ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2015, Vol. 41, No. 2, pp. 201–208. © Pleiades Publishing, Ltd., 2015.

Pyrimidooxadiazine and Triazolopyrimidooxadiazine Derivatives: Synthesis and Cytotoxic Evaluation in Human Cancer Cell Lines[1]

Seyed-Hadi Mousavi[a], Hoda Atapour-Mashhad[b], Mehdi Bakavoli[c], Ali Shiri[c, 2], Marzieh Akbarzadeh[c], and Zahra Tayarani-Najaran[a, 2]

[a] *Pharmacological Research Center of Medicinal Plants, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran*

[b] *Department of Chemistry, Payame Noor University, Mashhad, Iran*

[c] *Department of Chemistry, School of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran*

Received April 23, 2014; in final form, October 20, 2014

Abstract—In vitro antiproliferative activities of some pyrimido[4,5-*e*][1,3,4]oxadiazine and [1,2,4]triazolo[4',3':1,2]pyrimido[4,5-*e*][1,3,4]oxadiazine derivatives were examined in human malignant cancer cell lines. All synthesized compounds inhibited the growth of malignant cells in a dose dependent manner, but among them 1,5,7-trimethyl-3-phenyl-1*H*-[1,2,4]triazolo[4',3':1,2]pyrimido[4,5-*e*][1,3,4]oxadiazine and [(1,5-dimethyl-3-phenyl-1*H*-[1,2,4]triazolo[4',3':1,2]pyrimido[4,5-*e*][1,3,4]oxadiazin-7-yl)sulfanyl]acetonitrile, both with triazole moiety, were found to be more effective than other compounds; they also induced a sub-G1 peak in the flow cytometry histogram of treated cells compared to controls, indicating that apoptotic cell death is involved in toxicity they induce. The results showed that compounds with triazole moiety fused to pyrimido[4,5-*e*][1,3,4]oxadiazine derivatives are more active than those bearing chlorine or pyrrolidine groups at C-7 position.

Keywords: pyrimidooxadiazine, triazolopyrimidooxadiazine, cytotoxicity, tumor cell line, apoptosis

DOI: 10.1134/S1068162015020077

INTRODUCTION

Oxadiazines bearing heteroatoms at positions 1, 2, and 4 or 1, 3, and 4 are interesting and promising heterocyclic compounds, as they possess diverse biological activities [1]. There are some reports in the literature on their cardiovascular, antibacterial, insecticidal, and anticonvulsive activities [2, 3]. In addition, oxadiazines are useful intermediates in the synthesis of tenidap prodrugs or β-lactam antibiotics, particularly, the synthesis of carbapenems and penems [4, 5].

On the other hand, pyrimidine-containing compounds have been reported to offer a variety of anticancer potentials including antitumor [6], antineoplastic [7], antiproliferative [8, 9], and cyclin-dependent kinase [10], angiogenesis [11] and dihydrofolate reductase [12] inhibitory activities.

The promising therapeutic potential of this class of heterocycles prompted us to synthesize various pyrimidooxadiazine and triazolopyrimidooxadiazine derivatives and evaluate the antiproliferative profiles of the obtained derivatives against a panel of four human solid tumor cell lines: adenocarcinomic human alveolar basal epithelial (A549), human cervix carcinoma (HeLa), human liver carcinoma (HepG2), and human breast cancer (MCF-7) cell lines. The most active derivatives were further studied to determine the role of apoptosis in the cytotoxic activity.

RESULTS AND DISCUSSION

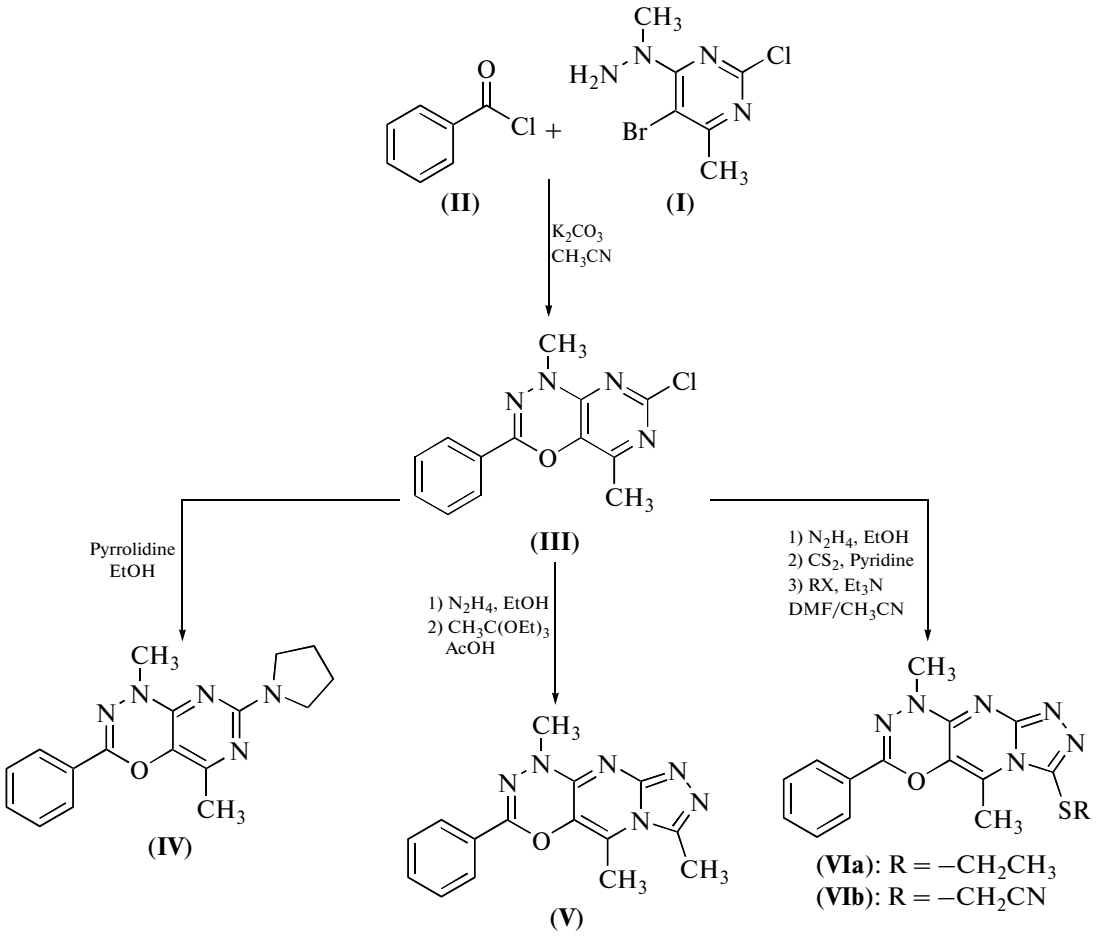
Chemical Synthesis

7-Chloro-1,5-dimethyl-3-phenyl-1*H*-pyrimido[4,5-*e*][1,3,4]oxadiazine (**III**) was prepared by the reaction of 5-bromo-2-chloro-6-methyl-4-(1-methylhydrazino)pyrimidine (**II**) with benzoyl chloride in the presence of K_2CO_3 according to previous published method [13]. Subsequent treatment of compound (**III**) with pyrrolidine in boiling ethanol led to the replacement of chorine atom to give 1,5-dimethyl-3-phenyl-7-(pyrrolidin-1-yl)-1*H*-pyrimido[4,5-*e*][1,3,4]oxadiazine (**IV**). On the other hand, compound (**III**) was treated with hydrazine hydrate and then with triethylorthoacetate in boiling acetic acid to give compound (**V**). Moreover, heating the hydrazino derivative of compound (**III**) and CS_2 in dry pyridine and conversion of the resulted product into the corresponding alkyl derivatives by reaction with ethylbromide and chloroac-

[1] The article is published in the original.

[2] Corresponding authors: e-mail: alishiri@um.ac.ir (A. Shiri); tayaraninz@mums.ac.ir.

etonitrile provided the desired compounds (**VIa**) and (**VIb**), respectively. The structural assignments of compounds (**III**)–(**VI**) are based upon the spectroscopic and microanalytical data (Scheme 1).



Scheme 1.

Cytotoxicity of the Synthesized Compounds (III)–(VI)

The cytotoxicities of the synthesized compounds (**III**)–(**VI**) were examined in malignant cells. At first, HeLa cells were incubated with various concentrations of compounds (**III**)–(**VI**) for 24 h. The results showed that these compounds decreased the viability of cells in a concentration-dependent manner. Among them, compounds (**V**) and (**VIb**) were found to be more effective than the other derivatives ($P < 0.001$) (Fig. 1). The doses of compounds (**III**)–(**V**), (**VIa**), and (**VIb**) that induced 50% cell growth inhibition (IC_{50}) against HeLa cells were 1092.0, 244.4, 91.06, 122.0, and 49.95 μmol L^{-1}, respectively (Fig. 1). Among all newly synthesized compounds, compounds (**V**) and (**VIb**) were selected to be evaluated via MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method for their in-vitro cytotoxic effect against a panel of four human tumor cell lines including A549, HeLa, Hep-G2, and MCF-7. The IC_{50} against A549 and HeLa for compound (**V**) were calculated to be 82.64 and 43.75 μmol L^{-1}, respectively (Fig. 2), while the IC_{50} for compound (**VIb**) against A549, HeLa, HepG2, and MCF-7 cells were 79.85, <25, 135.1, and 156.2 μmol L^{-1}, respectively, after 48 h (Fig. 3).

The Role of Apoptosis in HeLa Cells Treated with Compounds (III)–(VI)

To determine whether apoptosis is involved in cell toxicity of compounds (**V**) and (**VIb**) in A549 and HeLa cell lines, cellular morphological changes and DNA fragmentation were investigated in these cells. HeLa cells were exposed to 60 μmol L^{-1} of compound (**V**), incubated for 48 h, stained with DAPI, and examined with fluorescent microscopy. The same pro-

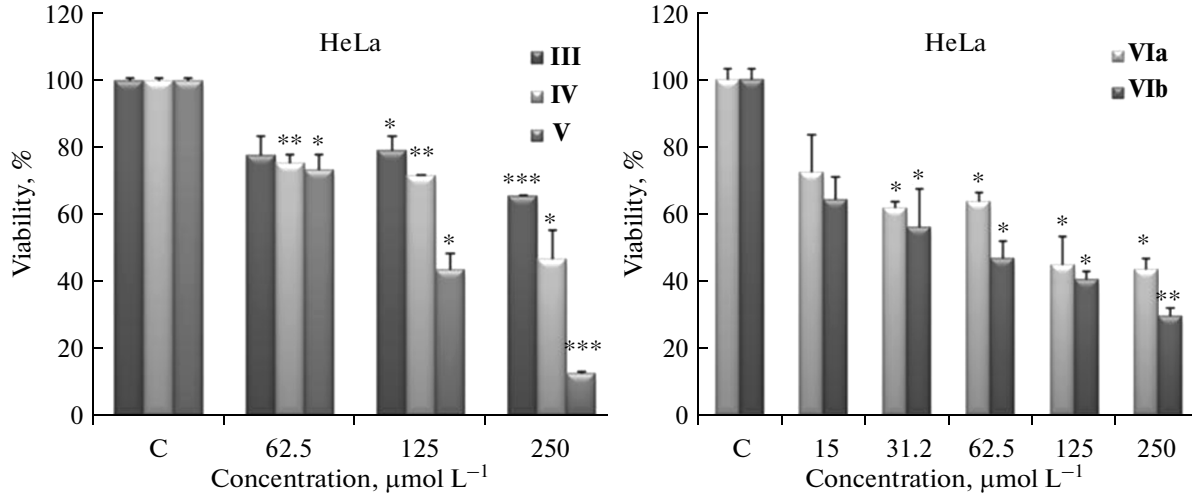


Fig. 1. Dose-dependent growth inhibition of HeLa cells by compounds (**III**)–(**VI**) at different concentrations (μmol L^{-1}) after 24 h. Viability was quantitated by MTT assay. IC_{50} against HeLa cells for compounds (**III**)–(**VI**) were 1092.0, 244.4, 91.06, 122.0, and 49.95 μmol L^{-1}, respectively. Results are mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared to control (C).

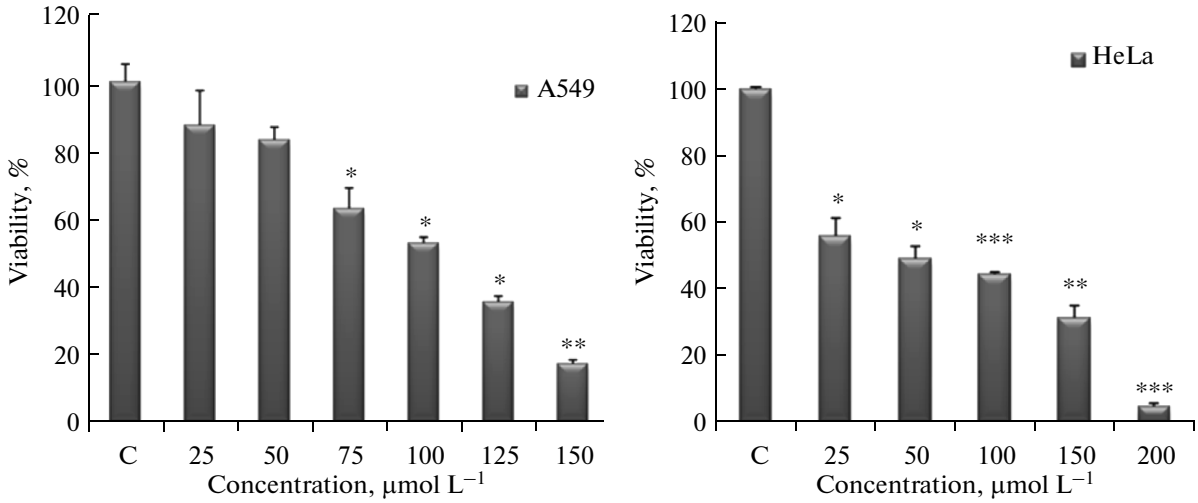


Fig. 2. Dose-dependent growth inhibition of A549 and HeLa cells by compound (**V**) at different concentration (μmol L^{-1}) after 48 h. Viability was quantitated by MTT assay. IC_{50} values against A549 and HeLa cells for compound (**V**) were 82.64 and 43.75 μmol L^{-1}, respectively. Results are mean ± SEM ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and ***$P < 0.001$ compared to control (C).

tocol was used for compound (**VIb**) (60 μmol L^{-1}) on A549 cells. As follows from Figure 4, after treatment with compounds (**V**) and (**VIb**), the cells stained with equal intensity of DAPI were condensed and presented strong blue staining under the fluorescent inverted microscope confirming that apoptosis had occurred. After the treatment with compound (**VIb**) (50 μmol L^{-1}), apoptosis of A549 cells was measured with PI staining and flow cytometry. The same protocol was used for compounds (**V**) and (**VIb**) in HeLa cells to detect the sub-G1 peak resulting from DNA fragmentation. Flow cytometry histograms of compounds (**V**) and (**VIb**)-treated cells exhibited a sub-G1 peak in A549 and HeLa cells. This indicates the involvement of an apoptotic process in compounds (**V**) and (**VIb**)-induced cell death (Figs. 5, 6).

In this study, we investigated the potential antitumor activity of fused heterocyclic compounds of pyrimidooxadiazine and triazolopyrimidooxadiazine derivatives. Different concentrations of compounds (**III**)–(**VI**) were tested for their antiproliferative activities. Structural changes on C-7 and a triazole moiety fused to pyrimido[4,5-*e*][1,3,4]oxadiazine heterocyclic ring appear to have considerable effect on the biological activity of the synthesized compounds. The results showed that among compounds (**III**)–(**VI**), compounds bearing fused triazole ring are more reactive than those bearing chlorine or pyrrolidine substituents on the C-7 position of the compound (**III**).

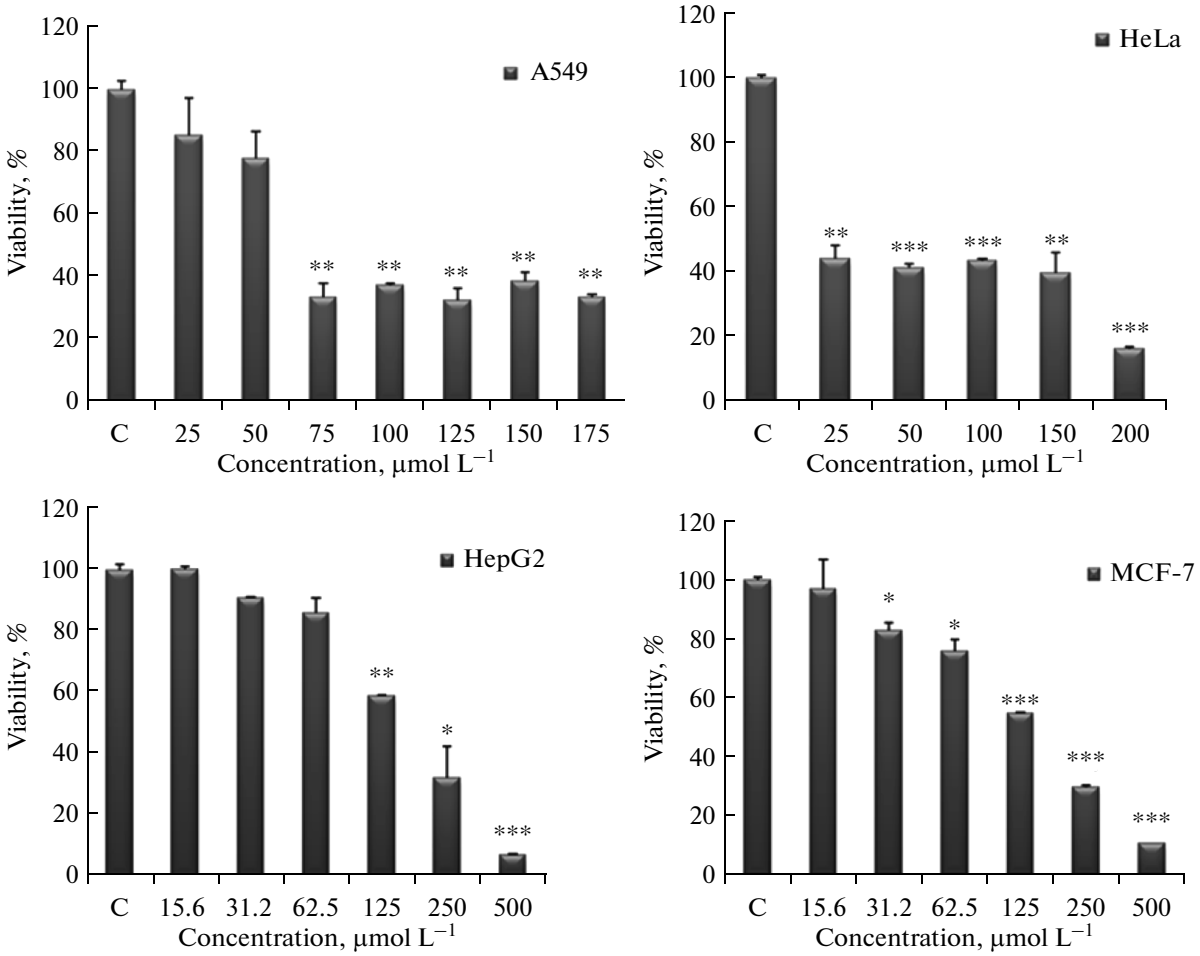


Fig. 3. Dose-dependent growth inhibition of A549, HeLa, HepG2, and MCF-7 cells by compound (**VIb**) at different concentrations (μmol L^{-1}) after 48 h. Viability was quantitated by MTT assay. IC_{50} values against A549, HeLa, HepG2, and MCF-7 for compound (**VIb**) were 79.85, <25, 135.1, and 156.2 μmol L^{-1}, respectively. Results are mean ± SEM (n = 3). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to control (C).

Based on these observations, our efforts were focused on compounds (**V**) and (**VIb**). They were evaluated for their inhibitory activity on A549, HepG2, and MCF-7 cell lines. We then explored the role of apoptosis in compounds (**V**) and (**VIb**)-induced toxicity. In the present study, compounds (**V**) and (**VIb**)-induced apoptosis was involved in the induction of cell death. Apoptotic cells exhibit several biochemical modifications such as protein cleavage, protein cross-linking, DNA fragmentation, and phagocytic recognition, which together result in the distinctive structural pathology [18]. PI-stained cell nuclei permit simple, quantitative, and reproducible measurement of apoptosis. The reduced DNA content of apoptotic nuclei resulted in an unequivocal hypodiploid DNA peak in the red fluorescence channels. According to the previous results [18], DNA fragmentation creates small fragments of DNA that can be eluted and incubated in a hypotonic phosphate–citrate buffer. When cells are stained with a quantitative DNA-binding dye, such as PI, in order to detect the sub-G1 peak resulted from DNA fragmentation, cells that have lost DNA take up less stain and appear to the left of the G1 peak.

CONCLUSION

In summary, we have identified 1,5,7-trimethyl-3-phenyl-1*H*-[1,2,4]triazolo[4',3':1,2]pyrimido[4,5-*e*][1,3,4]oxadiazine (**V**) and [(1,5-dimethyl-3-phenyl-1*H*-[1,2,4]triazolo[4',3':1,2]pyrimido[4,5-*e*][1,3,4]oxadiazin-7-yl)sulfanyl]acetonitrile (**VIb**) analogs as a novel class of antiproliferative agents by a cell-based screening method.

EXPERIMENTAL

Chemistry

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance DRX-400 Fourier transformer spectrometer in $CDCl_3$ at a resonant frequency of 100 MHz using tetramethylsilane (TMS) as internal standard; chemi-

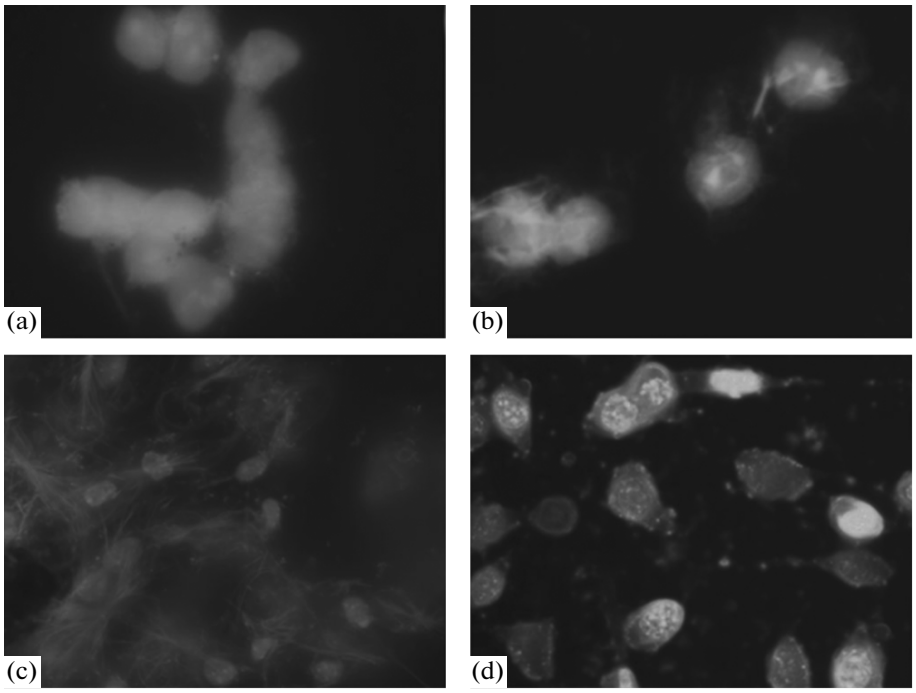


Fig. 4. Evaluation of morphological changes after administrating of compound (**VIb**) in HeLa and A549 cells. HeLa cells were exposed to 60 μmol L^{-1} of compound (**VIb**), incubated for 48 h, and examined for apoptosis by DAPI staining. The same protocol was used for compound (**VIb**) (60 μmol L^{-1}) in A549 cells. Cells stained with an equal intensity of DAPI were condensed and presented strong blue staining under the fluorescent inverted microscope (magnification 100×). (a) Control of A549 cells, (b) compound (**VIb**), 60 μmol L^{-1}, (c) control of HeLa cells, (d) compound (**VIb**), 60 μmol L^{-1}.

cal shifts are reported in δ, ppm. The infrared (IR) spectra (ν_{max}, cm^{-1}) were recorded on an Avatar 370 FT-IR Thermo Nicolet equipment using KBr discs. The mass spectra were recorded on a Varian Mat CH-7 instrument with 70 eV ionizing energy. Elemental analysis was performed on a Thermo Finnigan Flash EA microanalyzer.

Synthesis of 1,5-dimethyl-3-phenyl-7-pyrrolidino-1*H*-pyrimido[4,5-*e*][1,3,4]oxadiazine (IV). A mixture of 7-chloro-1,5-dimethyl-3-phenyl-1*H*-pyrimido[4,5-*e*][1,3,4]oxadiazine (**III**) (1 mmol, 0.27 g) [13] and pyrrolidine (2.2 mmol) in ethanol (5 mL) was heated under reflux for about 2 h. After the mixture was cooled, water was added to the solution. The resulting solid was collected by filtration, washed with water, and recrystallized in ethanol. Yield 79%; mp 168°C; ^{1}H NMR : 1.93 (t, J = 4 Hz, 4H, CH_2), 2.21 (s, 3H, CH_3-pyrimidine), 3.24 (s, 3H, CH_3-oxadiazine), 3.51 (t, 4H, CH_2), 7.4–7.8 (m, 5H, phenyl); ^{13}C NMR: 18.5 (CH_3), 25.5 (CH_2), 25.6 (CH_2), 42.7 (N–CH_3), 54.3 (NCH_2), 54.5 (NCH_2), 126.7 (O–C), 128.1, 128.2, 128.8, 128.9, 129.6, 131.1 (C of phenyl ring), 147.4 (O–C=N), 152.9 (N–C=N), 155.8 (C=N), 158.2 (N–C–N); IR: 3065, 2940, 1155; MS (*m/z*): 309 (M^+). Anal. calcd. for $C_{17}H_{19}N_5O$ (%): C, 66.00; H, 6.19; N, 22.64. Found: C, 65.79; H, 6.12; N, 22.50.

Synthesis of 1,5,7-trimethyl-3-phenyl-1*H*-[1,2,4]triazolo[4',3':1,2]pyrimido[4,5-*e*][1,3,4]oxadiazine (V). To a solution of 7-chloro-derivative (**III**) (3.7 mmol, 1.1 g) in ethanol (20 mL), hydrazine hydrate (2 mL) was added, and the solution was refluxed for 5 h. The resulting precipitate was filtered off and recrystallized from ethanol. Then, to a solution of the prepared hydrazine derivative in HOAc (4 mL), triethylorthoacetate (4 mmol) was added. The reaction solution was heated under reflux for 4 h. After the completion of the reaction which was monitored by TLC using chloroform–methanol (9 : 1), the mixture was cooled to room temperature. Water (10 mL) was added and the mixture was neutralized by saturated $NaHCO_3$ solution. The collected solid was recrystallized from ethanol. Yield 72%; mp 324–326°C; ^{1}H NMR: 2.60 (s, 3H, CH_3-pyrimidine), 2.75 (s, 3H,CH_3-triazol), 3.42 (s, 3H, CH_3–N), 7.32–7.81 (m, 5H, ph), ^{13}C NMR: 15.5 (CH_3-triazole), 16.7 (CH_3-pyrimidine), 42.4 (N–CH_3), 128.2, 128.4, 128.8, 128.9, 129.8, 131.3 (C of phenyl ring), 136.7 (O–C), 149.5 (N–C=N), 149.9 (O–C=N), 152.4 (N–C=N), 155.6 (C=N), 157.2 (N=C–N); IR: 3000, 2900, 1610, 1550, 1490, 1005; MS (*m/z*): 294 (M^+). Anal. calcd. for $C_{15}H_{14}N_6O$ (%): C, 61.22; H, 4.79; N, 28.55. Found: C, 61.10; H, 4.72; N, 28.48.

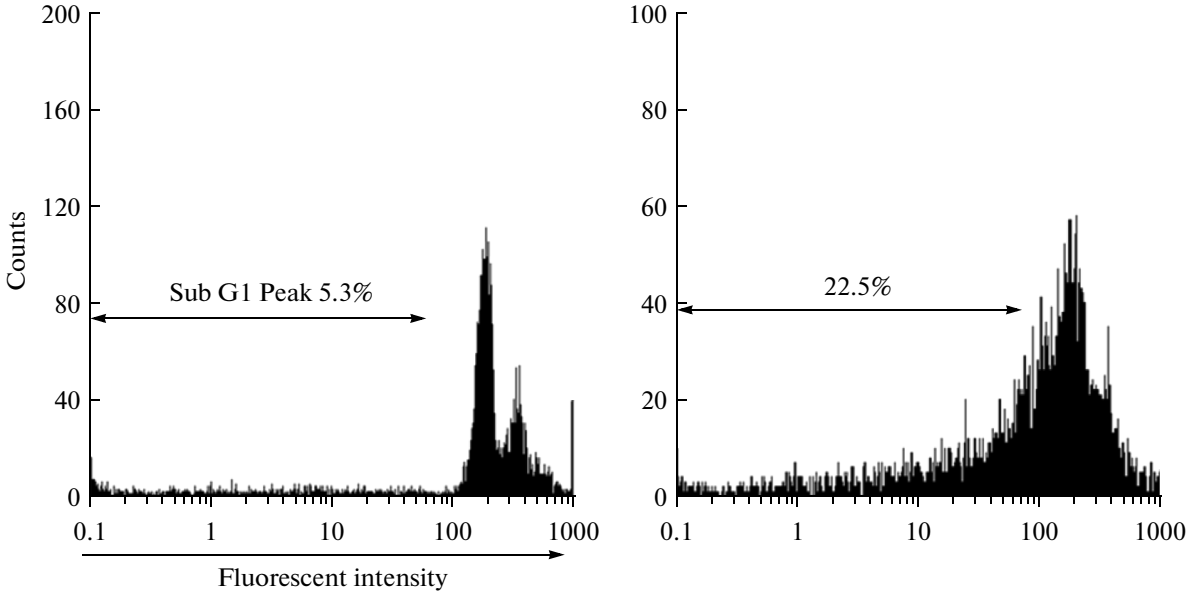


Fig. 5. Flow cytometry histograms of apoptosis assay by PI method in A549 cells. Cells were treated with compound (**VIb**) (50 μmol L^{-1}) for 48 h. Sub-G1 peak as an indicative of apoptotic cells was induced in compound (**VIb**)-treated, but not in control cells. (Left to right: control; compound (**VIb**).)

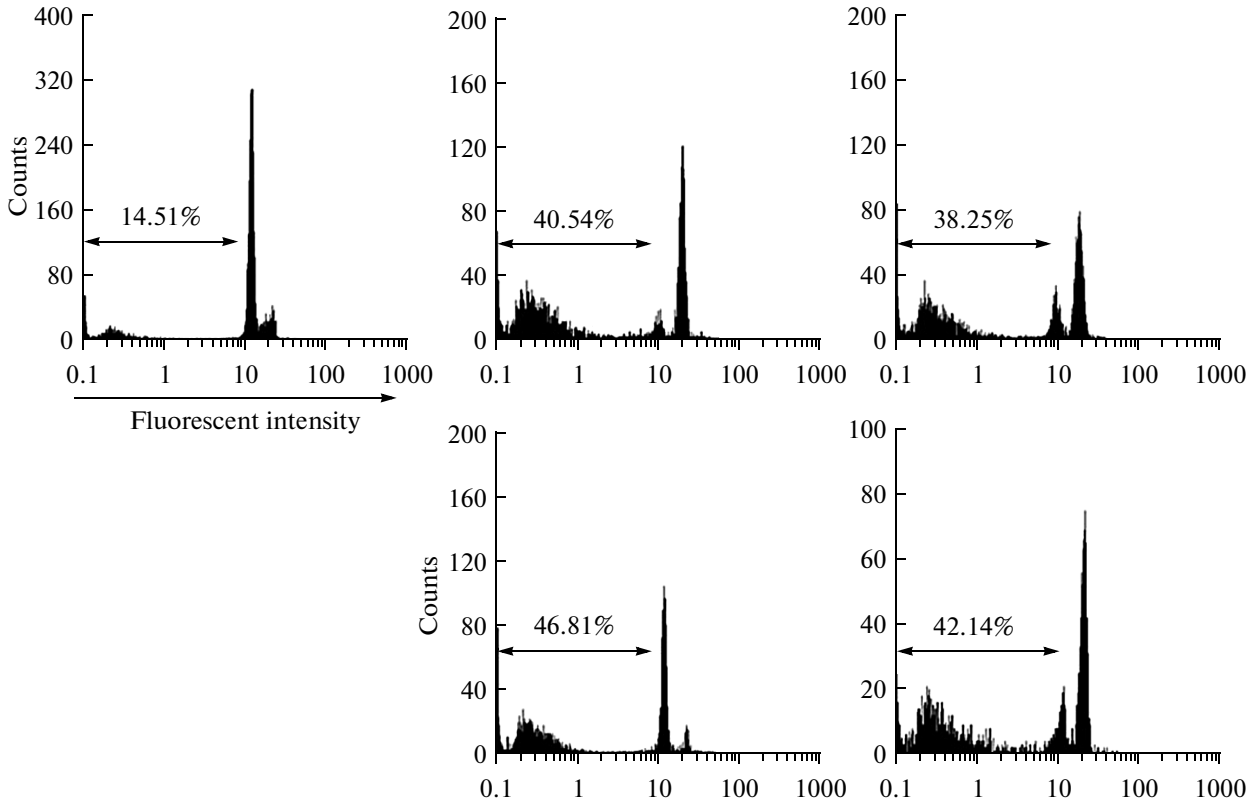


Fig. 6. Flow cytometry histograms of apoptosis assays by PI method in HeLa cells. Cells were treated with compounds (**V**) and (**VIb**) (30 and 60 μmol L^{-1}) for 48 h. Sub-G1 peak as an indicative of apoptotic cells was induced in compounds (**V**) and (**VIb**)-treated, but not in control cells. (Left to right (up): control; compound (**V**), 30 μmol L^{-1}; and compound (**V**), 60 μmol L^{-1}. Left to right (down): compound (**VIb**), 30 μmol L^{-1}, and compound (**VIb**), 60 μmol L^{-1}).)

*General Procedure for the Preparation of 7-Alkylsulfinyl-1,5-Dimethyl-3-Phenyl-1H-[1,2,4]triazolo[4',3':1,2]pyrimido[4,5-e][1,3,4]oxadiazines (**VIa**) and (**VIb**)*

3-Phenyl-1,5-dimethyl-7-hydrazino-1*H*-pyrimido[4,5-*e*][1,3,4]oxadiazine (1 mmol, 0.27 g) prepared from the previous part and CS_2 (1 mL) was refluxed in dry pyridine (7 mL) for 6 h. Then, the mixture was cooled to room temperature and the resulting solid was filtered off and recrystallized from ethanol. Consequently, the precipitant and an appropriate alkyl halide (0.9 mmol) and triethylamine (0.9 mmol) were heated in a mixture of DMF–MeCN (1 : 5) (12 mL) as solvent for 4 h. Then, the solvent was removed under reduced pressure. The crude solid was recrystallized from ethanol.

7-Ethylsulfinyl derivative (VIa). Yield 67%, mp 221–223°C, ^{1}H NMR: 1.43 (t, 3H, CH_3), 2.73 (s, 3H, CH_3-pyrimidine), 3.28 (q, 2H, CH_2), 3.45 (s, 3H, CH_3–N), 7.36–7.53 (m, 3H, ph), 7.74–7.88 (m, 2H, ph); ^{13}C NMR: 13.6 (CH_3), 15.3 (CH_3-pyrimidine), 30.4 (S–CH_2), 43.1 (N–CH_3), 128.2, 128.3, 128.7, 128.8, 129.7, 131.2 (C of phenyl ring), 136.9 (O–C), 146.5 (S–C=N), 149.8 (O–C=N), 152.3 (N–C=N), 155.7 (C=N), 157.3 (N=C–N); IR: 3010, 2980, 1590; MS (*m/z*): 340 (M^+), Anal. calcd. for $C_{16}H_{16}N_6OS$ (%): C, 56.45; H, 4.74; N, 24.69; S, 9.42. Found: C, 56.40; H, 4.62; N, 24.51; S, 9.33.

Cyanomethyl derivative (VIb). Yield 63%, mp 238–240°C, ^{1}H NMR: 2.77 (s, 3H, CH_3-pyrimidine), 3.47 (s, 3H, CH_3–N), 4.03 (s, 2H, CH_2CN), 7.40–7.85 (m, 5H, ph); ^{13}C NMR: 15.3 (CH_3-pyrimidine), 29.1 (S–CH_2), 42.8 (N–CH_3), 117.5 (CN), 128.1, 128.3, 128.6, 128.7, 129.8, 131.3 (C of phenyl ring), 137.3 (O–C), 146.7 (S–C=N), 149.7 (O–C=N), 152.4 (N–C=N), 155.6 (C=N), 157.5 (N=C-N); IR: 3020, 2980, 2220, 1570; MS (*m/z*): 351 (M^+); Anal. calcd. for $C_{16}H_{13}N_7OS$ (%): C, 54.69; H, 3.73; N, 27.90; S, 9.13. Found: C, 54.60; H, 3.70; N, 27.87; S, 9.02.

The newly synthesized compounds (**III**)–(**VI**) were dissolved at a concentration of 25 mmol L^{-1} in dimethylsulfoxide (DMSO) as a stock solution that was stored at –20°C and diluted with cell medium before each experiment.

Cell culture. The fluorescent probe propidium iodide (PI), sodium citrate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT), and Triton X-100 were purchased from Sigma (St Louis, MO, USA). RPMI and FCS were purchased from Gibco (Grand Island, USA). A549, HeLa, HepG2, and MCF-7 cell lines were obtained from Pasteur Institute (Tehran, Iran) and maintained at 37°C in a humidified atmosphere (90%) containing 5% CO_2. The cell lines were cultured in Dulbecco's modified Eagle's medium (RPMI) with 5% (v/v) fetal bovine serum (FBS), 100 units mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. The cells were seeded overnight and then incubated with various concentrations of compounds (**III**)–(**VI**) for 24 h and 48 h. The cells were seeded at 5000 cells/well onto 96-well culture plates for MTT assay and at 100000 cells/well onto a 24-well plate for apoptosis assay. There was a control sample for time course study in each concentration and they also received the equal volume of medium.

Cell viability. Cell viability was determined using a modified MTT assay [14, 15]. Briefly, the cells were seeded (5000 cells/well) onto flat-bottomed 96-well culture plates and allowed to grow for 24 h followed by treatment with compounds (**III**)–(**VI**). After removing the medium, the cells were labeled with MTT solution [5 mg mL^{-1} in phosphate buffered saline (PBS)] for 4 h and the resulting formazan was dissolved in DMSO (100 μL). The absorption was measured at 570 nm (620 nm as a reference) in an enzyme-linked immunosorbent assay (ELISA) reader.

DAPI staining. The cancer cells were incubated on glass cover slips in a six well plate. Twenty-four hours later, the cells were treated with compounds (**V**) and (**VIb**) for 48 h. After washing the cells with PBS for three times, they were fixed in 4% formaldehyde for 30 min and permeabilized with 3% Triton X-100 for 30 min. The cell nuclei were then stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) and examined under the fluorescent inverted microscope. Cells with condensed or fragmented nuclei were considered to be apoptotic.

Apoptosis. Apoptotic cells were detected using PI staining of treated cells followed by flow cytometry to detect the so-called sub-G1 peak [16, 17]. It has been reported that DNA fragmentation creates small fragments of DNA that can be eluted and incubated in a hypotonic phosphate-citrate buffer. When stained with a quantitative DNA-binding dye such as PI, cells that have lost DNA will take up less stain and appear to the left of the G1 peak. Briefly, A549 and HeLa cells were cultured overnight in a 24-well plate and treated with compounds (**V**) and (**VIb**) 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^{-1} PI in 0.1% sodium citrate plus 0.1% Triton X-100) before flow cytometric analysis. Cells were evaluated in a FACScalibur flow cytometer (Partec, Münster, Germany) using the Flomax software.

ACKNOWLEDGMENTS

The authors would like to thank Research Affairs of Mashhad University of Medical Sciences and Ferdowsi University of Mashhad for financially supporting this work. We also thank Dr. H. Nasirli for her kind assistance in flow cytometry. We are also grateful for the editorial assistance of Dr. N. Tayarani-Najaran.

REFERENCES

1. Berkowitz, P.T., Long, R.A., Dea, P., Robins, R.K., and Matthews, T.R., *J. Med. Chem.*, 1977, vol. 20, pp. 134–138.
2. Khan, K.M., Rahat, S., Choudhary, M.I., Rahman, A., Ghani, U., Perveen, S., Khatoon, S., Dar, A., and Malik, A., *Helv. Chim. Acta*, 2002, vol. 85, pp. 559–570.
3. Kornet, M.J., *J. Heterocycl. Chem.*, 1996, vol. 33, pp. 2047–2049.
4. Robinson, R.P. and Donahue, K.M., *J. Heterocycl. Chem.,* 1994, vol. 31, pp. 1541–1544.
5. Gravestock, M.B., Eur. Pat. Appl. No. EP 26516927, *Chem. Abstr.*, 1988, vol. 109, p. 468.
6. Li, J., Zhao, Y.F., Zhao, X.L., Yuan, X.Y., and Gong, P., *Arch. Pharm. Chem. Life Sci.*, 2006, vol. 339, pp. 593–597.
7. Abdel-hafez, A.A., *Arch. Pharm. Res.*, 2007, vol. 30, pp. 678–684.
8. Bartolini, S., Mai, A., Artico, M., Paesano, N., Rotili, D., Spadafora, C., and Sbardella, G., *J. Med. Chem.,* 2005, vol. 48, pp. 6776–6778.
9. Manetti, F., Brullo, C., Magnani, M., Mosci, F., Chelli, B., Crespan, E., Schenone, S., Naldini, A., Bruno, O., Trincavelli, M.L., Maga, G., Carraro, F., Martini, C., Bondavalli, F., and Botta, M., *J. Med. Chem.*, 2008, vol. 51, pp. 1252–1259.
10. Verma, S., Nagarathnam, D., Shao, J., Zhang, L., Zhao, J., Wang, Y., Li, T., Mull, E., Enyedy, I., Wang, C., Zhu, Q., Altieri, M., Jordan, J., Dang, T.T., and Reddy, S., *Bioorg. Med. Chem. Lett.*, 2005, vol. 15, pp. 1973–1977.
11. Gangjee, A., Zeng, Y., Ihnat, M., Warnke, L.A., Green, D.W., Kisliuk, R.L., and Lin, F.T., *Bioorg. Med. Chem.,* 2005, vol. 13, pp. 5475–5491.
12. Gangjee, A., Jain, H.D., Phan, J., Lin, X., Song, X., Mc Guire, J.J., and Kisliuk, R.L., *J. Med. Chem.*, 2006, vol. 49, pp. 1055–1065.
13. Bakavoli, M., Rahimizadeh, M., Shiri, A., Eshghi, H., Vaziri-Mehr, S., Pordeli, P., and Nikpour, M., *Heterocycl. Commun.*, 2011, vol. 17, pp. 49–52.
14. Mosmann, T., *J. Immunol. Methods*, 1983, vol. 16, pp. 55–63.
15. Mousavi, S.H., Tavakkol-Afshari, J., Brook, A., and Jafari-Anarkooli, I., *Food Chem. Toxicol.*, 2009, vol. 47, pp. 1909–1913.
16. Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F., and Riccardi, C.A., *J. Immunol. Methods*, 1991, vol. 139, pp. 271–279.
17. Tayarani-Najaran, Z., Mousavi, S.H., Asili, J., and Emami, S.A., *Food Chem. Toxicol.*, 2010, vol. 48, pp. 599–604.
18. Mousavi, S.H., Tayarani-Najaran, Z., and Hersey, P., *Iran J. Basic Med. Sci.*, 2008, vol. 11, pp. 121–142.