Full Paper

Heterocycles 27. Microwave Assisted Synthesis and Antitumour Activity of Novel Phenothiazinyl-Thiazolyl-Hydrazine Derivatives

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A series of new phenothiazinyl-thiazolyl-hydrazine derivatives were synthesized by Hantzsch cyclization of 1-(10-ethyl-10*H*-phenothiazin-3-yl)-methylidene-thiosemicarbazide with α-halocarbonyl derivatives. Comparison between classical and microwave assisted synthesis emphasizes the great advantages induced by microwaves irradiation which afforded high reaction yields in much shorter reaction time. Structural assignments were based on spectroscopic methods (high resolution NMR, FTIR, MS). The new compounds were tested *in vitro* for their antiproliferative activity against tumor cell lines using spectrometric methods. Most of the compounds exhibit cytotoxicity against hepatic and colon tumor cells in a dose-dependent mode and a relationship between the structure and their biological activity was observed.

Keywords: Cytotoxicity / Hepatocarcinoma / Hydrazine / Microwave assisted synthesis / Phenothiazine / Thiazole

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Introduction

Compounds containing hydrazino-thiazole pharmacophore group may develop antimicrobial [1, 2], antifungal [2], anti-inflammatory [3] or antioxidative [4] activity. Some synthetic hydrazino-thiazole derivatives also exhibited antitumour activity [5–8].

Phenothiazine derivatives are well known due to their widespread therapeutic applications [9, 10], but more recently, their cytotoxic potential was also investigated and certain phenothiazine derivatives were found to be effective against ovarian cancer cells [11], human cervical cancer cells [12], lymphomas and COLO 320 cells [13] and were proved to induce apoptosis in neuroblastoma, glioma [14] and lung fibroblast [15] cell lines. Moreover, some phenothiazine derivatives induced antiproliferative effects on multi-drug-resistant cancer cells [16, 17] and exhibited a synergic effect in combination with some antineoplastic drugs.

Our target was to obtain new compounds with antiproliferative activity against tumor cell lines, by assembling the hydrazino-thiazolyl group and the phenothiazine nucleus in the same molecular structure, encouraged by our previous results in the synthesis and evaluation of biological activity of some hydrazinothiazoles [18], sulphonyl-hydrazinothiazoles [19], aroyl-hydrazinothiazoles used as intermediates in the synthesis of thiazolo[2,3-c][1,2,4]triazoles [20, 21] and *p*-toluenesulphonyl-hydrazinothiazoles [22]. For the optimization of the reaction conditions and an ecofriendly synthetic approached in the synthesis of the new phenothiazinyl-thiazolyl-hydrazine derivatives, our goal was to test the

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possibility of using the microwave assisted synthesis, a technique which, applied in the preparation of various heterocycles including phenothiazine [23–25] and thiazole [26–28], showed major advantages such as enhanced reaction rates, easy workup of the reaction mixture and ensured high yields of products. The new phenothiazinyl-thiazolyl-hydrazine derivatives thus obtained gave promising results by *in vitro* test of antiproliferative activity against hepatic and colon tumor cell lines, as presented below.

Results and discussion

Chemical synthesis

The strategy applied for the chemical synthesis of the target heterocyclic hydrazine derivatives **2a–g** containing thiazole and phenothiazine units in the same molecular structure, is based on two reaction steps: (i) the condensation of 10-alkyl-10*H*-phenothiazine-3-carbaldehyde with thiosemicarbazide, followed by (ii) the Hantzsch thiazole synthesis using several α-haloketones. In order to find the most suitable reaction conditions which generate 1-((10-alkyl-10*H*-phenothiazin-3-yl)methylidene)thiosemicarbazide **1** [29–31] and also for a more ecofriendly synthetic approach, the syntheses were performed both under classical convective and microwave assisted heating techniques (Scheme 1).

Under classical convective heating to reflux in ethanol, the condensation of 10-ethyl-10*H*-phenothiazine-3-carbaldehyde with thiosemicarbazide required 4 h reaction time in order to obtain good yields (87%) of thiosemicarbazone **1**.

Alternative conditions were applied during the optimization of the microwave assisted synthesis of **1**, as presented in Table 1. Good to excellent yields were obtained by dielectric heating in the presence of ethanol solvent (Table 1, entries 1–3) in significantly shorter reaction time, as compared to the convective heating technique. For a greener synthesis, we attempted to replace the organic solvent with water, but unfortunately in such conditions **1** was recovered only in traces (Table 1, entry 6). Moderate yields of **1** can be obtained in ethanol/water mixture (Table 1, entry 5), thus indicating the possibility of using water at most as a co-solvent.

The phenothiazinyl-thiazolyl-hydrazine derivatives **2a–g** were obtained by both classical and microwave assisted Hantzsch cyclization of thiosemicarbazone **1** with several α-halocarbonyl derivatives (such as chloroacetone, 1,3-dichloroacetone, α-bromoacetophenone, 3-chloroacetylacetone, ethyl-α-bromoacetylacetate, ethyl-γ-bromoacetylacetate, ethyl-bromopyruvate), as shown in Scheme 2. Acetylhydrazides **3a–h** were obtained in high yields by stirring **2a–g** with acetic anhydride for 15 min at reflux, in the presence of catalytic amounts of pyridine (Scheme 2).

Table 1. Optimization of the microwave[a)] assisted synthesis of thiosemicarbazone **1**

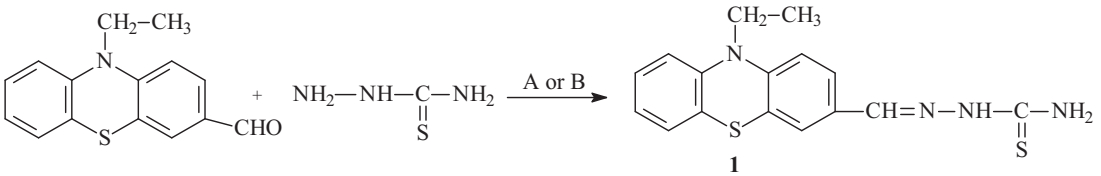
Entry	Solvent	Reaction time (min.)	Temperature (°C)	Yield (%)
1	Ethanol	30	100	95
2	Ethanol	20	100	65
3	Ethanol	10	100	48
4	Ethanol	10	80	61
5	Ethanol/Water	20	100	51
6	Water	20	100	5

[a)] Power $P = 200$ W.

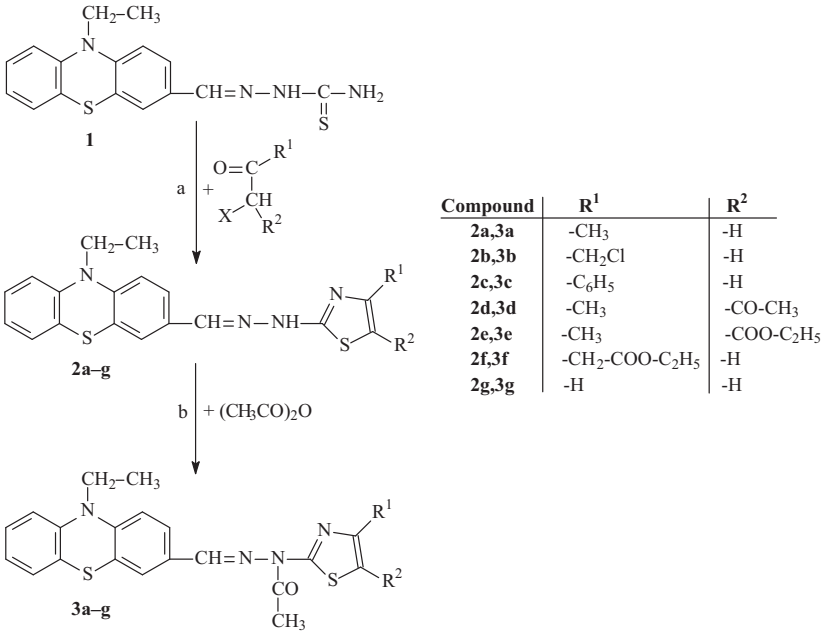
The target phenothiazyl-thiazolyl-hydrazine derivatives **2a–g** were obtained in moderate yields by the classical Hantzsch cyclization at room temperature, but excellent reaction yields were observed when microwave irradiation was applied (Table 2). Optimal reaction conditions for the microwave assisted Hantzsch cyclization shown in Table 2 method B, were established after several experiments of irradiation at different reaction temperatures (40 or 60°C) and times (30, 60 or 90 min) in the presence of dimethylformamide solvent. Very high yields of each compound **2a–g** were obtained by microwave assisted synthesis after 90 min at 60°C.

The comparison between classical and microwave assisted reaction conditions presented in Table 2 shows that Hantzsch cyclization afforded only 43–68% yields of target compounds **2a–b** after a very long reaction time (24 h) at room temperature, while important improvements of the reactions yields (89–99%) were observed when microwave irradiation was applied for much shorter reaction times (1.5 h), thus recommending the microwave assisted synthesis as the most suitable technique for obtaining the phenothiazinyl-thiazolyl-hydrazine derivatives **2a–g**.

The structures of the obtained compounds **1, 2a–g, 3a–g** were assigned based on their recorded high resolution



Scheme 1. Condensation of 10-ethyl-10*H*-phenothiazine-3-carbaldehyde with thiosemicarbazide. Reaction conditions: A) abs. ethanol, 78°C, convective heating; B) abs. ethanol or ethanol/water mixture, 100°C, MW irradiation.



Compound	R^1	R^2
2a,3a	$-CH_3$	-H
2b,3b	$-CH_2Cl$	-H
2c,3c	$-C_6H_5$	-H
2d,3d	$-CH_3$	$-CO-CH_3$
2e,3e	$-CH_3$	$-COO-C_2H_5$
2f,3f	$-CH_2-COO-C_2H_5$	-H
2g,3g	-H	-H

Scheme 2. Synthesis of phenothiazyl-thiazolyl-hydrazine derivatives **2a–g** and acetyl derivatives **3a–g**. (a) Hantzsch cyclization, DMF, ethanol, reaction conditions as given in Table 2. (b) Acetic anhydride, pyridine (catalytic amount), reflux 15 min.

Table 2. Experimental conditions for Hantzsch cyclization applied in the synthesis of phenothiazinyl-thiazolyl-hydrazine derivatives **2a–g**

Compound	Method[a)]	Temperature (°C)	Time (min)	Pressure (bar)	Power (W)	Yield (%)
2a	A	25	1440	1	–	57
	B	60	90	1.7	100	98
2b	A	25	1440	1	–	43
	B	60	90	1.7	100	92
2c	A	25	1440	1	–	62
	B	60	90	1.7	100	99
2d	A	25	1440	1	–	63
	B	60	90	1.7	100	89
2e	A	25	1440	1	–	60
	B	60	90	1.7	100	92
2f	A	25	1440	1	–	68
	B	60	90	1.7	100	94
2g	A	25	1440	1	–	59
	B	60	90	1.7	100	91

[a)] A, classical conditions; B, microwave irradiation.

^{1}H NMR, ^{13}C NMR, MS and FT-IR spectra. In the ^{1}H NMR spectra, the heteroaromatic rings and the carbonyl substituents determine characteristic deshielding effects which can be observed on key signals such as for example the signal of the proton in the azomethine group (–CH=N–) which appears as a singlet situated at 7.68 ppm in the thiosemicarbazone **1**, 7.76 ppm in the phenothiazinyl-thiazolyl-hydrazine **2a** and, respectively 8.21 ppm in the corresponding acetyl-derivative **3a**. Complete assignment of the structures was based on 2D homo- and heteronuclear correlation NMR experiments COSY, HMQC and HMBC.

Biological activity

The mathematic parameter used to quantify the compounds antiproliferative effect against the tumor cells was the half maximal inhibitory concentration (IC_{50}). These data were obtained by generating dose–response curves for every compound using the biostatistics software. The values IC_{50} obtained for each compound tested for both HepG2 and CC531S cell lines are shown in Table 3.

The hepatic HepG2 tumor cell line growth was inhibited by all compounds **1**, **2a–f** and **3b–g** (Fig. 1b). We compared the treated cells proliferation with the untreated, control tumor

Table 3. *In vitro* antiproliferative activity of phenothiazinyl-thiazolyl-hydrazine derivatives against hepatic HepG2 tumor cells and colon carcinoma CC531S cells (determined by colorimetric MTT method).

Cpd	HepG2 cells IC_{50} μg/mL			CC531S cells IC_{50} μg/mL		
1	5.64	3.42	2.98	4.21	6.10	8.45
2a	2.40	4.22	3.67	4.36	7.01	5.00
2b	12.72	8.64	8.04	7.03	9.15	10.80
2c	29.87	37.39	31.75	3.61	4.12	6.77
2d	24.95	19.58	14.72	9.88	7.18	14.75
2e	4.25	4.74	6.77	10.94	16.61	12.44
2f	2.78	2.21	3.26	8.18	9.42	10.27
3b	1.83	2.60	2.57	16.84	20.12	13.34
3c	3.84	4.93	4.77	6.01	13.49	8.18
3d	7.92	14.14	13.49	9.00	9.35	11.62
3e	14.7	11.65	10.28	13.28	9.82	21.15
3g	16.35	13.34	13.19	9.86	9.27	5.50
Cisplatin	2.39	2.12	2.65	2.60	1.638	1.87

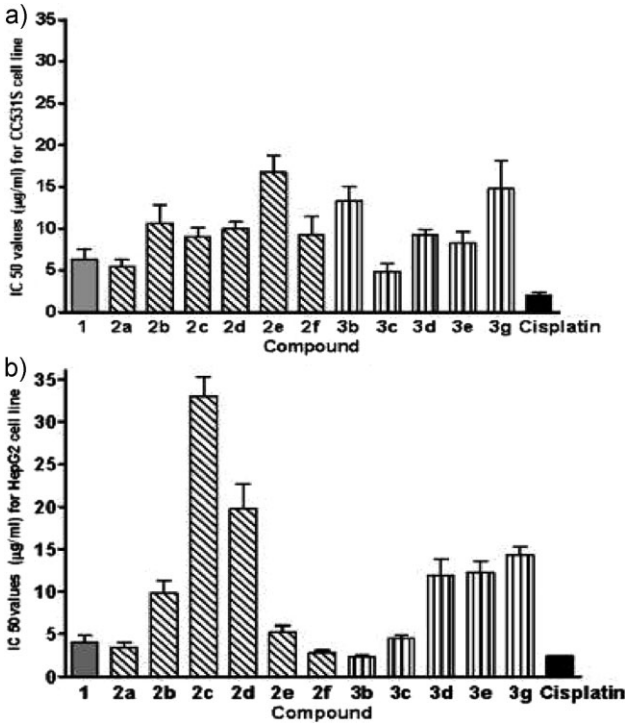


Figure 1. Cytotoxic activity of phenothiazinyl-thiazolyl-hydrazine derivatives quantified by IC_{50} values upon (a) CC531S cell lines (b) HepG2 cell lines.

cells. As positive reference values we determined the IC_{50} values of cisplatin, a commonly used chemotherapy drug, which showed effectiveness in tumor cell inhibition, and was proven to be active against HepG2 [32, 33] and CC531S [34] cells. Cytotoxicity upon cells treated with compounds **1**, **2g** and **3g** was significant in each case; Graph Pad Prism column statistics two-tailed *t*-test indicates significant inhibition (95% Cl of discrepancy, two-tailed p value <0.0395 for each compound). ANOVA one-way analysis of variance and Dunnett's multiple comparison test indicate that the activity of compounds **1**, **2a**, **2e**, **2f**, **3b** and **3c** is comparable to cisplatin cytotoxicity, while the activity of **2b**, **2c**, **2d**, **3d**, **3e** and **3g** is significantly lower ($p < 0.05$, p value extremely significant).

The colon carcinoma CC531S cells proliferation was better inhibited by compounds **1**, **2a** and **3c** (Fig. 1a). According to the two-tailed *t*-test and the ANOVA analysis the cytotoxicity of **1**, **2a–f** and **3b–g** is in each case significant relatively to the untreated cells (95% confidence interval, two-tailed p value not higher than 0.043). The biological effect of **1**, **2a**, **3c**, and **3e** is similar to cisplatin, while for the other compounds the activity shows significant differences ($p < 0.05$). Within this group, the compounds fall in two categories: **2c**, **3d**, **2f** and **2d** with cytotoxicities which differ slightly from the chemotherapy drug (Dunnett's multiple comparison test, p value summary significant), and **2b**, **3b**, **2e** and **3g** with significantly lower activity (p value summary extremely significant).

The comparison of the cytotoxicity of compounds **2b–e** with their acetylated analogues **3b–e** upon HepG2 cell line show that the presence of the acetyl group causes a decrease of IC_{50} value as compared to unacetylated parent compound, except for **3e**, which exhibits a larger IC_{50} value. The cytotoxicities of compounds **2b** and **3b** differ significantly (Wilcoxon matched pair test, two-tailed p value 0.0074, very significant), the difference between the activities of **2c** and **3c** is extremely significant ($p = 0.0002$), in the pair **2e–3e** there is a significant difference between IC_{50} values ($p = 0.0105$), while the activities of **2d** and **3d** do not diverge significantly.

The comparison of the cytotoxic activity of compounds **2b–e** with their acetylated analogues **3b–e** and the statistic analysis showed that against the CC531S cell line there are no notable differences between **2b** and **3b**, **2d** and **3d** (Wilcoxon matched pair test, Spearman two-tailed p value summary not

significant), while for the pairs **2c–3c** and **2e–3e** the IC_{50} values differ significantly ($p < 0.05$). The presence of the acetyl group in the molecule improved significantly the antiproliferative potential in compounds **3c** and **3e**.

The presence of acetyl group in **3e** gives a divergent outcome; the compound is more cytotoxic against colorectal tumor cells, but less toxic against hepatic cells as compared to its non-acetylated parent **2e**.

The cytotoxicity of the new compounds against the colorectal cells; is higher as compared to the cytotoxicity against hepatic cells (two-way ANOVA test, Bonferroni post-test, $p < 0.0001$).

The two groups of acetylated (**3b**, **3c**, **3d**, **3e**, **3g**) and non-acetylated (**2a**, **2b**, **2c**, **2d**, **2e**) phenothiazinyl-thiazolyl-hydrazine analogues were analyzed using grouped statistics; the two-way ANOVA test indicates that IC_{50} values for non-acetylated compounds are significantly higher for the HepG2 liver cells (Bonferroni post test, $p < 0.01$), while in the case of CC531S cell line the differences are irrelevant between the two groups ($p > 0.05$).

The biological activity of phenothiazinyl-thiazolyl-hydrazine derivatives slightly varies according to the substituent attached in positions 4, 5 of the thiazole ring and acetylation of hydrazine unit. Compound **2a** containing the methyl substituent in position 4 of the thiazole ring exhibits the highest cytotoxicity against both cell lines. Compound **2b** which contains a chloromethyl and **2c** which bears a phenyl group exhibit lower toxicity against tumor cells, but the effect is somehow enhanced for their acetylated analogues **3b** and **3c**. The compound **2d** containing a methyl substituent in position 4 and an acetyl group in position 5 of the thiazole ring does not exhibit a notable antiproliferative activity upon the studied cell lines, and the introduction of acetyl group in its analogue **3d** slightly increases this biological effect. Among the four structures containing ethoxycarbonyl groups (**2e**,**f** and **3e**,**g**), **2f** has a notable activity against Hep2G cell lines, while **3e** and **3g**, bearing two bulky groups with high steric hindrance, are characterized by a reduced biological activity.

Conclusions

New phenothiazinyl-thiazolyl-hydrazine derivatives were successfully prepared by Hantzsch cyclization of 1-(10-ethyl-10*H*-phenothiazin-3-yl)-methylidene-thiosemicarbazide with α-halocarbonyl compounds under microwave irradiation in sealed reaction vessels. This technique affords excellent reaction yields in much shorter reaction times as compared to classical reaction conditions and thus it may be recommended for laboratory scale preparation of compounds **1**, **2**.

The novel synthesized phenothiazinyl-thiazolyl-hydrazine derivatives exhibit *in vitro* antiproliferative activity against hepatic and colon carcinoma cells. A structure–activity relationship survey of phenothiazinyl-thiazolyl-hydrazine derivatives shows that the presence of substituents such as methyl or chloromethyl in position 4 of the thiazole ring enhanced the cytotoxic potential of the compounds, while the steric hindrance induced by the presence of bulk substituents in position 4 and 5 of the thiazole moiety weakened the antiproliferative activity. The acetylation of hydrazone unit does not affect significantly the antiproliferative effect, but slightly enhances the cytotoxicity against HepG2 and CC531S cell lines. Among the studied compounds **2a**, **2b**, **2f**, **3b**, **3c** and **3e** exhibit a distinct therapeutical potential.

Experimental

Chemistry

Melting points were determined with an Electrothermal IA 9200 digital melting point apparatus and are uncorrected. Elemental analyses were performed by a Vario EL III instrument. The mass spectra were recorded with a Shimadzu QP 2010 EI mass spectrometer. IR spectra were recorded on a Bruker Vector 22 FT-IR spectrometer fitted with a Golden Gate ATR accessory. ^1H and ^{13}C NMR spectra were recorded in $CHCl_3$-d_1 or DMSO-d_6, in 5 mm tubes at RT, on a WM-400 MHz Bruker NMR spectrometer. Assignments were made based on standard 2D HSQC and HMBC techniques. The spectral data are listed in the experimental part. Microwave assisted syntheses were performed in sealed vessels using a CEM Discover LabMate instrument, insured with on-line inside temperature and pressure control.

All α-halocarbonyl-derivatives were purchased from Sigma–Aldrich. All reactions as well as column chromatography were followed by TLC using Merck pre-coated silica gel 60 F_{254} aluminium sheets. Column chromatography was performed with Merck silica gel 60 (63–200 mesh).

10-Ethyl-10*H*-phenothiazine-3-carbaldehyde with m.p. = 98°C, was prepared according to described literature procedures [35].

*1-((10-Ethyl-10H-phenothiazin-3-yl)methylidene)-thiosemicarbazide (**1**)*

(a) To a solution of 10-ethyl-10*H*-phenothiazine-3-carbaldehyde (250 mg, 1 mmol) in ethanol (10 mL), thiosemicarbazide (90 mg, 1 mmol) was added in one portion. The reaction mixture was heated to reflux for 4 h. The solution was cooled to room temperature and then the solvent was evaporated in vacuum. The residue was separated by column chromatography on silica gel (silica gel 60, mesh 0.063–0.2 mm), by eluting with hexane/acetone (2:1 v/v) or toluene/EtOAc (1:1 v/v) to afford 270 mg (86%) of the title compound as yellow crystals with m.p. 170–172°C;

(b) The reaction mixture containing 10-ethyl-10*H*-phenothiazine-3-carbaldehyde (255 mg, 1 mmol) ethanol (5 mL) and thiosemicarbazide (90 mg, 1 mmol), was introduced in the quartz reaction vessel, which was sealed and then subjected to MW irradiation for 30 min at internal temperature of 100°C (Table 1). The product was filtered. The residue was purified by recrystallization from ethanol and afforded 310 mg (95%) of *i*th m.p. 171°C.

IR (ATR) ν_{max} (cm^{-1}): 3425, 3276 (νNH_2), 3163 (νNH), 3045 (νCH), 1597 ($\nu C{=}C$), 1543 ($\nu C{=}C$), 1247($\nu C{=}S$). MS (EI) *m/z* 328 (M^+), 311, 225 (100%), 198; ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.43 (t, 3H, $-CH_3$), 3.94 (q, 2H, $N-CH_2$), 6.82 (d, 1H, H_1), 6.85 (d, 1H, H_9), 6.93 (t, 1H, H_7), 7.09 (dd, 1H, H_6), 7.15 (t, 1H, H_8), 7.33 (dd, 1H, H_2), 7.42 (s, 1H, H_4), 7.68 (s, 1H, −CH=N), 9.34 (s, 3H, NH, NH_2). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.86 ($-CH_3$), 42.13 ($-CH_2$), 114.69 (C_9), 115.31 (C_1), 123.00 (C_7), 123.27 (C_{4a}), 124.90 (C_{5a}), 125.36 (C_2), 126.89 (C_8), 127.41 (C_6), 127.50 (C_4), 127.60 (C_3), 142.77 (−CH), 143.71 (C_{9a}), 147.14 (C_{10a}), 206.9 (−C=S); Elemental Anal. Calcd. for $C_{16}H_{16}N_4S_2$ (%): C, 58.51; H, 4.91; N, 17.06; S, 19.52. Found (%): C, 58.43; H, 4.62; N, 16.96; S, 19.45.

*General procedure for the synthesis of phenothiazinyl-thiazolyl-hydrazine derivatives (**2a–g**)*

(a) To a solution of thiosemicarbazone **1** (330 mg, 1 mmol) in a mixture of ethanol (5 mL) and DMF (2 mL), the appropriate α-halocarbonyl derivative (1 mmol) was added at room temperature. The reaction mixture was stirred at ambient temperature for 24 h, then poured on ice and neutralized with $NaHCO_3$ and the resulting precipitate was collected by suction filtration. The product was purified by crystallization or column chromatography.

(b) To a solution of thiosemicarbazone **1** (330 mg, 1 mmol) in DMF (5 mL), the appropriate α-halocarbonyl derivative (1 mmol) was added and the obtained reaction mixture was introduced in the reaction vessel which was sealed and then subjected to MW irradiation according to conditions presented in Table 2. The reaction product was poured on ice and then treated as presented above.

*1-((10-Ethyl-10H-phenothiazin-3-yl)methylidene)-2-(4-methylthiazol-2-yl)hydrazine (**2a**)*

Purification by column chromatography on silica gel 60, eluent hexane/ethyl acetate (2:1 v/v). Yellow-green crystals, yield A 57% (0.21 g), B 98%, m.p. = 149–151°C; IR (ATR) ν_{max} (cm^{-1}): 3165 (νNH), 3055 (νCH), 1576 ($\nu C{=}C$), 1496 ($\nu C{=}C$). MS (EI) *m/z*: 366 (M^+), 252, 239, 226, 223 (100%), 197, 114. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.42 (t, 3H, ^3J = 7.1 Hz, $-CH_3$), 2.26 (s, 3H, Th−CH_3), 3.95 (q, 2H, ^3J = 7.1 Hz, $N-CH_2$), 6.16 (s, 1H, Th−H_5), 6.80 (d, 1H, ^3J = 8.6 Hz, H_1), 6.86 (d, 1H, ^3J = 8.6 Hz, H_9), 6.93 (t, 1H, ^3J = 7.3 Hz, H_7), 7.09 (dd, 1H, ^3J = 7.3 Hz, H_6), 7.14 (t, 1H, ^3J = 8.6 Hz, H_8), 7.35 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.42 (s, 1H, H_4), 7.76 (s, 1H, −CH=N), 8.87 (s, 1H, −NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 13.01 ($-CH_3$), 16.8 (Th−CH_3), 42.03 ($-CH_2$), 102 (Th−C_5), 114.64 (C_9), 115.15 (C_1), 122 (C_7), 123.59 (C_{4a}), 124.62 (C_{5a}), 125.05 (C_2), 126.50 (C_8), 127.35 (C_6), 128.00 (C_4), 128.20 (C_3), 142.13 (Th−C_4), 144.10 (C_{9a}), 145.85 (C_{10a}), 146.09 (CH=N), 169.05 (Th−C_2). Anal. Calcd. for $C_{19}H_{18}N_4S_2$ (%): C, 62.27; H, 4.95; N 15.29; S 17.50. Found (%): C, 62.18; H, 4.73; N, 15.12; S, 17.41.

*1-((10-Ethyl-10H-phenothiazin-3-yl)methylidene)-2-(4-chloromethylthiazol-2-yl)hydrazine (**2b**)*

Purification by column chromatography on silica gel 60, eluent hexane/ethylacetate (1:1 v/v). Yellow-brown crystals, yield A 43% (0.18 g), B 92%, m.p. = 237–239°C; IR (ATR) ν_{max} (cm^{-1}): 3165 (νNH), 3050 (νCH), 2920 (νCH), 1599 ($\nu C{=}C$), 1559 ($\nu C{=}C$), 1497 ($\nu C{=}C$), 708 ($\nu C{-}Cl$). MS (EI) *m/z*: 400 (M^+), 269, 252, 237, 225, 223 (100%), 169, 147. ^1H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.42 (t, 3H, ^3J = 7.1 Hz, $-CH_3$), 3.95 (q, 2H, ^3J = 7.1 Hz, $N-CH_2$), 4.62 (s, 2H, $-CH_2Cl$), 6.80 (d, 1H, ^3J = 8.6 Hz, H_1), 6.84 (s, 1H, Th−H_5), 6.86 (d, 1H, ^3J = 8.6 Hz, H_9), 6.93 (t, 1H, ^3J = 7.3 Hz, H_7), 7.09 (dd, 1H, ^3J = 7.3 Hz, H_6), 7.14 (t, 1H, ^3J = 8.6 Hz, H_8), 7.35 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.42 (s, 1H, H_4), 7.93 (s, 1H, −CH=N), 8.91 (s, 1H, −NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 13.01 ($-CH_3$), 40.12 ($-CH_2$), 43.39 ($-CH_2Cl$), 109.00 (Th−C_5), 114.31 (C_9), 115.25 (C_1), 122.10 (C_7), 123.52 (C_{4a}), 124.64 (C_{5a}), 125.15 (C_2), 126.31 (C_8), 127.25 (C_6), 128.00 (C_4), 128.20 (C_3), 132.21 (Th−C_4), 140.73 (*CH=N*), 144.11 (C_{9a}), 145.72 (C_{10a}), 168.15 (Th−C_2). Anal. Calcd. for $C_{19}H_{17}ClN_4S_2$ (%): C, 56.92; H, 4.27; N, 13.97; S 16.00%. Found (%): C, 56.91; H, 4.12; N, 13.92; S, 16.00.

*1-((10-Ethyl-10H-phenothiazin-3-yl)methylidene)-2-(4-phenylthiazol-2-yl)hydrazine (**2c**)*

Purification by column chromatography on silica gel 60, eluent hexane/ethyl acetate (2:1 v/v) or crystallization from ethanol. Green crystals, yield A 62% (0.27 g), B 99%, m.p. = 201–202°C; IR (ATR) ν_{max} (cm^{-1}): 3215 (νNH), 3020 ($\nu C{-}H$), 1684 ($\nu C{=}N$), 1600 ($\nu C{=}C$), 1575 ($\nu C{=}C$), 1496 ($\nu C{=}C$). MS (EI) *m/z*: 428 (M^+), 399, 252, 239, 226, 223 (100%), 176, 134. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.41 (t, 3H, ^3J = 7.1 Hz, $-CH_3$), 3.92 (q, 2H, ^3J = 7.1 Hz, $N-CH_2$), 6.76 (d, 1H, ^3J = 8.6 Hz, H_1), 6.86 (d, 1H, ^3J = 8.6 Hz, H_9), 6.93 (t, 1H, ^3J = 7.3 Hz, H_7), 7.09 (s, 1H, Th−H5), 7.15 (dd, 1H, ^3J = 7.3 Hz, H_6), 7.25 (t, 1H, ^3J = 8.6 Hz, H_8), 7.35 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.42 (s, 1H, H_4), 7.60–7.72 (m, 5H, Ar−H), 8.76 (s, 1H, −CH=N), 9.78 (s, 1H, −NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.90 ($-CH_3$), 42.02 ($-CH_2$), 103 (Th−C_5), 114.31 (C_9), 115.25 (C_1), 122.1 (C_7), 123.52 (C_{4a}), 124.64 (C_{5a}), 125.15 (C_2, C_{meta}), 126.31 (C_8, C_{orto}), 126.41 (C_{para}), 127.25 (C_6), 127.91 (Th−C_4), 128 (C_4) 128.2 (C_3), 142.36 (−CH=N), 144.08 (C_{9a}), 146.08 (C_{10a}), 169.3 (Th−C_2). Anal. Calcd. for $C_{24}H_{20}N_4S_2$ (%): C, 67.26; H, 4.70; N, 13.07; S, 14.96. Found (%): C, 67.18; H, 4.63; N, 13.02; S, 14.82.

*5-Acetyl-((10-methyl-10H-phenothiazin-3-yl)methylidene)-2-(4-methylthiazol-2-yl)hydrazine (**2d**)*

Purification by column chromatography on silica gel 60, toluene/ethyl acetate (2:1 v/v) or crystallization from ethanol. Yellow crystals, yield A 63% (0.26 g), B 89%, m.p. = 235–236°C; IR (ATR) ν_{max} (cm^{-1}): 3265 (νNH), 3053 ($\nu C{-}H$), 2987 ($\nu C{-}H$), 2956 ($\nu C{-}H$), 1688 ($\nu C{=}O$), 1651 ($\nu C{=}N$), 1579 ($\nu C{=}C$), 1495 ($\nu C{=}C$), 1370. MS (EI) *m/z*: 408 (M^+), 332, 286, 268, 252, 239, 225, 223(100%), 197, 141. ^1H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.43 (t, 3H, ^3J = 7.1 Hz, $-CH_3$), 2.48 (s, 3H, Th−*CH*$_3$), 2.59 (s, 3H, $-CO-CH_3$), 3.95 (q, 2H, ^3J = 7.1 Hz, $N-CH_2$), 6.84 (d, 1H, ^3J = 8.6 Hz, H_1), 6.87 (d, 1H, ^3J = 7.6 Hz, H_9), 6.93 (t, 1H, ^3J = 7.6 Hz, H_8), 7.12 (dd, 1H, ^3J = 7.6 Hz, H_6), 7.15 (t, 1H, ^3J = 7.6 Hz, H_7), 7.39 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.45 (s, 1H, H_4), 7.76 (s, 1H, −CH=N), 8.83 (s, 1H, −NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.9 ($-CH_3$), 17.62 (Th−CH_3), 29.07 ($-CO-CH_3$), 42.02 ($-CH_2$), 111.63 (Th−C_5), 114.31 (C_9), 115.25 (C_1), 122.10 (C_7), 123.52 (C_{4a}), 124.64 (C_{5a}), 125.15 (C_2), 126.31 (C_8), 127.25 (C_6), 127.86 (Th−C_4), 128.00 (C_4), 128.20 (C_3), 141.00 (−CH=N), 144.08 (C_{9a}), 146.08 (C_{10a}), 170.00 (Th−C_2), 189.70 (C=O). Anal. Calcd. for $C_{21}H_{20}N_4OS_2$ (%): C, 61.74; H, 4.93; N, 13.71; S, 15.70. Found (%): C, 61.65; H, 4.89; N, 13.64; S, 15.62.

*5-Ethoxycarbonyl-((10-methyl-10H-phenothiazin-3-yl)methylidene)-2-(4-methylthiazol-2-yl)hydrazine (**2e**)*

Purification by column chromatography on silica gel 60, hexane/ethyl acetate (1:1 v/v) or crystallization from ethanol. Yellow-

green crystals, yield A 60% (0.26 g), B 92%, m.p. = 219–220°C; IR (ATR) ν_{max} (cm^{-1}): 3285 (νNH), 3021 (νC–H), 2952, 2850 (νC–H), 1662 (νC=O), 1599 (νC=N), 1566 (νC=C), 1523 (νC=C), 1494 (νC=C), 1244, 1094 (νC–O). MS (EI) *m/z*: 438 (M^+), 388, 342, 282, 268 (100%), 252, 239, 199, 149, 119, 97. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.37 (t, 3H, ^3J = 7.3 Hz, –CH_3), 1.43 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.59 (s, 3H, Th–CH_3), 3.94 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 4.31 (q, 2H, ^3J = 7.3 Hz, O–CH_2), 6.83 (d, 1H, ^3J = 8.6 Hz, H_1), 6.89 (d, 1H, ^3J = 7.6 Hz, H_9), 6.92 (t, 1H, ^3J = 7.6 Hz, H_7), 7.11 (dd, 1H, ^3J = 7.6 Hz, H_6), 7.16 (t, 1H, ^3J = 7.6 Hz, H_8), 7.37 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.45 (s, 1H, H_4), 7.79 (s, 1H, CH=N), 9.14 (s, 1H, –NH). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 12.84 (–CH_3), 14.41 (–CH_3), 16.62 (Th–CH_3), 42.11 (–CH_2), 60.87 (–CH_2), 112.33 (Th–C_5), 114.31 (C_9), 115.25 (C_1), 122.10 (C_7), 123.52 (C_{4a}), 124.64 (C_{5a}), 124.71 (Th–C_4), 125.15 (C_2), 126.31 (C_8), 127.25 (C_6), 128.00 (C_4) 128.20 (C_3), 143.00 (–CH=N), 144.58 (C_{9a}), 146.80 (C_{10a}), 162.20 (Th–C_2), 168.93 (–COO). Anal. Calcd. for $C_{22}H_{22}N_4O_2S_2$ (%): C, 60.25; H, 5.06; N, 12.78; S, 14.62. Found (%): C, 59.95; H, 4.99; N, 12.61; S, 14.61.

*Ethyl 2-(2-(2-((10-methyl-10H-phenothiazin-3-yl)-methylene)hydrazinyl)thiazol-4-yl)acetate (**2f**)*

Purification by column chromatography on silica gel 60, hexane/ethyl acetate (1:1 v/v). Green crystals, yield A 68% (0.29 g), B 94%, m.p. = 220–221°C; IR (ATR) ν_{max} (cm^{-1}): 3243 (νNH), 3030 (νC–H), 2956 (νC–H), 2852 (νC–H), 1722 (νC=O), 1596 (νC=C), 1237, 1024 (νC–O). MS (EI) *m/z*: 438 (M^+), 365, 352, 268 (100%), 252, 226, 199, 149, 119, 97, 73. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.28 (t, 3H, ^3J = 7.3 Hz, –CH_3), 1.42 (t, 3H, ^3J = 7.1 Hz, –CH_3), 3.58 (s, 2H, Th–CH_2–COO), 3.94 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 4.19 (q, 2H, ^3J = 7.3 Hz, O–CH_2), 6.44 (s, 1H, Th–H_5), 6.82 (d, 1H, ^3J = 8.6 Hz, H_1), 6.86 (d, 1H, ^3J = 8.1 Hz, H_9), 6.92 (t, 1H, ^3J = 7.6 Hz, H_7), 7.11 (dd, 1H, ^3J = 7.6 Hz, H_6), 7.14 ppm (t, 1H, ^3J = 8.1 Hz, H_8), 7.36 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.44 (s, 1H, H_4), 7.76 (s, 1H, –CH=N), 9.78 (s, 1H, –NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.90 (–CH_3), 14.12 (–CH_3), 36.15 (–CH_2), 42.06 (–CH_2), 61.26 (–CH_2), 105.23 (Th–C_5), 114.71 (C_9), 115.17 (C_1), 122.74 (C_7), 125.06 (C_{4a}), 126.79 (C_{5a}), 126.93 (Th–C_4), 127.31 (C_2), 127.37 (C_8), 127.85 (C_6), 128.00 (C_4), 128.20 (C_3), 143.08 (–CH=N), 144.03 (C_{9a}), 146.28 (C_{10a}), 163 (Th–C_2), 169.32 (–COO). Anal. Calcd. for $C_{22}H_{22}N_4O_2S_2$ (%): C, 60.25; H, 5.06; N, 12.78; S, 14.62. Found (%): C, 59.88; H, 4.99; N, 12.71; S, 14.52.

*4-Ethoxycarbonyl-((10-methyl-10H-phenothiazin-3-yl)-methylidene)-2-(thiazol-2-yl)hydrazine (**2g**)*

Purification by column chromatography on silica gel 60, hexane/acetone (1:1 v/v) or crystallization from ethanol. Yellow crystals, yield A 59% (0.25 g), B 91%, m.p. = 204–205°C; IR (ATR) ν_{max} (cm^{-1}): 3221 (νNH), 3135 (νC–H), 3079 (νC–H), 2978 (νC–H), 2935 (νC–H), 2867 (νC–H), 1681 (νC=O), 1577 (νC=C), 1493 (νC=C), 1241, 1095 (νC–O). MS (EI) *m/z*: 424 (M^+), 268, 252, 223 (100%), 199, 156, 149, 73. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.37 (t, 3H, ^3J = 7.1 Hz, –CH_3), 1.42 ppm (t, 3H, ^3J = 6.8 Hz, –CH_3), 3.93 (q, 2H, ^3J = 6.8 Hz, N–CH_2), 4.36 (q, 2H, ^3J = 7.1 Hz, O–CH_2), 6.83 (d, 1H, ^3J = 8.6 Hz, H_1), 6.87 (d, 1H, ^3J = 7.6 Hz, H_9), 6.92 (t, 1H, ^3J = 7.8 Hz, H_7), 7.12 (dd, 1H, ^3J = 7.8 Hz, H_6), 7.15 (t, 1H, ^3J = 7.6 Hz, H_8), 7.36 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.43 (s, 1H, H_4), 7.57 (s, 1H, Th–H_5), 7.76 (s, 1H, –CH=N), 9.17 (s, 1H, –NH). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.90 (–CH_3), 14.34 (–CH_3), 42.05 (–CH_2), 61.25 (–CH_2), 114.74 (C_9), 115.18 (C_1), 118.48 (C_7), 122.75 (Th–C_5), 123 (C_{4a}), 124.69 (C_{5a}), 125.38 (C_2), 126.56 (C_8), 127.38 (C_6), 128.09 (C_4) 128.20 (C_3), 142.21 (–CH=N), 144.05 (C_{9a}), 146.22 (C_{10a}), 161.64 (Th–C_2), 168.49 (–COO). Anal. Calcd. for $C_{21}H_{20}N_4O_2S_2$ (%): C, 59.41; H, 4.75; N, 13.20; S, 15.10. Found (%): C, 59.35; H, 4.69; N, 13.11; S, 15.01.

*General procedure for acetylation of **2a–g***

One millimole of phenothiazinyl-thiazolyl-hydrazine derivative (**2a–g**) was treated with 5 mL of acetic anhydride and catalytic amounts of pyridine. The resulting mixture was heated for 15 min to 130°C under stirring, then the acetic anhydride was evaporated under reduced pressure. The obtained solid was purified by recrystallization or column chromatography.

*N′-((10-Methyl-10H-phenothiazin-3-yl)methylene)-N-(4-methylthiazol-2-yl)acetohydrazide (**3a**)*

Purification by column chromatography on silica gel 60, toluene/ethyl acetate (1:1 v/v). Yellow crystals, yield 58.2% (0.20 g), m.p. = 73–75°C; IR (ATR) ν_{max} (cm^{-1}): 3054, 2970, 2850 (νCH), 1698 (νC=O), 1587 (νC=C), 1453 (νC=C), 1363 (–$COCH_3$). MS (EI) *m/z*: 408 (M^+), 366, 252, 239, 226, 223, 197, 114, 43 (100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.43 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.46 (s, 3H, –CH_3), 2.48 (s, 3H, –CH_3), 3.95 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 6.68 (s, 1H, Th–H_5), 6.81 (d, 1H, ^3J = 8.6 Hz, H_1), 6.89 (d, 1H, ^3J = 8.6 Hz, H_9), 7.01 (t, 1H, ^3J = 7.3 Hz, H_7), 7.12 (dd, 1H, ^3J = 7.3 Hz, H_6), 7.16 ppm (t, 1H, ^3J = 8.6 Hz, H_8), 7.38 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.49 (s, 1H, H_4), 8.21 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 13.01 (–CH_3), 16.8 (Th–CH_3), 22.36 (–CH_3), 42.03 (–CH_2), 106.00 (Th–C_5), 114.64 (C_9), 115.45 (C_1), 123.00 (C_7), 123.59 (C_{4a}), 124.68 (C_{5a}), 125.05 (C_2), 126.55 (C_8), 127.65 (C_6), 128.00 (C_4), 128.20 (C_3), 143.16 (Th–C_4), 145.22 (C_{9a}), 146.03 (C_{10a}), 147.17 (CH=N), 167.23 (Th–C_2), 169.38 (C=O). Anal. Calcd. for $C_{21}H_{20}N_4OS_2$ (%): C, 61.74; H, 4.93; N, 13.71; S, 15.70. Found (%): C, 61.64; H, 4.91; N, 13.71; S, 15.66.

*N-(4-(Chloromethyl)thiazol-2-yl)-N′-((10-methyl-10H-phenothiazin-3-yl)methylene)acetohydrazide (**3b**)*

Purification by column chromatography on silica gel 60, hexane/ethyl acetate (1:2, v/v). Yellow-brown crystals yield 49.3% (0.19 g), m.p. = 116–117°C; IR (ATR) ν_{max} (cm^{-1}): 3058 (νC–H), 2981 (νC–H), 2934 (νC–H), 2872 (νC–H), 1696 (νC=O), 1599 (νC=N), 1574 (νC=C), 1367. MS (EI) *m/z*: 443 (M^+), 399, 267, 253, 226, 223, 197, 169, 147, 43 (100%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 1.44 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.55 (s, 3H, –CH_3), 3.97 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 4.64 (s, 2H, –CH_2Cl), 6.87 (d, 1H, ^3J = 8.6 Hz, H_1), 6.71 (s, 1H, Th–H_5), 6.93 (d, 1H, ^3J = 8.6 Hz, H_9), 6.98 (t, 1H, ^3J = 7.3 Hz, H_7), 7.16 (dd, 1H, ^3J = 7.3 Hz, H_6), 7.20 (t, 1H, ^3J = 8.6 Hz, H_8), 7.56 (s, 1H, H_4), 7.63 (dd, 1H, ^3J = 8.6 Hz, H_2), 9.78 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): 13.01 (–CH_3), 22.32 (–CH_3), 41.22 (–CH_2), 43.39 (–CH_2Cl), 112 (Th–C_5), 114.32 (C_9), 115.65 (C_1), 122.12 (C_7), 123.52 (C_{4a}), 124.64 (C_{5a}), 125.15 (C_2), 126.31 (C_8), 127.25 (C_6), 129.47 (C_4), 129.65 (C_3), 145.23 (Th–C_4), 145.75 (C_{9a}), 146.02 (C_{10a}), 147.73 (CH=N), 162.34 (Th–C_2), 169.17 (C=O). Anal. Calcd. for $C_{21}H_{19}ClN_4OS_2$ (%): C, 56.94; H, 4.32; N, 12.65; S, 14.47. Found (%): C, 56.71; H, 4.27; N, 12.54; S, 14.42.

*N′-((10-Ethyl-10H-phenothiazin-3-yl)methylene)-N-(4-phenylthiazol-2-yl) acetohydrazide (**3c**)*

Purification by column chromatography on silica gel 60, toluene/ethyl acetate (1:1 v/v) or crystallization from ethanol. Green

crystals, yield 65% (0.30 g), m.p. = 170–172°C; IR (ATR) ν_{max} (cm^{-1}): 3107 (νC–H), 3057 (νC–H), 2985 (νC–H), 1679 (νC=O), 1599 (νC=N), 1575 (νC=C), 1548 (νC=C), 1362. MS (EI) *m/z*: 470 (M^+), 427, 398, 267, 252, 239, 226, 223, 176, 134, 43 (100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.44 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.59 (s, 3H, –CH_3), 3.96 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 6.88 (d, 1H, ^3J = 8.6 Hz, H_1), 6.90 (d, 1H, ^3J = 8.6 Hz, H_9), 6.95 (t, 1H, ^3J = 7.3 Hz, H_7), 7.09 (dd, 1H, ^3J = 7.3 Hz, H_6), 7.15 (t, 1H, ^3J = 8.6 Hz, H_8), 7.21 (s, 1H, Th–H_5), 7.41–7.43 (m, 5H, Ar–H), 7.57 (s, 1H, H_4), 7.62 (dd, 1H, ^3J = 8.6 Hz, H_2), 9.78 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.85 (–CH_3), 22.86 (–CH_3), 42.45 (–CH_2), 111.7 (Th–C_5), 114.73 (C_9), 115.56 (C_1), 122.98 (C_7), 123.22 (C_{4a}), 123.53 (C_{5a}), 126 (C_2), 126.44 (C_{meta}), 127.4 (C_8), 127.57 (C_{orto}), 128.00 (C_{para}), 128.15 (C_6), 128.24 (Th–C_4), 128.70 (C_4), 128.77 (C_3), 130.17 (CH=N), 143.05 (C_{9a}), 150.87 (C_{10a}), 172.18 (Th–C_2), 190.06 (C=O). Anal. Calcd. for $C_{26}H_{22}N_4OS_2$ (%): C, 66.36; H, 4.71; N, 11.91; S, 13.62. Found (%): C, 66.28; H, 4.62; N, 11.91; S, 13.54.

*N-(5-Acetyl-4-methylthiazol-2-yl)-N′-((10-ethyl-10H-phenothiazin-3-yl)methylene)acetohydrazide (**3d**)*

Purification by column chromatography on silica gel 60, toluene/ethyl acetate (1:1 v/v) or crystallization from ethanol. Yellow crystals, yield 52.4% (0.26 g), m.p. = 173–174°C; IR (ATR) ν_{max} (cm^{-1}): 3058 (νC–H), 2969 (νC–H), 2928 (νC–H), 2860 (νC–H), 1681 (νC=O), 1657 (νC=O), 1597 (νC=N), 1572 (νC=C), 1502 (νC=C), 1365, 1328. MS (EI) *m/z*: 450 (M^+), 407, 286, 268, 252, 239, 225, 223, 197, 141, 43 (100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.44 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.51 (s, 3H, Th–CH_3), 2.62 (s, 3H, –CH_3), 2.65 (s, 3H, –CH_3), 3.97 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 6.89 (d, 1H, ^3J = 7.6 Hz, H_9), 6.90 (d, 1H, ^3J = 8.6 Hz, H_1), 6.95 (t, 1H, ^3J = 7.6 Hz, H_8), 7.09 (dd, 1H, ^3J = 7.6 Hz, H_6), 7.15 (t, 1H, ^3J = 7.6 Hz, H_7), 7.57 (s, 1H, H_4), 7.63 (dd, 1H, ^3J = 8.6 Hz, H_2), 9.78 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 13.00 (–CH_3), 17.69 (Th–CH_3), 29.07 (–CO–CH_3), 31.56 (–CO–CH_3), 42.60 (–CH_2), 111.93 (Th–C_5), 114.51 (C_9), 115.35 (C_1), 122.58 (C_7), 123.82 (C_{4a}), 124.94 (C_{5a}), 125.45 (C_2), 126.85 (C_8), 127.69 (C_6), 128.00 (C_4), 128.20 (C_3), 141.83 (Th–C_4), 144.08 (C_{9a}), 146.08 (C_{10a}), 145.31 (–CH=N), 169.32 (Th–C_2), 188.72 (–C=O), 189.31 (–C=O). Anal. Calcd. for $C_{23}H_{22}N_4O_2S_2$ (%): C, 61.31; H, 4.92; N, 12.43; S, 14.23. Found (%): C, 61.22; H, 4.91; N, 12.36; S, 14.18.

*Ethyl 2-(1-acetyl-2-((10-ethyl-10H-phenothiazin-3-yl)methylene)hydrazinyl)-4-methylthiazole-5-carboxylate (**3e**)*

Purification by column chromatography on silica gel 60, toluene/ethyl acetate (1:2 v/v) or crystallization from ethanol. Yellow-green crystals, yield 62.6% (0.29 g), m.p. = 163–164°C; IR (ATR) ν_{max} (cm^{-1}): 3050 (νC–H), 2987 (νC–H), 2956 (νC–H), 2902 (νC–H), 1707 (νC=O ester), 1680 (νC=O ketone), 1597 (νC=N), 1574 (νC=C), 1544 (νC=C), 1496 (νC=C), 1362, 1296 (νC–O), 1096 (νC–O). MS (EI) *m/z*: 480 (M^+), 437, 392, 388, 282, 267, 252, 225, 199, 149, 119, 97, 43 (100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.38 (t, 3H, ^3J = 7.3 Hz, –CH_3), 1.44 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.62 (s, 3H, Th–CH_3), 2.65 (s, 3H, –CH_3), 3.96 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 4.33 (q, 2H, ^3J = 7.3 Hz, O–CH_2), 6.89 (d, 1H, ^3J = 8.6 Hz, H_1), 6.91 (d, 1H, ^3J = 8.6 Hz, H_9), 6.96 (t, 1H, ^3J = 7.6 Hz, H_7), 7.09 (dd, 1H, ^3J = 7.6 Hz, H_6), 7.15 (t, 1H, ^3J = 8.6 Hz, H_8), 7.56 (s, 1H, H_4), 7.63 (dd, 1H, ^3J = 8.6 Hz, H_2), 9.78 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.85 (–CH_3), 14.34 (–CH_3), 29.68 (–CH_3), 32.39 (Th–CH_3), 42.44 (–CH_2), 60.90 (–CH_2), 114.37 (Th–C_5), 115.31 (C_9), 115.42 (C_1), 123.10 (C_7), 123.53 (C_{4a}), 123.99 (C_{5a}), 124.45 (Th–C_4), 125.15 (C_2), 126.51 (C_8), 127.45 (C_6), 128.24 (C_4), 130.12 (C_3), 144.86 (C_{9a}), 146.12 (C_{10a}), 146.32 (–CH=N), 169.53 (Th–C_2), 188.05 (–CO), 190.02 (–COO). Anal. Calcd. for $C_{24}H_{24}N_4O_3S_2$ (%): C, 59.98; H, 5.03; N, 11.66; S, 13.34. Found (%): C, 59.85; H, 4.99; N, 11.58; S, 13.28.

*Ethyl 2-(2-(1-acetyl-2-((10-ethyl-10H-phenothiazin-3-yl)-methylene)hydrazinyl)-thiazol-4-yl)-acetate (**3f**)*

Purification by column chromatography on silica gel 60, toluene/ethyl acetate (1:1, v/v). Yellow crystals, yield 54.8% (0.26 g), m.p. = 169–170°C; IR (ATR) ν_{max} (cm^{-1}): 3080 (νC–H), 2970 (νC–H), 2860 (νC–H), 1740 (νC=O ester), 1680 (νC=O ketone), 1590 (νC=N), 1575 (νC=C), 1544 (νC=C), 1496 (νC=C), 1362, 1285 (νC–O), 1080 (νC–O). MS (EI) *m/z*: 480 (M^+), 437, 351, 268, 252 (100%), 226, 198, 185, 199, 170, 149, 119, 97, 43. ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.32 (t, 3H, ^3J = 7.3 Hz, –CH_3), 1.43 (t, 3H, ^3J = 7.1 Hz, –CH_3), 2.64 (s, 3H, –CH_3), 3.83 (s, 2H, Th–CH_2–COO), 3.94 (q, 2H, ^3J = 7.1 Hz, N–CH_2), 4.29 (q, 2H, ^3J = 7.3 Hz, O–CH_2), 6.83 (d, 1H, ^3J = 8.6 Hz, H_1), 6.89 (d, 1H, ^3J = 8.1 Hz, H_9), 6.96 (t, 1H, ^3J = 7.6 Hz, H_7), 7.12 (dd, 1H, ^3J = 7.6 Hz, H_6), 7.15 (t, 1H, ^3J = 8.1 Hz, H_8), 7.53 (s, 1H, H_4), 7.61 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.87 (s, 1H, Th–H_5), 9.73 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.91 (–CH_3), 14.32 (–CH_3), 29.68 (–CH_3), 36.25 (–CH_2), 42.36 (–CH_2), 61.26 (–CH_2), 105.23 (Th–C_5), 114.82 (C_9), 115.27 (C_1), 122.83 (C_7), 125.16 (C_{4a}), 126.80 (C_{5a}), 126.95 (Th–C_4), 127.33 (C_2), 127.39 (C_8), 127.86 (C_6), 128.05 (C_4), 129.22 (C_3), 144.73 (C_{9a}), 146.32 (C_{10a}), 146.38 (–CH=N), 169.26 (Th–C_2), 188.12 (–CO), 190.07 (–COO). Anal. Calcd. for $C_{24}H_{24}N_4O_3S_2$ (%): C, 59.98; H, 5.03; N, 11.66; S, 13.34. Found (%): C, 59.85; H, 4.98; N, 11.63; S, 13.28.

*Ethyl 2-(1-acetyl-2-((10-ethyl-10H-phenothiazin-3-yl)-methylene)hydrazinyl)thiazole-4-carboxylate (**3g**)*

Purification by column chromatography on silica gel 60, toluene/acetone (1:2 v/v). Yellow crystals, yield 60.5% (0.28 g), m.p. = 114–115°C; IR (ATR) ν_{max} (cm^{-1}): 3056 (νC–H), 2982 (νC–H), 2938 (νC–H), 2906 (νC–H), 1730 (νC=O ester), 1671 (νC=O ketone), 1600 (νC=N), 1575 (νC=C), 1548 (νC=C), 1362, 1203 (νC–O ester), 1070 (νC–O ester). MS (EI) *m/z*: 466 (M^+), 423, 350, 268, 252, 223, 199, 156, 149, 73, 43 (100%). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 1.38 (t, 3H, ^3J = 7.1 Hz, –CH_3), 1.43 (t, 3H, ^3J = 6.8 Hz, –CH_3), 2.65 (s, 3H, –CH_3), 3.94 (q, 2H, ^3J = 6.8 Hz, N–CH_2), 4.38 (q, 2H, ^3J = 7.1 Hz, O–CH_2), 6.84 (d, 1H, ^3J = 8.6 Hz, H_1), 6.89 (d, 1H, ^3J = 7.6 Hz, H_9), 6.96 (t, 1H, ^3J = 7.8 Hz, H_7), 7.13 (dd, 1H, ^3J = 7.8 Hz, H_6), 7.16 (t, 1H, ^3J = 7.6 Hz, H_8), 7.49 (s, 1H, H_4), 7.58 (dd, 1H, ^3J = 8.6 Hz, H_2), 7.89 (s, 1H, Th–H_5), 9.78 (s, 1H, –CH=N). ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): 12.9 (–CH_3), 14.34 (–CH_3), 29.66 (–CH_3), 42.25 (–CH_2), 61.29 (–CH_2), 114.84 (C_9), 115.28 (C_1), 118.49 (C_7), 122.85 (Th–C_5), 123.65 (C_{4a}), 124.79 (C_{5a}), 126.38 (C_2), 126.66 (C_8), 127.48 (C_6), 128.19 (C_4), 128.22 (C_3), 144.15 (C_{9a}), 146.23 (C_{10a}), 146.36 (–CH=N), 169.26 (Th–C_2), 188.22 (–CO), 190.17 (–COO). Anal. Calcd. for $C_{23}H_{22}N_4O_3S_2$ (%): C, 59.21; H, 4.75; N, 12.01; S, 13.74. Found (%): C, 58.83; H, 4.65; N, 11.91; S, 13.71.

Biological activity

In vitro anticancer screening

Cell culture, treatment and cytotoxicity assessment

Cell cultures were performed using Class II LaminAir laminar hoods, Uniequip incubator, Heidolph Titramax 1000 small

vibration orbit platform shaker with reproducible temperature control, Hettich centrifuge with spin-out rotor.

Spectrometric measurements were completed with Biotek Synergy 2 Multi-Mode Microplate Reader with SQ Xenon Flash light source, by well-area colorimetric scanning.

Experimental results were evaluated with the Graph Pad Prism 5 biostatistics software.

Cell cultures

In order to assess the antiproliferative and the antimitotic properties of phenothiazine derivatives **1–3**, the biological systems we used were the tumor cell lines: the highly proliferating HepG2 and CC531S cells. The HepG2 human hepatoblastoma tumor cells were acquired from the European Collection of Cell Cultures (ECCAC). The CC531S colon adenocarcinoma cell line was a generous gift from Dr. Calin Precup from ''Iuliu Hatieganu'' University of Medicine and Pharmacy in Cluj Napoca.

Both cell lines are adherent, they were cultivated under sterile conditions on 25-cm^2 Cole-type culture flasks (Nunclon Easy Flask), in RPMI-1640 cell growth media, supplemented with fetal calf serum (FCS), Hepes, penicillin–streptomycin and glutamine (all chemicals from Sigma). Culture flasks were kept in sterile incubators having constant temperature (37°C), CO_2 level (5%) and humidified atmosphere. Passages were performed using mechanic-enzymatic methods.

Cell treatment and cytotoxicity evaluation

We used the colorimetric MTT method to complete the evaluation of biomaterial cytotoxicity. This cellular inhibition test applicable to the eukaryotic cells is based on the enzymatic turnover of mitochondrial dehydrogenase. The MTT tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma M5655) is a pale yellow water-soluble dye, which is easily taken up by viable cells and reduced [36, 37] to the purple formazan product 1-(4,5-dimethyl-1,3-thiazol-2-yl)-3,5-di(phenyl)-2H-tetrazole. A cleavage of the tetrazolium rings occurs due to the presence of mitochondrial reductases only in intact living cells. The insoluble purple formazan product does not cross the cellular membrane and remains accumulated in the cells. The crystals are dissolved into a colored solution with organic solvents such dimethyl sulfoxide (DMSO).

For the experiment, cells were placed on flat bottom 96-well microplates, in 200 μL media, and after 24 h of incubation the compounds **1–3** solutions were added, 10 μL in each well. The 12 compounds **1–3** were solubilized in DMSO, to obtain the stock solutions and then sequential dilutions were obtained using phosphate buffered saline solution (PBS-Sigma). In the treated wells the final concentrations of **1–3** were between 0.1 and 50 μg/mL for each compound, for every compound a series of 8–10 concentration were tested. For the cell proliferation, the reference values were the untreated cells. For every compound we used reagent blank (media and MTT), color control (wells contains media and phenothiazine derivatives **1–3** solution, but no cells). As positive control we used the antineoplastic drug cisplatin (Actavis Sindan Pharma) in the same concentrations as the studied compounds.

Three independent experiments were performed, all in triplicate. After 24 h of treatment with phenothiazine derivatives **1–3**, the culture media was removed from the wells, without disturbing the attached cells, and 100 μL of MTT-Hanks media solution was added to each well. After 1 h incubation at 37°C, MTT solution was removed, and formazan crystals were solubilized by adding DMSO, which leads to their release from the living cells.

The 96-well plates were measured at 492 nm with a multimode microplate reader, by monochromator-based absorbance detection. The optical density, quantified by colorimetric measurements, is in direct proportional relationship with the amount of formazan in the cells [38] and it is an indicator of the cellular proliferation [39].

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