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Synthesis and Evaluation of Cytotoxicity of 6-Amino-4-Aryl-2-Thioxo-1,2,3,4-Tetrahydropyrimidine-5-Carbonitriles[1]

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Abstract—Several derivatives of 6-amino-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitriles were synthesized via Biginelli type reaction and tested for their anti-proliferative activity on human breast cancer (MCF-7) and human colon carcinoma (HT29) cell lines. Malignant and non-malignant cells were cultivated in RPMI medium and incubated with different concentrations of these pyrimidines. Cell viability was evaluated by MTT assay. Apoptotic cells were determined using DAPI (4'-6-diamidino-2-phenylindole) and propidium iodide staining of DNA fragmentation by flow cytometry (sub-G1 peak). 6-Amino-4-(4-chlorophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile and 6-amino-4-[4-dimethylamino)phenyl]-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile decreased the viability of MCF-7 and HT29 cells, in contrast to L929 cells. These compounds induced a sub-G1 peak inflow cytometry histograms of treated cells indicating that apoptosis is involved in their toxicity.

Keywords: apoptosis, cytotoxic evaluation, pyrimidines, toxicity

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INTRODUCTION

Cancer is a group of diseases characterized by uncontrolled growth and spread of abnormal cells. If the spread is not controlled, it can result in a death. Breast cancer is the most common cause of cancer death among women (522000 deaths in 2012) and the most frequently diagnosed cancer among women in 140 of 184 countries worldwide [1]. Similarly, the colorectal cancer continues to be a major health problem worldwide [2, 3]. At present, with the development of molecular biology, chemotherapy is becoming a more important therapeutic method. Hence, the search for new drugs has always been in demand in order to improve the efficiency of response to anticancer chemotherapy.

Being a building unit of DNA and RNA, pyrimidine derivatives are found to be associated with a variety of chemotherapeutic effects, including antineoplastic [4, 5], angiogenic [6], and enzyme inhibitory effects [7, 8]. Literature survey revealed that 5-fluorouracil (5-FU), one of the early-prepared metabolites, has become one of the most widely used antineoplastic agents due to its inhibition of DNA synthesis [9, 10].

Thio-substituted pyrimidine derivatives occupy a special position among these compounds. They are also identified as potential antimicrobial and anticancer [11–18], as well as anti-inflammatory agents [19]. Prompted by these reports and in continuation of our endeavor on potential anticancer agents [20, 21], we have now focused our attention on preparing a series of 6-amino-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitriles (**I**)–(**X**) and screened them for antiproliferative activity against human breast cancer (MCF-7), human colon carcinoma (HT29), and non-malignant mouse fibroblast (L929) cell lines. The MCF-7 and HT29 cell lines are known as widely used model systems for the study of breast [22] and colorectal [23] cancers.

RESULTS AND DISCUSSION

Chemistry

Several methods are well known for the synthesis of pyrimidine derivatives. In the current study, Biginelli type reaction was utilized for the synthesis of substituted pyrimidine derivatives [24, 25]. According to the recent report by Ghodasara et al. [17], several derivatives of 6-amino-4-phenyl-2-

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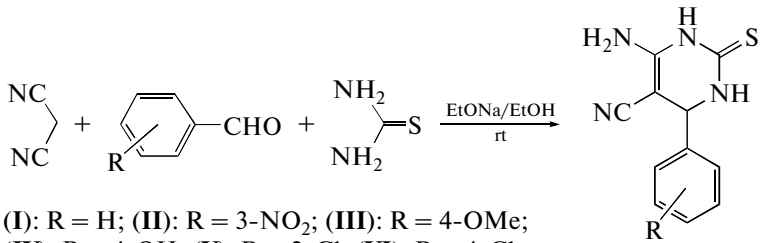
thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitriles were prepared by one-pot and three-component reaction between aryladehydes, malononitrile, and thiourea in EtOH in the presence of sodium ethoxide at room temperature as shown in Scheme.



(**I**): R = H; (**II**): R = 3-NO_2; (**III**): R = 4-OMe; (**IV**): R = 4-OH; (**V**): R = 2-Cl; (**VI**): R = 4-Cl; (**VII**): R = 4-Me; (**VIII**): R = 3-Br; (**IX**): R = 4-Br; (**X**): R = 4-$N(Me)_2$

(**I**)–(**X**)

Scheme.

The results show that the electron withdrawing substituents on phenyl ring can facilitate the cyclization process, while the electron donating substituents have adverse effects.

The structural assignments of the newly synthesized compounds were based upon spectral and elemental analysis data. The previously reported compounds were identified by comparing their physical and spectral data with those of the authentic samples. For example, the IR spectrum of compound (**X**) showed the stretching vibration bands of C=S at 1157 cm^{-1}, C≡N at 2179 cm^{-1}, NH_2 at 3406 and 3328 cm^{-1}, and NH at 3201 cm^{-1}, respectively. The ^1H NMR spectrum of compound (**X**) in DMSO-d_6 showed the D_2O exchangeable peaks of NH_2 and two NH at δ 6.08, 9.63, and 9.91 ppm, respectively; the signals of two methyl groups were assigned at δ 2.88 ppm and the singlet signal of C–H, at 4.84 ppm. Two characteristic doublet signals around δ 6.70–7.05 ppm were determined for aromatic ring protons. In ^{13}C NMR spectrum, the appearance of the carbon signals of the aryl moiety at δ 142.1, 130.4, 125.2, and 123.0 ppm, as well as the thiocarbonyl at δ 174.3, nitrile at δ 119.8, and methyl groups at δ 35.6 ppm, confirmed the heterocyclization. The mass spectrum of compound (**X**) showed the molecular ion peak at *m/z* 273 (M^+) corresponding to the molecular formula $C_{13}H_{15}N_5S$.

Pharmacology

Cytotoxicity of 6-amino-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile derivatives (I)–(X). Many nucleoside analogs have been reported to be potent growth inhibitors in cell cultures [26–28]. Correspondingly, in this study, the target products (**I**)–(**X**) were screened in vitro against MCF-7 and HT29 using MTT assay. First, malignant cells were incubated with various concentrations of compounds (**I**)–(**X**) (15–500 μM) for 48 and 72 h. The results showed that these compounds decreased cell viability in a time- and concentration-dependent manner. Dose inducing 50% cell growth inhibition (IC_{50}) for the tested compounds was calculated and presented in Table 1.

Structure–activity relationship studies revealed that biological activity is largely dependent on the R substituents. Compound (**I**) showed a dose- and time-dependent growth inhibition against MCF-7 and HT29 cell lines. Compounds (**II**) and (**VII**) showed similar effects. Our results reveal that compounds containing halogen groups showed good toxicity effects on two cell lines in comparison with the phenyl analog. Among halogenated derivatives, compounds (**V**) and (**VI**) demonstrated more potent antiproliferative activities than the other halogenated compounds. Similarly, compound (**X**), containing dimethylamino group, showed various effects on two cell lines and had better antiproliferative activity on HT29 cell line after both 48 and 72 h treatment. The other analogs showed no significant toxicity. Generally speaking, compounds (**VI**) and (**X**) were found to be more effective than other derivatives.

In order to compare the cytotoxicity of compounds (**VI**) and (**X**) against malignant and non-malignant cells, another MTT assay was carried out using mouse fibroblast (L929) cells. The results showed that both compounds decreased cell viability in malignant cells more than in non-malignant ones (Fig. 1).

The role of apoptosis in HT29 cells treated with 6-amino-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile derivatives (I)–(X). Recently pyrimidine derivatives were discovered as potent apoptosis inducers [29, 30]. Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA fragmentation, and phagocytic recognition that altogether result in the distinctive structural pathology [31]. Herein, to determine whether apoptosis is involved in the toxicity of compounds (**VI**) and (**X**) against MCF-7 cells, DNA fragmentation was investigated in these cells. The cells were incubated with

Table 1. Doses inducing 50% cell growth inhibition (IC_{50}) of MCF-7 and HT29 cells for compounds (**I**)–(**X**)

Entry	R	IC_{50}, μM			
		MCF-7		HT29	
		48 h	72 h	48 h	72 h
(I)	H	251	211	157	47
(II)	3-NO_2	791	143	154	70
(III)	4-OMe	353	267	364	87
(IV)	4-HO	521	289	453	309
(V)	2-Cl	95	92	42	29
(VI)	4-Cl	78	70	30	25
(VII)	4-Me	454	170	181	78
(VIII)	3-Br	120	72	61	35
(IX)	4-Br	129	111	74	59
(X)	4-$N(Me)_2$	453	80	21	3

Results are Mean ± SEM ($n = 3$).

30 μM of compound (**VI**) or (**X**) for 48 h, stained with DAPI, and evaluated by fluorescent microscopy. As shown in Fig. 2, cells stained with equal intensity of DAPI were condensed and presented strong blue staining under the fluorescent inverted microscope after treatment with compounds (**VI**) and (**X**), which confirmed the occurrence of apoptosis.

It has been reported that DNA fragmentation creates small fragments of DNA that can be eluted following incubation in a hypotonic phosphate–citrate buffer. When cells are stained with a quantitative DNA-binding dye, such as PI, to detect the sub-G1 peak resulting from DNA fragmentation, the cells that have lost DNA would take up less stain and appear to the left of the G1 peak [32]. So apoptosis that followed the treatment with compounds (**VI**) and (**X**) (15, 30, and 60 μM) was measured with PI staining and flow cytometry to detect the sub-G1 peak resulting from DNA fragmentation. Flow cytometry histograms of cells treated with compounds (**VI**) and (**X**) exhibited a sub-G1 peak in HT29 cells. It is the indication of the involvement of an apoptotic process in compound (**VI**)- and (**X**)-induced cell death (Fig. 3 and Table 2).

Table 2. Apoptotic cells determined using PI staining of DNA fragmentation and flow cytometry (sub-G1 peak)

Concentration, μM	Apoptosis, % HT29	
	(VI)	**(X)**
0	6.1	6.1
15	38.8	29.7
30	36.6	35.1
60	46.0	38.2

CONCLUSION

A series of 6-amino-4-aryl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitriles (**I**)–(**X**) were prepared and evaluated for the antiprolifrative activity against MCF-7, HT29, and L929 cell lines by a cell-based screening method. Compounds (**VI**) and (**X**) were found to be more potent than their other analogs, with apoptosis playing an important role in cell death induced by these compounds.

EXPERIMENTAL

Chemistry

Melting points were recorded on a Krüss Melting Point Meter in an open capillary tube and are uncorrected. The IR spectra (ν_{max}, cm^{-1}) were recorded in for KBr pellets on a Buck Scientific spectrophotometer. The ^1H NMR and ^{13}C NMR spectra (δ, ppm) were recorded on a Bruker (400 MHz) spectrometer using DMSO-d_6 or $CDCl_3$ as solvents and TMS as the internal reference standard. Mass spectra were taken on an LKB 9000 mass spectrometer. Chemical ionization mass spectra were recorded on an Eager 300 elemental analyzer. The purification of compounds was routinely checked by TLC using silica gel and the spots were exposed in iodine vapor for visualization.

General Procedure for Synthesis of 6-Amino-4-Aryl-2-Thioxo-1,2,3,4-Tetrahydropyrimidine-5-Carbonitriles (I)–(X)

To a magnetically stirred solution of appropriate benzaldehyde (1 mmol), malononitrile (1 mmol, 0.066 g), and thiourea (1 mmol, 0.076 g) in ethanol (5 mL), sodium ethoxide (2 mmol) was added and the mixture was stirred at room temperature. When the re-

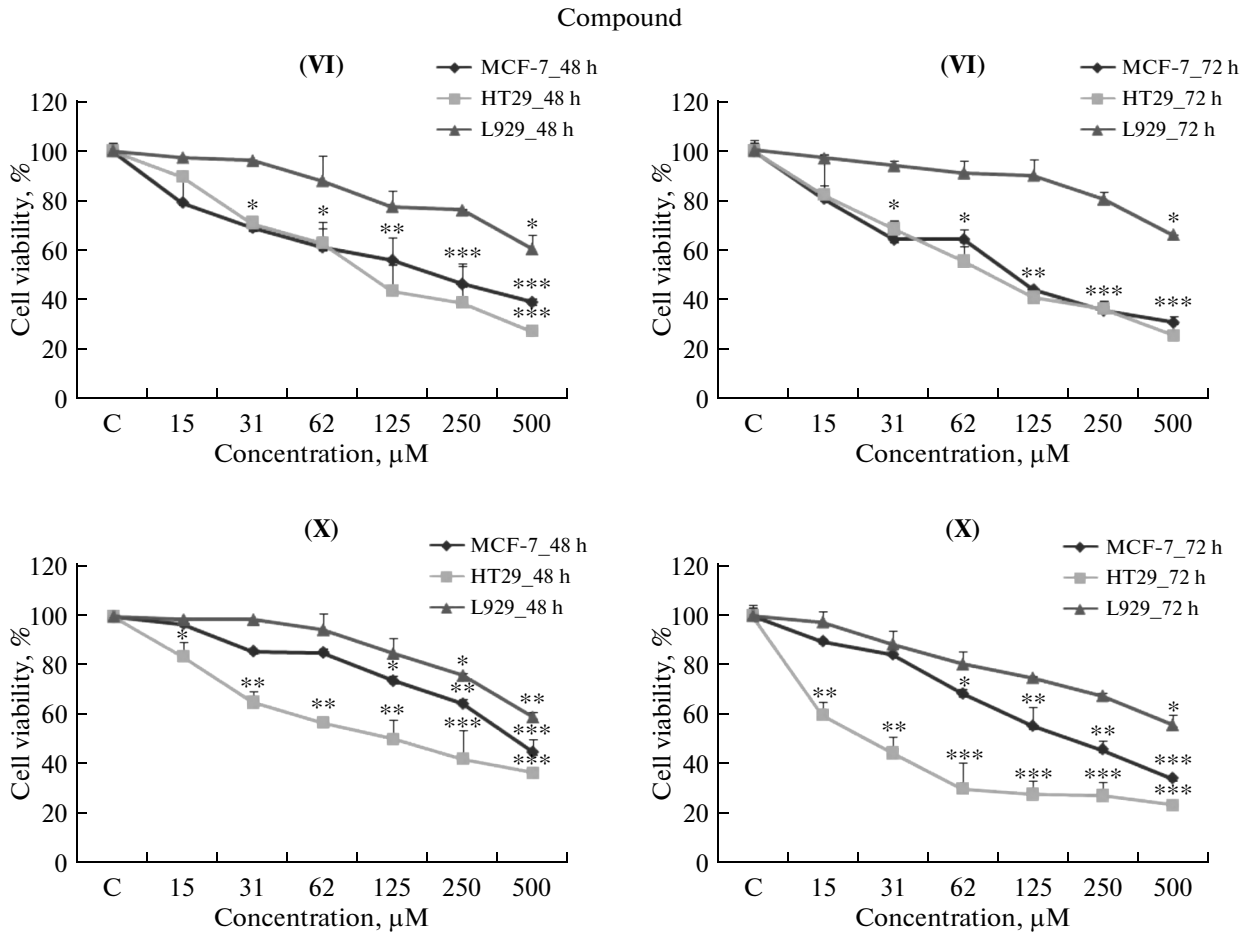


Fig. 1. Dose-dependent growth inhibition of MCF-7, HT29, and L929 cells by compounds **(VI)** and **(X)** (15–500 μM) after 48 and 72 h. Viability was quantified by MTT assay. Results are Mean ± SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with control.

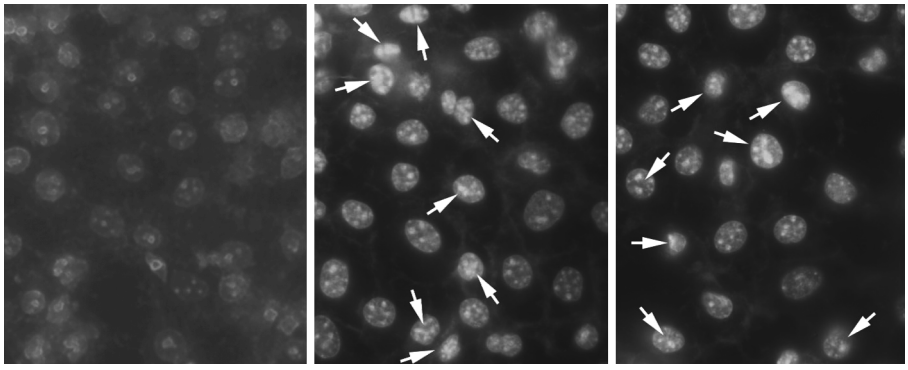


Fig. 2. The evaluation of morphological changes after administrating of compounds **(VI)** and **(X)** in MCF-7 cells. Cells were exposed to 30 μM of compounds **(VI)** and **(X)**, incubated for 48 h. Cells stained with an equal intensity of DAPI were condensed and presented strong blue staining under the fluorescent inverted microscope (magnification ×100). Left to right: control of MCF-7 cells; compound **(VI)** (30 μM); and compound **(X)** (30 μM).

action was completed, which was monitored by TLC using $CHCl_3$: MeOH (9 : 1), the solvent was removed under reduced pressure. Then, water (5 mL) was added to the resulting precipitate and the mixture was neutralized by 0.1 N HCl solution. The solid was filtered and washed with CH_2Cl_2 (2 × 10 mL).

6-Amino-4-phenyl-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (I). Yield 85%, mp 154°C (lit. 150–152°C [17, 25]).

6-Amino-4-(3-nitrophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (II). Yield 56%, mp 137°C (lit. 131–133°C [17]).

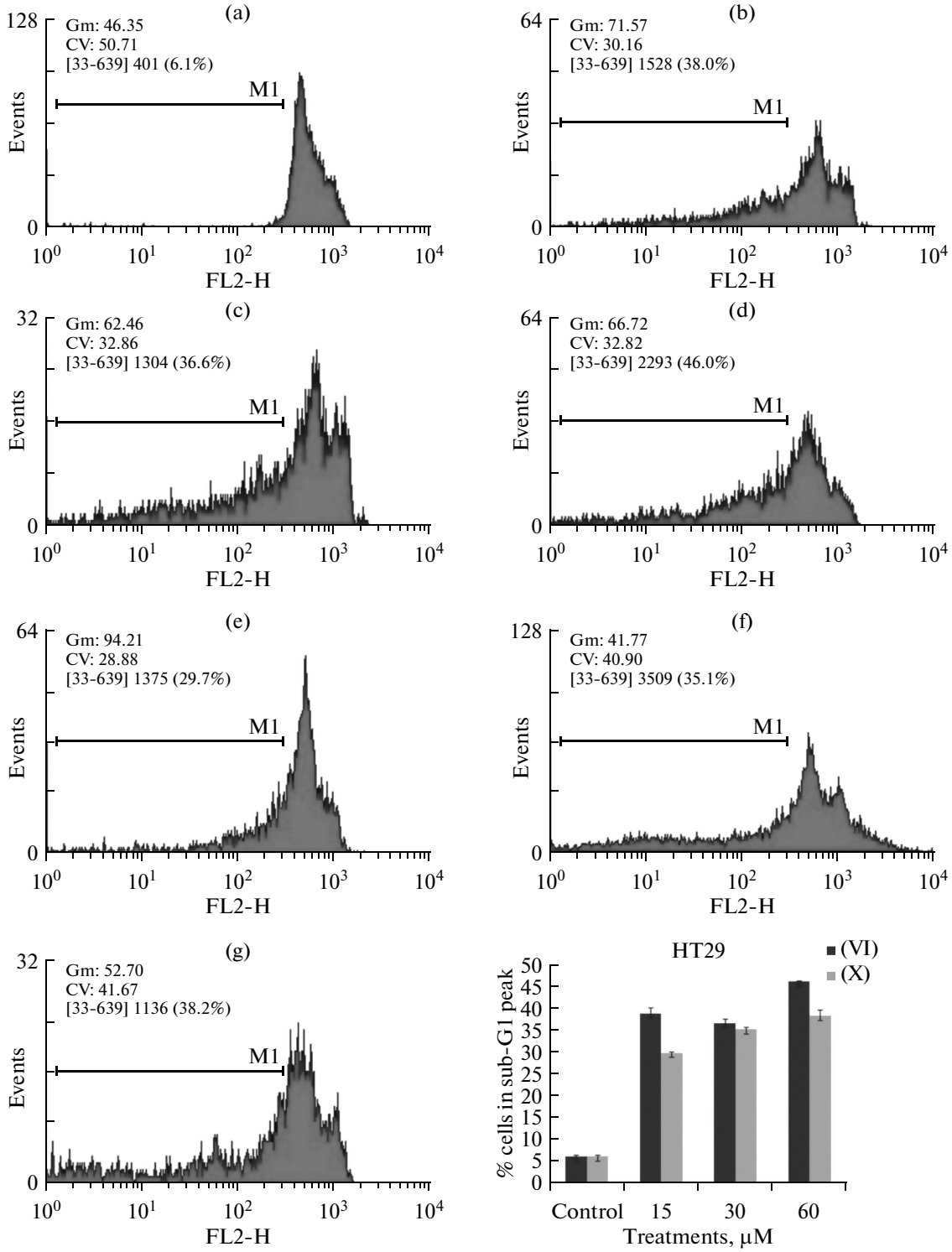


Fig. 3. Flow-cytometry histograms of apoptosis assays by PI method in HT29 cells. Cells were treated with compounds (**VI**) and (**X**) (15, 30, and 60 μM) for 48 h. Sub-G1 peak, as indication of apoptotic cells, was induced in cells treated with compounds (**VI**) and (**V**), but not in control cells. (a) control; (b) compound (**VI**) (15 μM); (c) compound (**VI**) (30 μM); (d) compound (**VI**) (60 μM); (e) compound (**X**) (15 μM); (f) compound (**X**) (30 μM); (g) compound (**X**) (60 μM).

6-Amino-4-(4-methoxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (III). Yield 41%, mp 124°C (lit.118–120°C [17, 25]).

6-Amino-4-(4-hydroxyphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (IV). Yield 37%, mp 146°C (lit. 141–143°C [17]).

6-Amino-4-(2-chlorophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (V). Yield 75%, mp 154°C (lit. 148–150°C [17]).

6-Amino-4-(4-chlorophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (VI). Yield 78%, mp 127°C (lit. 123–125°C [17]).

6-Amino-4-(4-methylphenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (VII). Yield 81%, mp 129°C (lit. 126–128°C [17]).

6-Amino-4-(3-bromophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (VIII). Yield 71%, mp 130°C; IR spectrum: 3417–3317 (NH_2), 3193 (NH), 2195 (C≡N), 1168 (C=S); ^{1}H NMR (DMSO-d_6, 400 MHz): 5.42 (s, 1H, CH-Ar), 6.17 (s, 2H, NH_2, D_2O exchangeable), 7.27–7.49 (m, 4H, Arom-H), 9.66 and 10.08 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz): 174.4 (C=S), 149.5 (C–NH_2), 143.3 (CH of Ph), 129.0 (2CH–CH–Br of Ph), 128.2 (CH of Ph), 122.1 (Br–CH of Ph), 120.0 (CN), 54.8 (C–CN), 39.9 (CH–Ph); EI-MS *m/z* 308 $[M]^+$ (100); anal. calcd. for $C_{11}H_9N_4SBr$ (%): C, 42.73; H, 2.91; N, 18.12; found: C, 42.59; H, 3.06; N, 18.03.

6-Amino-4-(4-bromophenyl)-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (IX). Yield 71%, mp 127°C; IR spectrum: 3406–3325 (NH_2), 3193 (NH), 2183 (C≡N), 1164 (C=S); ^{1}H NMR (DMSO-d_6, 400 MHz,): 5.003 (s, 1H, CH–Ar), 6.19 (s, 2H, NH_2, D_2O exchangeable), 7.16–7.60 (dd, 4H, Arom-H), 9.77 and 10.05 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz): 174.4 (C=S), 149.4 (C–NH_2), 143.3 (CH of Ph), 130.2 (2CH–CH-Br of Ph), 129.3 (2CH of Ph), 123.0 (Br–CH of Ph), 119.9 (CN), 54.7 (C–CN), 39.8 (CH–Ph); EI-MS *m/z* 308 $[M]^+$ (100); anal. calcd. for $C_{11}H_9N_4SBr$ (%): C, 42.73; H, 2.91; N, 18.12; found: C, 42.61; H, 2.89; N, 18.00.

6-Amino-4-[4-(dimethylamino)phenyl]-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (X). Yield 40%, mp 154°C; IR spectrum: 3159–2816 (NH_2), 2179 (C≡N), 1157 (C=S); ^{1}H NMR (DMSO-d_6, 400 MHz,): 2.88 (s, 6H, CH_3), 4.84 (s, 1H, CH–Ar), 6.08 (s, 2H, NH_2, D_2O exchangeable), 6.70–7.05 (dd, 4H, Arom-H), 9.63 and 9.91 (s, 1H, NH, D_2O exchangeable); ^{13}C NMR (DMSO-d_6, 100 MHz): 174.3 (C=S), 149.5 (C–NH_2), 142.1 (CH of Ph), 130.4 (2CH of Ph), 125.2 (2CH–CH–N of Ph), 123.0 (N–CH of Ph), 119.8 (CN), 54.9 (C–CN), 39.6 (CH–Ph), 35.6 (2CH_3–N); EI-MS *m/z* 273 $[M]^+$ (100); anal. calcd. for $C_{13}H_{15}N_5S$ (%): C, 57.14; H, 5.49; N, 25.64; found: C, 57.01; H, 5.63; N, 25.58.

Cell Assays

Each of compounds (**I**)–(**X**) were dissolved at a concentration of 50 mM in dimethyl sulfoxide (DMSO) as stock solution, stored at −20°C and diluted with medium before each experiment. The final DMSO concentration used as solvent control did not exceed 1% throughout the study. The fluorescent probes propidium iodide (PI) and 4'-6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), sodium citrate, and Triton X-100 were purchased from Sigma (St Louis, MO, USA). RPMI and fetal calf serum were purchased from Gibco (Grand Island, New York, USA).

Cell culture. Human breast cancer cell line (MCF-7) and human colon carcinoma cell line (HT29) cancerous and mouse fibroblast (L929) non-malignant cells were obtained from the Pasteur Institute (Tehran, Iran) and maintained at 37°C in a humidified atmosphere (90%) containing 5% CO_2. Cell line was cultured in RPMI-1640 medium with 5% (v/v) fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin. Cells were seeded overnight and then incubated with various concentrations of compounds (**I**)–(**X**) for 48 and 72 h. Cells were seeded at 5000 cells/well onto 96-well culture plates for MTT assay. For each concentration course study, there was a control sample that remained untreated.

Cell viability. Cell viability was determined using a modified MTT assay [33, 34]. Briefly, cells were seeded (5000 cells/well) onto flat-bottomed, 96-well culture plates, allowed to grow for 24 h, and treated with compounds (**I**)–(**X**) (15–500 μM) for 48 and 72 h. MTT solution (5 mg/mL in phosphate-buffered saline; PBS) was added to the wells, cells were incubated for 4 h, plates with adhesive cells were sharply turned upside down removing the medium. DMSO (100 μL) was added to dry wells. The absorption was measured at 570 nm (620 nm as a reference) in an enzyme-linked immunosorbent assay reader.

Apoptosis

DAPI staining. Nuclear morphology of MCF-7 cells was analyzed with DAPI (4'-6-diamidino-2-phenylindole) staining [35]. MCF-7 cells were seeded onto glass cover slips in a six well plate. Twenty-four hours later, cells were treated with compounds (**VI**) and (**X**) for 48 h.The cells were fixed in 4% formaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 30 min. Nuclei were then stained with DAPI, and examined under the fluorescent inverted microscope. Cells with condensed or fragmented nuclei were considered to be apoptotic.

PI staining. Apoptotic cells were detected using PI staining of treated cells followed by flow cytometry to detect the so-called sub-G1 peak [36, 37]. Briefly, HT29 cells were cultured overnight in a 24-well plate and treated with compounds (**VI**) and (**X**) for 48 h. Floating and adherent cells were then harvested and incubated at 4°C overnight in the dark with 750 μL of a hypotonic buffer (50 μg/mL of PI in 0.1% sodium citrate plus 0.1% Triton X-100) before the flow cytometry analysis using a FACS can flow cytometer (BD

Biosciences, Franklin Lakes, New Jersey, USA) was conducted. A total of 10000 events were acquired with fluorescence-activated cell sorting (FACS).

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