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Benzothiazole Thiourea Derivatives as Anticancer Agents: Design, Synthesis, and Biological Screening[1]

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Abstract—In a systematic effort to identify a potent anticancer agent, we synthesized benzothiazole thiourea derivatives and examined their cytotoxic activity against five different human and animal cancer cell lines. Benzothiazolylthiocarbamides have been prepared in excellent yields by reaction of substituted 2-amino benzothiazoles with carbon disulfide and dimethyl sulfate followed by their ammonolysis. Cytotoxicity of the four compounds were screened for antitumor activity against human breast cancer cells (MCF-7), human cervix epithelial carcinoma (HeLa), human colon cancer cell line (HT-29), human leukemia cell line (K-562), and mouse neuroblastoma cell line (Neuro-2a) using cisplatin as a reference by MTT assay. Our results presented herein provide experimental evidence that benzothiazolylthiocarbamides induce apoptosis in cancer cell lines. According to flow cytometry results, treatment of HT-29 cells with 1-(6-ethoxy-1,3-benzothiazol-2-yl)thiourea produced a large population of apoptotic cell (79.45%), which was 1.2-fold higher than that produced by cisplatin (65.28%) at the same concentration.

Keywords: anticancer activity, benzothiazolylthiocarbamides, MTT assay, flow cytometry

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INTRODUCTION

Cancer is a mass of cells with malignant transformation, which divide uncontrollably, invade and spread to distant organs, and develop secondary disease. Cancer is among the four major non-communicable diseases (heart disease, cancers, lung disease, and diabetes) that are the leading causes of deaths worldwide. A 2013 survey by the World Health Organization (WHO) estimated that non-communicable diseases (NCDs) kill more than 36 million people each year. Cancer is second in the list; killed approximately 21% of these 36 million people, so that 8.2 million people worldwide died from cancer in 2012. Even though the incidence of cancers is comparatively higher than other diseases, improvements in cancer therapy have significantly reduced the number of deaths due to cancer. Also improvement of quality of life and survival of cancer patients will be greatly enhanced by the development of highly effective drugs to selectively kill malignant cells. In the current study we tried to synthesize derivatives of benzothiazole thiourea and then investigate their anticancer activity.

Benzothiazoles are an important class of heterocycles, which can serve as unique and versatile scaffolds for experimental drug design [1–3]. 2-Aminobenzothiazoles have received considerable attention because of their interesting pharmacological activities, including anticonvulsant [4], analgesic [5], anti-tumor [6, 7], antibacterial [8, 9], antimicrobial [10, 11], and muscle relaxant activities [12]. The combinations of urea and thiourea derivatives with benzothiazole have produced DNA topoisomerase [13, 14] or HIV reverse transcriptase inhibitors [15, 16]. Frentizole (**I**) (Fig. 1) is a non-toxic antiviral and immunosuppressive agent used clinically in rheumatoid arthritis and systemic lupus erythematosus [17] and thiourea derivative YH3945 (**II**) (Fig. 1), a selective and potent inhibitor of farnesylprotein transferase, is being developed for the treatment of cancer [18].

Thiourea and its derivatives have been widely used in research and technological applications such as in the pharmaceutical industry [13, 19], as catalysts in chemical reactions [20–22], and for extraction of toxic metals using a solid supported liquid membrane system [23]. The antiviral [24], cytotoxic [13, 19], and antifungal [25] activities of these compounds are well known. In addition, the corrosion of some metals can be determined using thiourea and its derivatives [26].

Our results presented herein provide experimental evidence that benzothiazolethiourea derivatives induce apoptosis in human cancer cells. These compounds, especially the lead compound (**Vd**), may be useful in the treatment of cancer.

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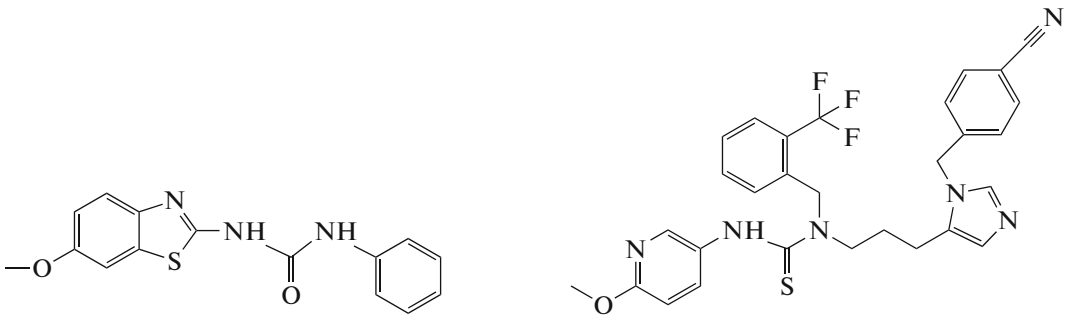
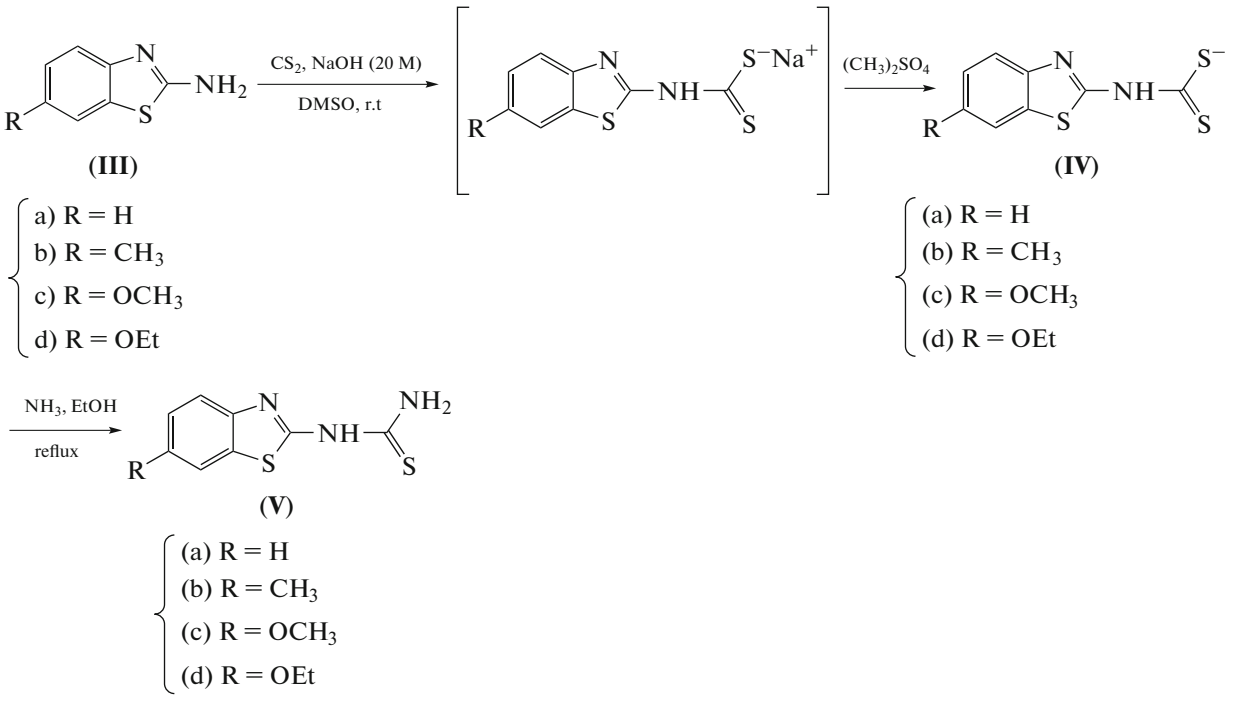


Fig. 1. Structures of frentizole and YH3945, two derivatives of benzothiazoles.

RESULTS AND DISCUSSION

In this study, the potential of four benzothiazole-thiourea derivatives as effective anticancer compounds in vitro were investigated. The interesting pharmacological activities of 2-aminobenzothiazoles, especially as anti-tumor agents, provide a strong rationale for continued research into the development of new compounds containing this structure for improved therapeutic performance.

Benzothiazole thiourea derivatives were prepared by an easy protocol, as shown in Scheme. Treatment of substituted 2-amino benzothiazole (**III**) with carbon disulfide in alkali media gave an intermediate, which was methylated with dimethyl sulfate in excellent yields. Ammonolysis of compounds (**IVa–d**) in ethanol under reflux conditions gave benzothiazole thiourea derivatives (**Va–d**) in excellent yields. Structures of synthesized title compounds (**Va–d**) were confirmed by FT-IR, NMR, and mass spectrometry techniques.



Scheme 1. General synthesis of the benzothiazole thiourea derivatives.

Newly synthesized four compounds were screened for their in vitro growth inhibitory activities against five human and animal cultured cell lines, namely, human breast cancer cells (MCF-7), human cervix epithelial carcinoma (HeLa), human colon cancer cell line (HT-29), human leukemia cell line (K-562), and mouse neuroblastoma cell line (Neuro-2a) by MTT assay using cisplatin as a comparative standard.

Table 1. Cytotoxic activities of compounds (**Va–d**) against MCF-7, HeLa, HT-29, K-562, and Neuro-2a cancer cell lines after 72 h of continuous exposure

	Cell lines					
Compound	MCF-7	HeLa	HT-29	K-562	Neuro-2a	L-929
(**Va**)	8.55 ± 2.33	21.7 ± 3.15	8.1 ± 2.44	69.7 ± 8.18	57.4 ± 6.47	134.1 ± 9.5
(**Vb**)	11.9 ± 2.75	47.3 ± 4.98	14.8 ± 2.23	85.3 ± 9.45	235.3 ± 16.5	135 ± 6
(**Vc**)	12.8 ± 3.25	45.2 ± 5.17	15.0 ± 3.11	98.1 ± 12.5	200.5 ± 18.7	230.7 ± 18.2
(**Vd**)	6.72 ± 3.00	4.97 ± 1.89	3.90 ± 2.05	40.5 ± 5.59	22.7 ± 2.90	107.6 ± 11.7
Cisplatin	6.25 ± 2.13	0.39 ± 0.11	16.2 ± 2.97	25.8 ± 2.93	211.0 ± 20.9	0.7 ± 0.2

[a]The concentration of the complex required to inhibit cell growth by 50%. The experiments were done in triplicate. Data were expressed as the mean of the triplicate. The agent with $IC_{50} > 100$ μM is considered to be inactive.

Table 2. Percentages of the cell death pathways observed by the flow cytometry assay

Treatment	Vital cells, %	Apoptotic cells, %	Late apoptotic/necrotic cells, %	Necrotic cells, %
Control	81.13	8.68	9.45	0.74
Cisplatin	33.74	34.65	30.63	0.98
Compound (**Vd**)	19.73	43.91	35.54	0.82

Results are shown as IC_{50} after 72 h of continuous exposure (Table 1).

For compounds (**Va–d**) and cisplatin as a comparative standard, we have measured their IC_{50} values in all cell lines at concentrations in the range between 20 nM and 200 μM. For different cell lines, the values determined for these compounds spanned the range from 3.90 to 98.1 μM (IC_{50} over 200 μM was ignored), while those found for the comparative standard ranged between 0.39 an up to 200 μM (Table 1). These values confirmed that we prepared weakly-to-moderately cytotoxic compounds, with compound (**Vd**) showing the strongest cytotoxicity in both cell lines. If we set aside some cases in Table 1, cytotoxicity in this assay followed the general trend: (**Vd**) > (**Va**) > (**Vb**), (**Vc**). The highest cytotoxic activity was observed for compound (**Vd**), which was approximately the same and four times more potent than that of cisplatin against the two examined cancer cell lines (MCF-7 and HT-29, respectively). The effect of synthesized compound on mouse fibroblast cell line (L-929) was evaluated as control, simultaneously. Our results confirmed that the synthesized compound produced no cytotoxic effects on L-929 cells. The compounds exhibited no cytotoxicity effects upon 24 and 48 h of exposure.

Based on the results of in vitro cytotoxicity studies of newly synthesized benzothiazole thiourea derivatives, the highly active compound was selected for apoptosis assay by flow cytometry. The results are presented in Table 2 and Fig. 2. Four areas in the diagrams stand for necrotic cells (Q1, left square on the top), late apoptosis or necrosis cells (Q2, right square on the top), live cells (Q3, left square at the bottom), and apoptosis cells (Q4, right square at the bottom), respectively. As it follows from Table 2 and Fig. 2, compound (**Vd**) produced a large population of apoptotic cell (79.45%), which is nearly 1.2-fold larger than that produced by cisplatin (65.28%) at the same concentration. Therefore, the newly synthesized compounds, particularly the lead compound (**Vd**), could induce apoptosis of HT-29 cancer cells. But the proapoptotic property needs further investigation to better understand the precise mechanism of action of these compounds and basic pre-clinical research is needed before they could be recommended for human administration.

In this work the synthesis, spectroscopic characterization, and anticancer activity of benzothiazole thiourea derivatives, are reported. Because of the interesting pharmacological activities of this important class of heterocycles, we used four novel compounds of this family in this investigation. The results obtained can be summarized as follows:

(1) In addition to anticonvulsant, analgesic, antibacterial, antimicrobial, and muscle relaxing properties, for which they are now so well known, benzothiazoles also exhibit a number of other therapeutic effects including anti-cancer activities, which our results confirm by the excellent cytotoxic activities of the newbenzothiazole thiourea derivatives, particularly against human colon cancer cell line (HT-29).

(2) Our results predict that the highest cytotoxic activity of these compounds belongs to compound (**Vd**), which reflects the higher electron donating nature of OEt group in compound (**Vd**) and its steric effects.

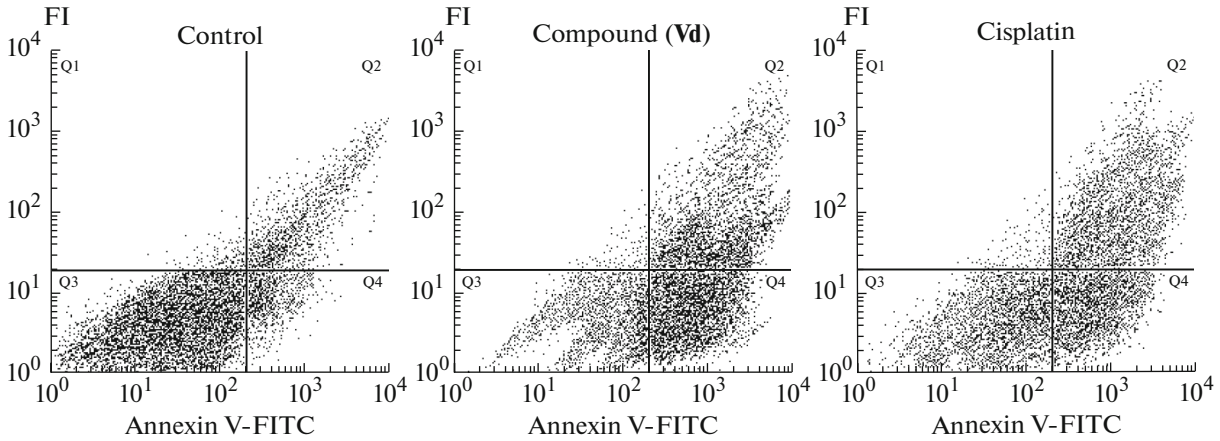


Fig. 2. Flow cytometry results after the exposure of HT-29 cancer cells to the active compound (**Vd**) and cisplatin. Four areas in the diagrams represent four different cell states: necrotic cells (Q1), late apoptotic or necrotic cells (Q2), living cells (Q3), and apoptotic cells (Q4).

(3) Our results confirm that compound (**Vd**) produces a higher population of apoptotic cells than other investigated compounds in this study, even higher than cisplatin. This is the desirable death mechanism for a cytotoxic drug.

EXPERIMENTAL

All solvents, reagents, and compounds were purchased from Merck and Fluka companies. RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were purchased from GIBCO (Gaithersburg, USA). Penicillin and streptomycin were purchased from Biochrom AG (Berlin, Germany). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) was purchased from Sigma Co., Ltd. Cisplatin was purchased from Sigma Aldrich.

NMR spectra were recorded on Avance Brucker-400 MHz spectrometers; ^{1}H spectra were scanned at 400 MHz and ^{13}C, at 100 MHz. All chemical shifts in NMR experiments are reported as δ, ppm, and were referenced to residual solvent. FT-IR spectra (KBr; ν, cm^{-1}) were recorded on an AVATAR-370-FTIR ThermoNicolet. All mass spectra were scanned on a Varian Mat CH-7 at 70 eV. Reaction was monitored by TLC using silica gel plates and the products were identified by comparison of their spectra and physical data with those of the authentic samples. Melting points were measured on an Electrothermal 9100 apparatus.

*General Synthesis of Methyl-1,3-Benzothiazol-2-ylcarbamodithioate Derivatives (**IVa–d**)*

A solution of 2-aminobenzothiazole derivative (**III**) (0.02 mol) in dimethylsulfoxide (10 mL) was stirred vigorously. To this carbon disulfide (1.6 mL) and 1.2 mL of 20 M aqueous sodium hydroxide were added dropwise during 30 min with stirring. Dimethyl sulfate (0.02 mol) was added gradually, keeping the reaction mixture stirring in freezing mixture for 2 h. The reaction mixture was then poured into ice water. The solid obtained was filtered, washed with water, dried under high vacuum, and recrystallized from ethanol.

Methyl-1,3-benzothiazol-2-ylcarbamodithioate (IVa). $C_9H_8N_2S_3$. Yellow solid (85% yield), mp 196–200°C; IR: 3230, 3188, 3050, 1605, 1525, 1448, 748; ^{1}H NMR ($CDCl_3$): 2.75 (s, 3H, CH_3), 4.9 (br s, 1H, NH), 7.45–7.49 (t, J = 7.6 Hz, 1H), 7.53–7.58 (t, J = 7.2 Hz, 1H), 7.68–7.88 (d, J = 8.0 Hz, 1H), 7.83–7.85 (d, J = 8.0 Hz, 1H);^{13}C NMR: 199.9, 174.5, 153.2, 130.8, 125.3, 124.5, 121.8, 118.3, 19.0; MS (m/z): 240 (M^+), 238, 191, 165, 134, 90.

Methyl(6-methyl-1,3-benzothiazol-2-yl)carbamodithioate (IVb). $C_{10}H_{10}N_2S_3$. Yellow solid (78% yield), mp 197–199°C; IR: 3247, 3199, 2987, 3060, 2971, 1681, 1581, 1527, 1358, 743; ^{1}H NMR (DMSO-d_6): 2.39 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 3.38 (br s, 1H, NH) 7.30–7.32(d, J = 8.4 Hz, 1H), 7.40–7.42 (d, J = 8.0 Hz, 1H),7.71 (s, 1H); ^{13}C NMR (DMSO): 185.2, 134.5, 129.1, 126.5, 123.1, 114.6, 21.4, 18.4; MS (m/z): 254 (M^+), 252, 206, 163, 147, 91, 43.

Methyl(6-methoxy-1,3-benzothiazol-2-yl)carbamodithioate (IVc). $C_{10}H_{10}N_2OS_3$. Yellow solid (81% yeild), mp 197°C; IR: 3145, 3071, 2987, 2826, 1603, 1573, 1330, 834; ^{1}H NMR ($CDCl_3$): 2.70 (s, 3H, CH_3), 3.88 (s, 3H, CH_3), 7.07–7.09 (d, J = 9.2 Hz, 1H), 7.27 (s, 1H), 7.69–7.71 (d, J = 7.6 Hz, 1H), 12.30 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): 157.2, 153.9,

146.1, 142.2, 130.9, 120.0, 115.7, 104.4, 55.8, 18.6, MS (*m/z*): 270 (M^+), 268, 220, 196, 178, 134, 91, 28.

Methyl(6-ethoxy-1,3-benzothiazol-2-yl)carbamodithioate (IVd). $C_{11}H_{12}N_2OS_3$. Yellow solid (73% yeild), mp 197°C; IR: 3186, 3068, 2975, 2913, 2880, 1602, 1568, 1323, 988, 816; ^{1}H NMR (DMSO-d_6): 1.32–1.36 (t, J = 8.0 Hz, 3H, CH_3), 2.55 (s, 3H, CH_3), 4.03–4.08 (q, J = 6.8 Hz, 2H, CH_2), 7.07–7.09 (d, J = 8.0 Hz, 1H), 7.44 (s,1H), 7.53 (d, 1H), 13.76 (br s, 1H, NH); ^{13}C NMR (DMSO): 156.3, 128.2, 116.7, 116.3, 107.1, 64.2, 18.3, 15.0, MS (*m/z*): 284 (M^+), 282, 235, 206, 178, 164, 57.

*General Synthesis of 1-(1,3-Benzothiazol-2-yl)thiourea Derivatives (**Va–d**)*

Methyl benzo[*d*]thiazol-2-ylcarbamodithioate derivatives (**IV**) (0.01 mol) were dissolved in ethanol (25 mL). To this ammonia (0.1 mol) was added and refluxed for 4 h. The reaction mixture was cooled and poured into ice water. The solid obtained was filtered, washed with water, dried under high vacuum, and recrystallized from ethanol.

1-(1,3-Benzothiazol-2-yl)thiourea (Va). $C_8H_7N_3S_2$. Light yellow solid (68% yield), mp 215–220°C; IR: 3271, 3185, 3121, 3024, 1614, 1567, 1523, 1187, 754; ^{1}H NMR (DMSO-d_6): 7.14–7.18 (t, J = 16 Hz, 1H), 7.27–7.31 (t, J = 16 Hz, 1H), 7.56–7.58 (d, J = 8.0 Hz, 1H), 7.61–7.63 (d, J = 8.0 Hz, 1H), 9.48–9.54 (br s, 2H, NH_2), 10.50–10.63 (br s, 1H, NH); ^{13}C NMR (DMSO): 181.4, 174.5, 153.2, 130.8, 125.3, 124.5, 121.8, 118.3; MS (*m/z*): 209 (M^+), 207, 149, 122, 95, 60; Anal. calcd for $C_8H_7N_3S_2$: C, 45.91; H, 3.37; N, 20.08; S, 30.64. Found: C, 45.71; H, 3.40; N, 19.00; S, 30.96.

1-(6-Methyl-1,3-benzothiazol-2-yl)thiourea (Vb). $C_9H_9N_3S_2$. Light green solid (65% yield), mp 215°C; IR: 3269, 3182, 3113, 2974, 1617, 1558, 1527, 1182, 812; ^{1}H NMR (DMSO-d_6): 2.25 (s, 3H, CH_3), 7.24–7.22(d, J = 8.0 Hz, 1H), 7.60–7.56 (d, J = 16.0 Hz, 1H), 7.72 (s, 1H, NH), 9.10 (s, 2H, NH_2), 11.79 (br s, 1H, NH); ^{13}C NMR (DMSO): 181.4, 174.5, 150.2, 134.1, 130.7, 126.6, 121.3, 117.1, 20.9; MS (*m/z*): 223 (M^+), 221, 204, 163, 135, 91, 76, 60, 28.

1-(6-Methoxy-1,3-benzothiazol-2-yl)thiourea (Vc). $C_9H_9N_3OS_2$. Light yellow solid (60% yield), mp 160°C; IR: 3272, 3177, 3125, 3025, 2965, 2835, 1600, 1529, 1487, 1225, 830; ^{1}H NMR (DMSO-d_6): 3.83 (s, 3H, CH_3), 7.07–7.09 (d, J = 9.2 Hz, 1H), 7.27 (s, 1H), 7.69–7.71(d, J = 7.6 Hz, 1H), 9.08 (s, 2H, NH_2), 11.70 (br s, 1H, NH); ^{13}C NMR (DMSO):181.4, 174.5, 156.7, 145.5, 131.9, 118.2, 114.6, 104.9, 55.8; MS (*m/z*): 239 (M^+), 235, 221, 204, 196, 181, 164, 137, 95, 69, 29.

1-(6-Ethoxy-1,3-benzothiazol-2-yl)thiourea (Vd). $C_{10}H_{11}N_3OS_2$. Yellow solid (64% yield), mp 220–225°C; IR: 3450, 3205, 3158, 3037, 2970, 2931, 1610, 1592, 1531, 1212, 1059, 804; ^{1}H NMR (DMSO-d_6): 1.32–1.35 (t, J = 12.0 Hz, 3H, CH_3), 4.02–4.07 (q, J = 20.0 Hz, 2H, CH_2), 6.97–7.00 (d, J = 12.0 Hz, 1H), 7.50 (s, 1H), 7.58–7.60 (d, J = 8.0 Hz, 1H), 9.03 (s, 2H, NH_2), 11.59 (br s, 1H, NH); ^{13}C NMR (DMSO): 179.9, 159.5, 155.9, 131.9, 121.0, 115.4, 107.9, 106.1, 64.0, 15.1; MS (*m/z*): 253 (M^+), 250, 234, 218, 193, 165, 138, 95, 69, 60, 43, 28.

Biological Studies

Cell culture methods. Human breast cancer cells MCF-7 (ATCC HTB-22), human cervix epithelial carcinoma HeLa (ATCC CCL-2), human colon cancer cell line HT-29 (ATCC HTB-38), human leukemia cell line K-562 (ATCC CCL-243), mouse neuroblastoma cell line Neuro-2a (ATCC CCL-131), and mouse fibroblast L-929 cell line (ATCC CCL-1) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 1.0 mM sodium pyruvate and 5% fetal bovine serum, at 37°C in an atmosphere of 5% CO_2. Cells were plated in 24-well sterile plates at a density of 1×10^4 cells/well in 100 μL of medium and incubated for 24 h. Also MCF-7 and HT-29 cells were cultured in DMEM containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. K-562 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin.

MTT assay in human cancer cell lines. Compounds (**Va–d**) were screened for antitumor activity against human cell lines MCF-7, HeLa, HT-29, and K-562 and mouse cell line Neuro-2a using cisplatin as a comparative standard. Cell viability was evaluated using a colorimetric method based on atetrazolium salt MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]), which is reduced by living cells to yield purple formazan crystals. Cells were seeded in 96-well plates at a density of (2–5) × 10^4 MCF-7, HeLa, HT-29, K-562, and Neuro-2a cells per well in 200 μL of culture medium and left to incubate overnight for optimal adherence. After careful removal of the medium, 200 μL of a dilution series of the compounds in fresh medium were added and incubation was performed at 37°C/5% CO_2 for 24 h or 72 h. Compounds (**Va–d**) were first solubilized in DMSO,

diluted in medium, and added to the cells at final concentrations between 20 nM and 200 μM. The percentage of DMSO in cell culture medium did not exceed 1%. Cisplatin was first solubilized in saline and then added at the same concentrations used for the other compounds. At the end of the incubation period, the compounds were removed and the cells were incubated with 200 μL of MTT solution (500 μg/mL). After 3–4 h at 37°C/5% CO_2, the medium was removed and the purple formazan crystals were dissolved in 200 μL of DMSO by shaking. The cell viability was evaluated by measurement of the absorbance at 570 nm by using a STAT FAX-2100 microplate reader (Awareness Technology, Palm City, FL, USA).The cell viability was calculated dividing the absorbance of each well by that of the control wells (cells treated with medium containing 1% DMSO). Each experiment was repeated at least three times and each point was determined in at least three replicates.

Apoptosis assay for compound (Vd) by flow cytometry. Evaluation of apoptosis by flow cytometry is generally accomplished by methods that use annexin V-FITC as vital dye, which accesses phosphatidylserine exposed on the external membrane at the beginning of apoptosisprocess. The differentiation between apoptotic and necrotic cells can be performed by simultaneous staining with propidium iodide (PI). Therefore, annexin V-FITC was used as a marker of phosphatidylserine exposure and PI as a marker for dead cells. This combination allows differentiation among early apoptotic cells (annexin V-positive, PI-negative), late apoptotic/necrotic cells (annexin V-positive, PI-positive), and viable cells (annexin V-negative, PI-negative) [27].

Cells (5×10^5) were seeded and treated with compound (**Vd**)and incubated for 24 h at a concentration close to the IC_{50} at 37°C. Following treatment, the cells were harvested by trypsinization and centrifugation at 1000 rpm for 5 min. The supernatant was removed and the cell pellet was washed in PBS followed by two washes in binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$). The cells were incubated with an annexin V-FITC antibody (5 mL in 100 mL binding buffer) and incubated at 4°C for 15 min in the dark. Samples were washed in binding buffer and the supernatant was discarded. The pellet was resuspended in 490 mL binding buffer and 10 mL propidium iodide (10 mg/mL PBS) was added to the samples before analysis by flow cytometry. Flow cytometry was done using Partec PAS flow cytometer (Partec GmbH, Germany) with cisplatin as a reference.

Statistical analysis. IC_{50} values expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical tests, including one-way ANOVA, Tukey multiple comparison, or unpaired Student's *t*-tests, were performed using SPSS, ver.17 software. A *p*-value of less than 0.05 was considered as significant.

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