12, 138.81, 144.31, 153.88, 163.32 (d, JCF = 249.38 Hz), 190.32. Anal. Calcd for C₁₈H₁₇FNO₄S₂: C, 57.28; H, 4.42; N, 3.71. Found: C, 57.25; H, 4.32; N, 3.70.

6.1.5.7. 3-(3,4,5-Trimethoxyphenyl)-4-(4-chlorophenyl)thiazole-2(3H)-thione (5d). Yield 70%; mp 214–215 °C; IR (KBr, cm⁻¹) %: 2928, 1597, 1505, 1274, 1262, 1231, 1133, 1092, 1050, 821. ¹H NMR (500 MHz, CDCl₃) δ: 3.76 (s, 6H, 3-OCH₃ and 5-OCH₃), 3.90 (s, 3H, 4-OCH₃), 6.43 (s, 2H, H-2 and H-6), 6.66 (s, 1H, Thiazole-H), 7.08 (d, 2H, J = 8.22 Hz, H-3' and H-5'), 7.27 (d, 2H, J = 8.03 Hz, H-2' and H-6'). MS (m/z, %): 395 (M⁺ + 2, 45), 393 (M⁺, 100), 378 (40), 346 (14), 329 (12).
1504, 1417, 1231, 1127, 1004, 753.
H NMR (500 MHz, CDCl3) δ: 3.74 (s, 3H, CH3), 3.79 (s, 3H, OCH3), 3.85 (s, 3H, OCH3)
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1599, 1505, 1463, 1417, 1275, 1249, 1052, 833.
H NMR (500 MHz, CDCl3) δ: 3.74 (s, 3H, OCH3), 3.79 (s, 3H, OCH3)
3.88 (s, 3H, 4-OCH3), 6.50 (dd, 1H, J = 8.8 Hz, H-2 and H-6), 6.67 (s, 1H, Thiazole-H), 7.11 (d, 1H, J = 8.24 Hz, H-6), 7.19 (dd, 1H, J = 8.24 and 1.92 Hz, H-5), 7.38 (3H, 1H, J = 1.9 Hz, H-3).
13C NMR (125 MHz, CDCl3) δ: 36.60, 61.23, 106.16, 116.12, 116.78, 125.74, 130.12, 133.29, 135.72, 137.24, 138.79, 140.57, 153.7.
MS (m/z, %): 247 (M+, 8), 412 (29), 382 (12), 326 (13), 167 (18), 149 (31), 137 (30), 124 (44), 109 (54), 97 (85), 83 (86), 69 (93), 57 (100), 43 (85). Anal. Calcd for C18H17NO5S2: C, 60.17; H, 4.19; N, 3.58. Found: C, 59.98; H, 4.32; N, 3.42.
H NMR (500 MHz, CDCl3) δ: 3.85 (s, 3H, OCH3), 6.66 (s, 1H, Thiazole-H), 6.93 (d, 2H, J = 8.89 Hz, H-3 and H-5), 7.12–7.15 (m, 4H, H-2, H-6 and H-6'), 7.27 (2H, J = 7.61 Hz, H-3 and H-5'), 7.32 (1H, J = 7.35 Hz, H-4'). Anal. Calcd for C14H16N2O4S2: C, 64.18; H, 4.38; N, 4.68. Found: C, 64.10; H, 4.49; N, 4.81.

6.1.5.15. 3-(3,4-Dimethoxyphenyl)-4-phenylthiazole-2(3H)-thione (6b).
Yield 24%; mp 168–169°C; IR (KBr, cm–1): max: 3038, 2927, 1609, 1522, 1488, 1367, 1260, 1155, 1026, 996, 877, 852. 1H NMR (500 MHz, CDCl3) δ: 3.76 (s, 3H, OCH3), 3.79 (s, 3H, OCH3), 6.66 (s, 1H, 4-OCH3), 6.93 (d, 2H, J = 8.89 Hz, H-3 and H-5), 7.12–7.15 (m, 4H, H-2, H-6 and H-6'), 7.27 (2H, J = 7.61 Hz, H-3 and H-5'), 7.32 (1H, J = 7.35 Hz, H-4'). Anal. Calcd for C14H16N2O4S2: C, 64.18; H, 4.38; N, 4.68. Found: C, 64.10; H, 4.49; N, 4.81.

6.2. Biological activity

6.2.1. Cell lines and cell culture

6.2.2. Cytotoxicity assay

6.2.3. Tubulin polymerization assay

Sheep brain microtubule protein was isolated by two cycles of polymerization-depolymerization in the PEM buffer [100 mMPIPES, pH 6.9, 1 mM MgSO4 and 1 mM ethylene glycol tetraacetic acid (EGTA)], according to the method described by Sengupta et al. [21]. Tubulin was purified from the microtubule protein by phosphocellulose chromatography [22]. The tubulin solution was rapidly frozen as drops in liquid nitrogen and stored at –70°C until used. Protein concentration was determined by the method of Bradford with bovine serum albumin as the standard [23]. The purity of tubulin was determined using polyacrylamide gel electrophoresis, which was performed by the Laemmli method [24]. The tubulin polymerization assay was carried out based on reported method with some modifications [25]. Tubulin pellets were thawed and centrifuged at 0°C to remove any aggregated or denatured tubulin. A drug/DMSO-tubulin pre-incubation, without
GTP, was performed for 15 min at 0 °C in ice. After adding three μL GTP (final concentration, 1 mM), the drug/DMSO-tubulin samples were immediately placed in a Varian Cary 100 UV/visible spectrophotometer, initiated polymerization by setting the temperature controller at 37 °C. The absorbance (A 350 nm) for a period of 30 min was recorded and the results were compared to the DMSO-treated control cells to evaluate the relative degree of change in optical density. DMSO was used at the final concentrations of <4% (v/v).

6.2.4. Acidine orange/ethidium bromide staining test
T47D cells grown in 12-well plates (50,000 cells/well) were treated with and without IC50 concentration of compounds 4b and 5f for 24 h. After treatment, cells were harvested and washed three times with phosphate buffer saline (PBS). Then, the cells were stained with 100 μL of a mixture of acidine orange and ethidium bromide (1:1, 100 μg/mL) solutions. Stained cell suspension (10 μL) were placed on a clean microscope slide and covered with a coverslip. The cells were immediately analyzed by Axioscope 2 plus fluorescence microscope (Zeiss, Germany).

6.2.5. Annexin V-PE and 7-AAD double staining test and flow cytometric analysis
Annexin V-PE/7-AAD double staining test was performed using an Annexin-V-PE kit (BD Pharmingen product) as described in protocol. The MCF-7 cells were treated with IC50 concentrations of the compounds 4b, 5f and etoposide. After incubation, the cells were collected and washed twice with cold PBS and resuspended in the binding buffer (100 μL of calcium buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2). Then, the cells were double stained with 5 μL of Annexin V-PE and 5 μL of 7-AAD solution. Finally, the samples were incubated for 15 min at room temperature and then analyzed by flow cytometry [18].

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.10.046.

References