Original Article

Synthesis and Cytotoxicity Evaluation of *N*-(5-mercapto-4*H*-1,2,4-triazol-3-yl)-2-phenylacetamide Derivatives as Apoptosis Inducers with Potential Anticancer Effects

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Abstract

Background: Discovery of new anticancer drugs is one of the urgent issues in the medicinal chemistry researches. Incidence of severe side effects and acquired resistance to the current medications are the logical reasons for the development of novel antineoplastic agents. **Methods:** Herein, a new series of 4H-1,2,4-triazole derivatives was synthesized and subsequently their cytotoxicity was assessed using dimethylthiazol diphenyltetrazolium bromide assay. Furthermore, activity of caspase 3, mitochondrial membrane potential (MMP), and generation of reactive oxygen species (ROS) were investigated. All synthesized derivatives (3a–3o) were tested against Hela (cervical cancer), A549 (lung carcinoma), and U87 (glioblastoma), and the obtained data were compared with doxorubicin. **Results:** Among the chlorinated derivatives, compound 3c with para positioning of the chlorine on the phenyl residue possessed higher cytotoxicity (IC50 = s3.2 ± 0.6 μM) than compounds 3a and 3b, which positioned chlorine at ortho and meta position, respectively. Chlorine as electron-withdrawing moiety caused enhancement in cytotoxicity. **Conclusion:** Fortunately, most of the tested compounds showed remarkable cytotoxic activity toward applied cells, especially Hela. Activation of caspase 3, MMP reduction, and ROS generation were also observed for the studied compounds.

Keywords: *Anticancer, cytotoxic, synthesis, triazole*

Introduction

Cancer as a major public health problem in the world is one of the most important leading causes of mortality and morbidity in the recent years. Most of the cases were reported in Africa, Central, and South America. These countries consisting the 70% of the cancer associated deaths occur in these countries. It is expected that the numbers will be raised until the next two decades.[1,2] Unfortunately, successful treatment of cancer has not been achieved, and this issue is one of the most challenging area in the current medical research. High incidence of undesirable side effects such as nausea, vomiting, kidney failure, and bone marrow suppression as well as acquired resistance to the current antineoplastic medications tempted the efforts for the development of novel anticancer agents.[3-6] Most of the anticancer drugs perform their corresponding cytotoxic activity through induction of apoptosis. Apoptosis or programmed cell death is one of the critical phenomena observed in all tissues for the remission of the unnecessary and damaged cells. Apoptosis is responsible for hemostasis and normal development. Dysfunction of this highly regulated process in the cells is a hallmark of the neoplastic diseases. Deficiency in this fundamental process led to tumor progression, tumorigenesis, and resistance to the anticancer pharmacotherapy.[7,8] Induction of the apoptotic pathways could be very beneficial for the development of new anticancer drugs. DNA fragmentation, chromatin condensation, cell shrinkage, and finally membrane blebbing are the distinguished features of apoptosis that proceed to the formation of the apoptotic bodies.[7,8] Caspases are important enzymes in the apoptotic pathways, especially caspase 3, which is presented in the crossroad of intrinsic and extrinsic pathway. Activation of caspase 3 could induce apoptosis in cancerous cells.[9]

1,2,4-Triazole heterocyclic derivatives with diverse pharmacological activities have positive background in possessing remarkable

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anticancer effects [Figure 1]. These heterocyclic derivatives have exerted different biological effects such as anti-inflammatory, antituberculosis, antibacterial, antifungal, and anti-seizure[10-18] and because of this positive background, they have appeared in some current drugs such as trazodone, tazobactam, triazolam, estazolam, and fluconazole. Besides, synthetic importance and interest of these heterocycles as present, nucleus in the pharmacophore of some well-known anticancer drugs such as letrozole and anastrozole also has attracted a special attention to this scaffold[18] [Figure 2].

In the previous literature, we reported the potential cytotoxic activity of 1,3,4-thiadiazole derivatives bearing thiol functional group.[19] In continuation of our previous research and interest on the synthesis of 1,2,4-triazole,[10] the design of novel 4*H*-1,2,4-triazole was carried out via a bioisosteric change of 1,3,4-thiadiazole nucleus with 4*H*-1,2,4-triazole congener [Figure 3]. Increasing of the cytotoxicity is appealed according to the more hydrophilicity and more solubility of triazole derivatives compared to thiadiazole derivatives as well as superior capability of nitrogen atom compared to sulfur atom.

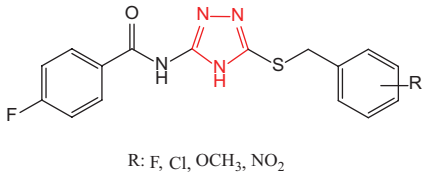


Figure 1: Chemical structures of 4*H*-1,2,4-triazole derivatives that were reported previously as anticancer agents

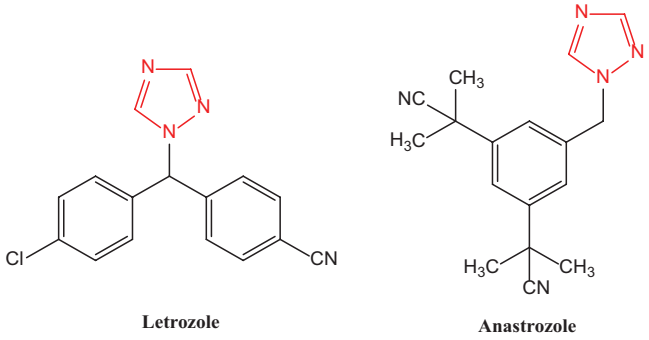


Figure 2: Structures of letrozole and anastrozole as two anticancer drugs with 1,2,4-triazole ring

Besides, phenylacetamide residue and thiol functional group are also conserved in the designed derivatives as cytotoxic-enhancing portions.[20]

Cytotoxicity was investigated *in vitro* using dimethylthiazol diphenyltetrazolium bromide (MTT) assay. In addition, other related experimental tests consisting mitochondrial membrane potential (MMP), reactive oxygen species (ROS), and investigation of caspase 3 activity were also explored.

Materials and Methods

Chemistry

Chemical reagents that are used for the synthesis of target compounds were purchased from the commercial companies such as Sigma-Aldrich, St. Louis, Missouri, United States and Merck, Burlington, Massachusett, United States. All solvents were bought from the Merck, Scharlau, Scharlau, Barcelona, Spain and Duksan companies, Seoul, South Korea. Thin layer chromatography (TLC) was performed using aluminum-based TLC sheets. Purification of the intermediate and final compounds was implemented by silica gel (70–230 mesh). Nuclear magnetic resonance (NMR) Bruker 250 MHz apparatus was used for 1HNMR spectra acquisition. All prepared compounds were dissolved in deuterated chloroform ($CDCl_3$), and tetramethylsilane (TMS) was applied for comparison as internal standard. Chemical shifts for each proton were presented as δ (parts per million [ppm]) related to TMS. Potassium bromide (KBr) disk was prepared for infrared (IR) spectra in Shimadzu 470 spectrophotometer, Japan. Mass spectroscopy was performed using a FiniganTSQ-70 spectrometer (Finigan, USA) at 70 eV and mass of each fragment was provided with its frequency percentage. Melting points for final compounds were also obtained using melting point analyzer apparatus Electrothermal 9001A model, England in open capillary tubes.

General protocol for the synthesis of compounds 3a–3o

All target compounds 3a–3o were synthesized according to Scheme 1. In a flat bottom flask, 0.2 g (1.7 mmol) of 5-amino-4*H*-1,2,4-triazole-3-thiol was mixed with equimolar quantity of appropriate phenylacetic acid derivative in 20 mL tetrahydrofuran (THF). Then, equimolar quantities of dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole

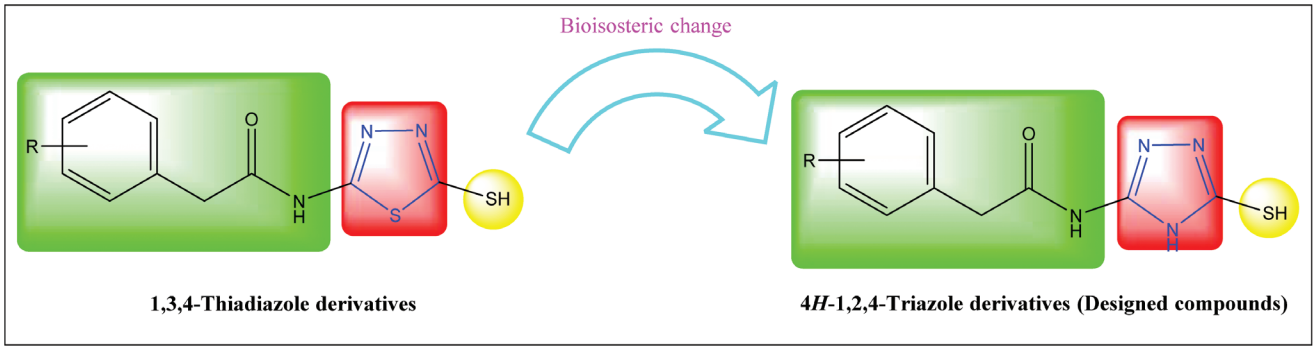
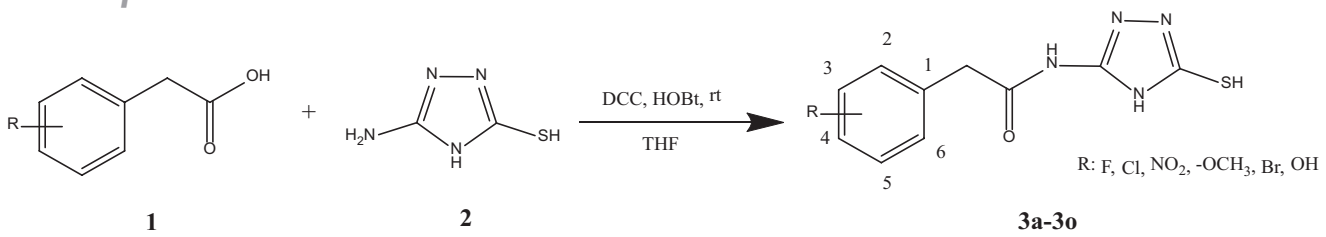


Figure 3: Bioisosteric change of 1,3,4-thiadiazole ring with 4*H*-1,2,4-triazole ring



1 **2** **3a-3o**

Scheme 1: Synthetic protocol for target compounds 3a–3o

(HOBt) were added to the reaction medium. The reaction mixture was stirred for 1 h in ice bath and subsequently stirring was continued for 24 h. THF was evaporated under reduced pressure by rotary evaporator and ethyl acetate/water was added to the obtained residue for extraction. Aqueous layer was removed and brine was added to the organic layer twice. Anhydrous sodium sulfate was applied for dryness and subsequently filtered off. Finally, the ethyl acetate phase was evaporated and afforded powder was washed using diethyl ether (Et_2O) and *n*-hexane.[19-21]

2-(2-Chlorophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3a)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.40 (broad singlet, SH), 3.81 (singlet, 2H, -CH_2-), 5.92 (brs, H_4-triazole), 7.29 (triplet, 1H, *J* = 8 Hz, H_4-2-chlorophenyl), 7.41 (t, 1H, *J* = 8 Hz, H_5-2-chlorophenyl), 7.73 (doublet, 1H, *J* = 7.5 Hz, H_6-2-chlorophenyl), 7.86 (d, 1H, *J* = 7.5 Hz, H_3-2-chlorophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). Mass (*m/z*, %): 268 (M^+, 30), 266 (45), 224 (40), 152 (35), 143 (30), 125 (10), 116 (35), 99 (55), 56 (100).

2-(3-Chlorophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3b)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.37 (brs, SH), 3.61 (s, -CH_2-), 6.98 (brs, H_4-triazole), 7.30 (s, 1H, H_2-3-chlorophenyl), 7.37 (t, 1H, *J* = 8Hz, H_5-3-chlorophenyl), 7.72 (d, 1H, *J* = 7.25 Hz, H_4-3-chlorophenyl), 7.83 (d, 1H, *J* = 7.25 Hz, H_6-3-chlorophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 268 (M^+, 15), 266 (25), 224 (50), 152 (50), 143 (25), 125 (30), 116 (40), 99 (35), 56 (100).

2-(4-Chlorophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3c)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.46 (brs, SH), 4.12 (s, 2H, -CH_2-), 7.67 (d, 2H, *J* = 8 Hz, $H_{2,6}$-4-chlorophenyl), 7.88 (d, 2H, *J* = 8 Hz, $H_{3,5}$-4-chlorophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2924, 2850 (C-H, stretch, aliphatic), 1624 (C = O, stretch). MS (*m/z*, %): 268 (M^+, 20), 266 (35), 224 (80), 152 (40), 143 (60), 125 (30), 116 (55), 99 (75), 56 (100).

2-(2-Fluorophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3d)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.41 (brs, SH), 3.69 (s, 2H, -CH_2-), 7.10 (m, 1H, H_4-2-fluorophenyl), 7.39 (m, 1H, H_5-2-fluorophenyl), 7.70 (d, 1H, *J* = 7.75 Hz, H_4-2-fluorophenyl), 7.86 (d, 1H, *J* = 8 Hz, H_4-2-fluorophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 252 (M^+, 10), 250 (25), 224 (40), 143 (55), 136 (30), 109 (35), 99 (70), 56 (100).

2-(3-Fluorophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3e)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.37 (brs, SH), 3.64 (s, 2H, -CH_2-), 7.05 (m, 1H, H_5-3-fluorophenyl), 7.29 (m, 1H, H_6-3-fluorophenyl), 7.41 (m, 1H, H_2-3-fluorophenyl), 7.76 (m, 1H, H_4-3-fluorophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 252 (M^+, 15), 250 (30), 224 (30), 143 (85), 136 (20), 109 (60), 99 (55), 56 (100).

2-(4-Fluorophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3f)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.37 (brs, SH), 3.61 (s, 2H, -CH_2-), 6.97 (m, 2H, 4-fluorophenyl), 7.75 (m, 2H, 4-fluorophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1631 (C = O, stretch). MS (*m/z*, %): 252 (M^+, 5), 250 (20), 224 (80), 143 (65), 136 (40), 109 (50), 99 (70), 56 (100).

N-(5-Mercapto-4H-1,2,4-triazol-3-yl)-2-(2-methoxyphenyl) acetamide (3g)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.44 (brs, SH), 3.65 (s, 2H, -CH_2), 3.76 (s, 3H, -OCH_3), 6.90 (s, 1H, H_4-triazole), 7.12–7.43 (m, 2H, 2-methoxyphenyl), 7.68 (d, 1H, *J* = 8 Hz, H_3-2-methoxyphenyl), 7.90 (d, 1H, *J* = 8 Hz, H_6-2-methoxyphenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 264 (M^+, weak), 224 (30), 143 (55), 121 (45), 107 (30), 99 (65), 90 (25), 56 (100).

N-(5-Mercapto-4H-1,2,4-triazol-3-yl)-2-(3-methoxyphenyl) acetamide (3h)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.37 (brs, SH), 3.61 (s, 2H, -CH_2), 3.77 (s, 3H, -OCH_3), 7.02 (s, 1H, H_4-triazole),

6.82 (t, 1H, J = 8 Hz, 3-methoxyphenyl), 6.84 (s, 1H, H_2-3-methoxyphenyl), 7.02 (s, 1H, H_4-triazole), 7.24 (d, 1H, J = 8 Hz, H3-3-methoxyphenyl), 7.41 (m, 1H, H_6-3-methoxyphenyl), 7.68 (d, 1H, J = 8 Hz, H_3-3-methoxyphenyl), 7.90 (d, 1H, J = 8 Hz, H_6-3-methoxyphenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 264 (M^+, weak), 224 (45), 143 (20), 121 (50), 107 (60), 99 (25), 90 (55), 56 (100).

N-(5-Mercapto-4H-1,2,4-triazol-3-yl)-2-(4-methoxyphenyl) acetamide (3i)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.37 (brs, SH), 3.57 (s, 2H, -CH_2-), 3.78 (s, 3H, -OCH_3), 5.75 (brs, -CONH-), 6.85 (d, 2H, J = 7 Hz, $H_{3,5}$-4-methoxyphenyl), 7.20 (d, 2H, J = 7 Hz, $H_{2,6}$-4-methoxyphenyl), 7.39 (s, 1H, H_4-triazole). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 264 (M^+, weak), 224 (40), 143 (40), 121 (85), 107 (40), 99 (45), 90 (35), 56 (100).

N-(5-Mercapto-4H-1,2,4-triazol-3-yl)-2-(2-nitrophenyl) acetamide (3j)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.36 (s, SH), 4.05 (s, 2H, -CH_2-), 7.10 (1H, H_4-triazole), 7.35 (d, 1H, J = 8 Hz, H_6-2-nitrophenyl), 7.46 (t, 1H, J = 8 Hz, H_4-2-nitrophenyl), 7.46 (t, 1H, J = 8 Hz, H_5-2-nitrophenyl), 8.10 (d, 1H, J = 8 Hz, H_3-2-nitrophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch), 1527 (NO_2, stretch, asymmetric), 1346 (NO_2, stretch, symmetric). MS (*m/z*, %): 279 (M^+, weak), 251 (15), 224 (35), 181 (40), 137 (100), 116 (15), 107 (65), 90 (20), 56 (30).

N-(5-Mercapto-4H-1,2,4-triazol-3-yl)-2-(4-nitrophenyl) acetamide (3k)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.34 (brs, SH), 3.76 (s, 2H, -CH_2-), 7.46 (d, 2H, J = 8.75 Hz, $H_{2,6}$-4-nitrophenyl), 7.78 (m, 1H, H_4-triazole), 8.17 (d, 2H, J = 8.75 Hz, $H_{3,5}$-4-nitrophenyl), 9.40 (brs, -CONH-). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch), 1516 (NO_2, stretch, asymmetric), 1350 (NO_2, stretch, symmetric). MS (*m/z*, %): 279 (M^+, weak), 251 (20), 224 (15), 181 (30), 137 (100), 116 (40), 107 (45), 90 (45), 56 (60).

2-(2-Hydroxyphenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3l)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.40 (s, 2H, -CH_2), 3.68 (brs, SH), 5.32 (brs, OH), 7.36–7.47 (m, 2H, $H_{4,5}$-2-hydroxyphenyl), 7.73 (d, 2H, J = 8 Hz, H_3-2-hydroxyphenyl), 7.85 (d, 2H, J = 8 Hz, H_6-2-hydroxyphenyl). IR (KBr, cm^{-1}) ῡ: 3329 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1624 (C = O, stretch). MS (*m/z*, %): 250 (M^+, weak), 224 (40), 152 (25), 143 (40), 135 (100), 107 (60), 99 (60), 90 (20), 77 (30), 56 (90).

2-(3-Hydroxyphenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3m)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.41 (brs, SH), 3.61 (s, 2H, -CH_2-), 5.08 (brs, OH), 6.77–7.43 (m, 4H, 3-hydroxyphenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3051 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 2500–3500 (broad, stretch, OH), 1625 (C = O, stretch). MS (*m/z*, %): 250 (M^+, weak), 224 (60), 152 (45), 143 (30), 135 (100), 107 (75), 99 (40), 90 (25), 77 (40), 56 (60).

N-(5-Mercapto-4H-1,2,4-triazol-3-yl)-2-(o-tolyl) acetamide (3n)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.39 (brs, SH), 2.38 (s, 3H, -CH_3), 3.66 (s, 2H, -CH_2-), 7.51 (t, 1H, J = 8Hz, H_5-2-methylphenyl), 7.69 (t, 1H, J = 8 Hz, H_4-2-methylphenyl), 8.04 (d, 1H, J = 8 Hz, H_3-2-methylphenyl), 8.37 (d, 1H, J = 8 Hz, H_6-2-methylphenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3035 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 248 (M^+, 15), 133 (100), 105 (65), 91 (25).

2-(4-Bromophenyl)-N-(5-mercapto-4H-1,2,4-triazol-3-yl) acetamide (3o)

^{1}HNMR ($CDCl_3$, 250 MHz) δ (ppm): 3.36 (brs, SH), 6.90 (brs, H_4-triazole), 7.17 (d, 2H, J = 8 Hz, $H_{2,6}$-4-bromophenyl), 7.45 (d, 2H, J = 8 Hz, $H_{2',6'}$-4-bromophenyl). IR (KBr, cm^{-1}) ῡ: 3325 (N-H, stretch), 3066 (C-H, stretch, aromatic), 2927, 2850 (C-H, stretch, aliphatic), 1627 (C = O, stretch). MS (*m/z*, %): 311 (M^+, weak), 224 (40), 214 (20), 198 (20), 169 (90), 143 (40), 99 (55), 56 (100).

Cytotoxicity Evaluation

Cell culture

The cancerous cell lines were obtained from the Pasteur Institute of Iran (Tehran, Iran) and maintained in Dulbecco's modified Eagle's medium, which was supplemented with 10% fetal calf serum (Gibco, Grand Island, New York, USA), penicillin (100 U/mL), and streptomycin (100 µg/mL). The cells were incubated at 37°C in a 5% CO_2 atmosphere. The cells were seeded at a concentration of 5×10^5 in a 25 cm^2 flask containing 4 mL of complete culture medium.

Dimethylthiazol diphenyltetrazolium bromide assay

Cellular toxicities of the synthesized compounds were evaluated in three cancer cell lines A549, Hela, and U87 using the MTT method. Briefly, the cells were cultured in 96-well plate in a concentration of 6×10^3 cells/well. Chemicals were prepared in dimethyl sulfoxide (DMSO) solution in a way that the final concentration of the solvent was 0.1% in the culture medium. After 24 h, the cells were incubated with different concentrations of synthesized compounds 3a–3m for a 24-h period. Cells were washed with phosphate buffered saline (PBS) and then incubated with a solution of 0.5 mg/mL MTT for 3 h at 37°C. After removal of the medium, blue formazan

crystals were dissolved in 200 µL DMSO for 5 min. Inhibitory effects of drugs were determined by measuring the absorbance at λ = 450 nm using a microplate reader (Biotek Instruments, Winooski, Vermont, USA). Calculations of inhibition were based on the following equation: %Inhibition of growth = (1 − absorbance of treated cells/absorbance of control cells) × 100.

The half maximal inhibitory concentrations (IC_{50}) were calculated after performing a liner regression analysis using the GraphPad Prism 6.0 (GraphPad Software, San Diego, California).

Caspase-3 assay

The test procedure to assess the activity of selected compounds on caspase-3 activity was adapted from manufacturer's instructions. This test is based on the detection of *p*-nitroanilide (pNA), which is cleaved from its precursor DEVD-pNA by caspase 3. Briefly, 5×10^5 cells were cultured in six-well plates and were incubated with the IC_{50} concentration of chemicals for 24 h. Then the cells were washed with PBS, and lysed with 60 µL of lysis buffer while they were kept on ice. Cell lysate was centrifuged at 4°C for 5 min. Equal amounts (10 µL) of supernatant were added to the reaction buffer, which contained the caspase 3 substrate. This reaction was incubated for 2 h in 37°C and the amount of pNA was determined by measuring the intensity of light at 405 nm. Percentage of caspase activity was calculated based on the comparison of absorbance of each compound with a drug-free control.

Measurement of mitochondrial membrane potential

Apoptosis can initiate when mitochondrial membrane permeability changes. This can result in the reduction of MMP using another fluorescent dye, rhodamine 123. This dye is a cell-permeant, cationic dye that is sequestered by active mitochondria. Apoptosis of the cells decreases mitochondrial membrane potential and hence the release of rhodamine 123 from the mitochondria, which results in a decrease in intracellular fluorescence intensity. After the incubation of cells with IC_{50} concentration of the compounds, they were washed with PBS and treated with rhodamine 123 for 30 min at 37°C. Then, they were washed with PBS again, and fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

Determination of intracellular reactive oxygen species

Oxidative stress was assessed by measuring reactive oxygen generation using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). When oxidized, this molecule becomes a fluorochrome, which can be traced using the excitation and emission wavelengths of 485 and 530 nm, respectively. Cells were seeded (4×10^5) in six-well plates and incubated with the IC_{50} concentrations of each agent for 24 h. Then, the cells were washed with PBS and incubated for 30 min with 30 µL DCF-DA at 37°C. Next, they were lysed with Triton X-100 and 100 µL of the lysate was transferred to 96-well plate for fluorescence detection using the microplate reader (BioTek Instruments, H1M).

Results and Discussion

A new series of 4*H*-1,2,4-triazole derivatives was synthesized and subsequently their cytotoxicity was assessed using MTT assay [Table 1]. Furthermore, activity of caspase 3, MMP, and generation of ROS were investigated. All synthesized derivatives (3a–3o) were tested against Hela (cervical cancer), A549 (lung carcinoma), and U87 (glioblastoma), and the obtained data were compared with doxorubicin. Fortunately, most of the tested compounds showed remarkable cytotoxic activity toward applied cells, especially Hela. Activation of caspase 3, MMP reduction, and ROS generation were also observed for the studied compounds.

Cytotoxicity

The synthesized compounds 3a–3o were tested against three cancerous cell lines for the investigation of cytotoxicity. Hela (cervical carcinoma), A549 (lung carcinoma), and U87 (glioblastoma) were used for the implementation of the MTT assay. Diverse 1,2,4-triazole-based derivatives

Table 1: Properties of compounds 3a–3o

Code	*R*	Yield (%)	MW (g/mol)	Melting point (mp) (°C)	Chemical formula
3a	2-Cl	67	268	236	$C_{10}H_9ClN_4OS$
3b	3-Cl	77	268	202	$C_{10}H_9ClN_4OS$
3c	4-Cl	74	268	213	$C_{10}H_9ClN_4OS$
3d	2-F	72	252	185	$C_{10}H_9FN_4OS$
3e	3-F	63	252	192	$C_{10}H_9FN_4OS$
3f	4-F	69	252	191	$C_{10}H_9FN_4OS$
3g	2-OCH_3	71	264	188	$C_{11}H_{12}N_4O_2S$
3h	3-OCH_3	74	264	102	$C_{10}H_9FN_4OS$
3i	4-OCH_3	81	264	126	$C_{10}H_9FN_4OS$
3j	2-NO_2	86	279	107	$C_{10}H_9N_5O_3S$
3k	4-NO_2	87	279	105	$C_{10}H_9N_5O_3S$
3l	2-OH	84	250	154	$C_{10}H_{10}N_4O_2S$
3m	3-OH	78	250	158	$C_{10}H_{10}N_4O_2S$
3n	2-CH_3	73	236	139	$C_{11}H_{12}N_4OS$
3o	4-Br	71	313	120	$C_{10}H_9BrN_4OS$

R = Radical (Side chain); MW = Molecular Weight

Table 2: Cytotoxicity results (IC_{50} ± standard error of mean [SEM], µM) of compounds 3a–3o against cancerous cells

Compound	R	Hela	A549	U87
(3a)	2-Cl	**7.3 ± 2.3**	6.6 ± 1	18.8 ± 6.7
(3b)	3-Cl	**4.1 ± 1.1**	33.8 ± 8.2	>100
(3c)	4-Cl	**3.2 ± 0.6**	**2.5 ± 1.3**	31.8 ± 7.9
(3d)	2-F	**1.4 ± 0.01**	8.5 ± 2.8	4.7 ± 2.1
(3e)	3-F	88.5 ± 3.2	**3.2 ± 1**	4.9 ± 1.1
(3f)	4-F	**2.9 ± 0.89**	>100	>100
(3g)	2-OCH_3	**1 ± 0.02**	9 ± 2.3	>100
(3h)	3-OCH_3	51 ± 8.6	17.2 ± 4.6	>100
(3i)	4-OCH_3	>100	3.8 ± 1.8	68.5 ± 12.9
(3j)	2-NO_2	**3.15 ± 1.4**	28.5 ± 9.1	2.2 ± 1.7
(3k)	4-NO_2	**3.5 ± 1.2**	>100	27.7 ± 5.2
(3l)	2-OH	**3.5 ± 0.9**	23.1 ± 6.5	4.4 ± 2.9
(3m)	3-OH	>100	>100	>100
(3n)	2-CH_3	>100	**1.8 ± 0.01**	22.4 ± 6.7
(3o)	4-Br	**2.3 ± 0.8**	36 ± 7	26.2 ± 5.8
Doxorubicin	-	10.9 ± 1.3	3.4 ± 0.9	8.2 ± 1.8

R = Radical (side chain)

Bold values show remarkable cytotoxic activity

bearing various electron-withdrawing and electron-donating properties were synthesized to explore the role of electronic effect and other responsible parameters for exerting the cytotoxicity.

According to Table 2, evaluation data of the target compounds toward cancerous cell line showed that Hela cells were the most sensitive cell line with respect to the tested compounds. Most of the tested derivatives showed remarkable cytotoxic activity against Hela cells in comparison with doxorubicin as the standard anticancer drug. Among the chlorinated derivatives, compound 3c with *para* positioning of the chlorine on the phenyl residue possessed higher cytotoxicity (IC_{50} = s3.2 ± 0.6 µM) than compounds 3a and 3b, which positioned chlorine at *ortho* and *meta* position, respectively. Chlorine as electron-withdrawing moiety caused enhancement in cytotoxicity. According to the obtained data, it could be suggested that positioning at *para* is more beneficial for cytotoxicity due to other properties of this atom such as lipophilicity and steric effects. Increasing the electronegativity of the moiety as applied in fluorinated derivatives produced significant cytotoxic potency while the fluorine was introduced at positions *ortho* (IC_{50} = 1.4 ± 0.01 µM) and *para* (IC_{50} = 2.9 ± 0.89 µM). Fluorine atom reduced the cytotoxic activity significantly when introduced at position *meta* (IC_{50} = 88 ± 3.2 µM). Comparison of the obtained results of chlorinated and fluorinated derivatives showed that for position *meta*, electron-withdrawing effects with lipophilicity and steric effects could be presented for exerting an acceptable anticancer potency, and electronic effect was not responsible for merely cytotoxic effect. Utilization of the other electron-withdrawing substituents such as nitro group also confirmed the aforementioned trend. Substitution of the nitro moiety in *ortho* and *para* positions of the phenyl ring led to a favorable anticancer activity in Hela cells. Nitro group as electron-receiving group also is responsible for steric effect such as chlorine moiety and besides, may participate in electrostatic interaction. Hydrophilic feature of this moiety such as lipophilic feature of chlorine enhanced the anticancer activity. So, hydrophilic as well as lipophilic moieties are suitable for this position. Bromine atom at position *para* similar to other electron-withdrawing moieties increased the cytotoxicity (IC_{50} = 2.3 ± 0.8 µM). Using electron-donating substituents on the phenyl ring declined the cytotoxicity. It seems that potent electron-donating groups such as methoxy and hydroxyl are very beneficial for anticancer activity as observed in compounds 3g (IC_{50} = 1 ± 0.02 µM) and 3l (IC_{50} = 3.5 ± 0.9 µM), respectively.

A549 cell line was more resistant to the tested compounds compared to Hela cells. Only compounds 3c (4-Cl, IC_{50} = 2.5 ± 1.3 µM), 3e (3-F, IC_{50} = 3.2 ± 1 µM), and 3n (2-CH_3, IC_{50} = 1.8 ± 0.01 µM) displayed superior cytotoxicity toward this cell line compared to doxorubicin (IC_{50} = 3.4 ± 0.9 µM). Compounds 3a (2-Cl, IC_{50} = 6.6 ± 1 µM), 3d (2-F, IC_{50} = 8.5 ± 2.8 µM), 3g (2-OCH_3, IC_{50} = 9 ± 2.3 µM), and 3i (4-OCH_3, IC_{50} = 3.8 ± 1.8 µM) also showed remarkable activity against A549 cells.

U87 cell line was also more resistant than Hela cells to the tested derivatives. Only compounds 3d (2-F, IC_{50} = 4.7 ± 2.1 µM), 3e (3-F, IC_{50} = 4.9 ± 1.1 µM), 3j (2-NO_2, IC_{50} = 2.2 ± 1.7 µM), and 3l (2-OH, IC_{50} = 4.4 ± 2.9 µM) displayed superior cytotoxicity toward this cell line compared to doxorubicin (IC_{50} = 8.2 ± 1.8 µM).

Caspase 3 activation

As tested compounds showed remarkable anticancer activity against Hela cell line, more investigations were carried out for caspase 3 activity in these cells. According to Figure 4, all tested compounds caused activation of caspase 3 more than the control. All of them activated caspase 3 equal or more than doxorubicin as reference drug except compound 3k. Compound 3k caused less caspase 3 activation than the control. Compound 3d with

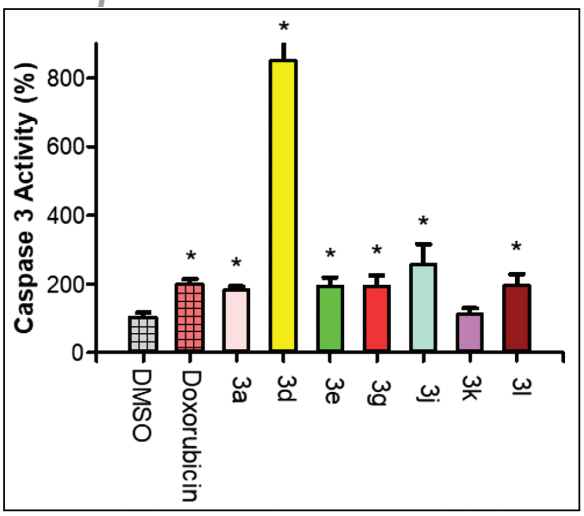


Figure 4: Results of activation of caspase 3 in Hela cells. *shows compounds which activated caspase 3 equal or more than doxorubicin as reference drug.

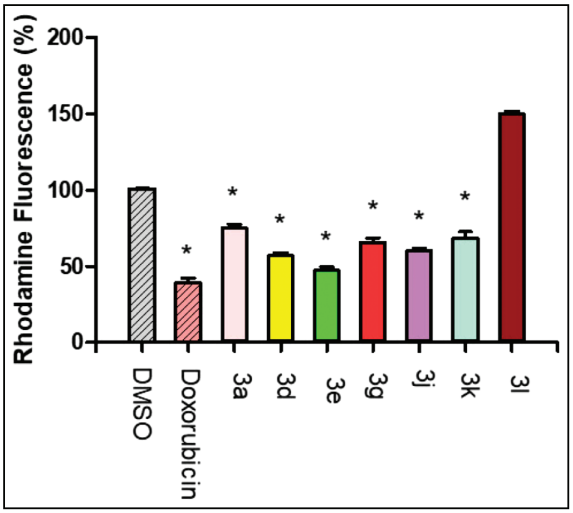


Figure 5: Results of mitochondrial membrane potential (MMP) in Hela cells *shows compounds caused a significant decline in MMP.

ortho-fluorine moiety on the phenyl ring that caused a high cytotoxicity against Hela cells (IC_{50} = 1.4 ± 0.01 µM) also exerted a high activation of caspase 3. This observation could prove strongly the hypothesis that compound 3d as a potent cytotoxic agent acts via apoptosis pathway, especially activation of caspases.

Mitochondrial membrane potential

Potential of the mitochondrial membrane is a determining factor for following up the activation of intrinsic pathway of apoptosis. Any reduction in this potential is an indicator for the activation of intrinsic apoptotic pathway. As shown in Figure 5, all tested compounds caused a significant decline in MMP except compound 3l. As compound 3l produced a significant raise in MMP and increase in caspase 3 activity [Figure 4], it could be suggested that this compound acts through the extrinsic pathway.

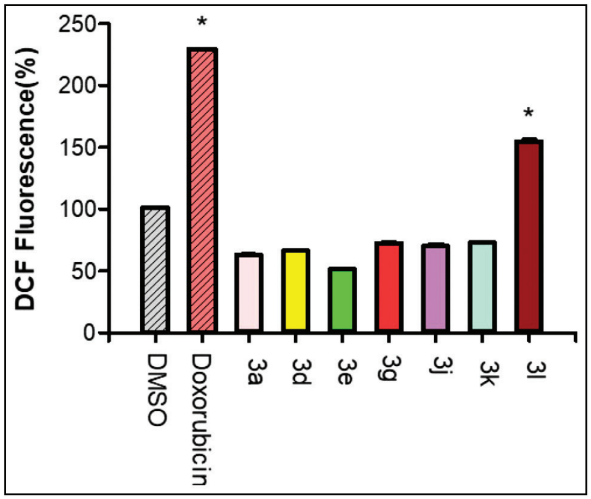


Figure 6: Results of capability of reactive oxygen species generation in Hela cells *shows compounds which generated ROS.

Reactive oxygen species generation

Capability of generation of ROS is an important index for the exploration of the likely mechanism of cytotoxicity. According to Figure 6, none of the investigated compounds generated ROS except for compound 3l. As discussed earlier about the compound 3l that acts as an apoptosis inducer via extrinsic pathway, herein we could propose that the generation of free radicals maybe a responsible factor for the induction of apoptosis.

Conclusion

4*H*-1,2,4-triazole derivatives that were synthesized in this research project showed favorable cytotoxic effect. The investigated compounds showed potential for caspase 3 activation. Furthermore, reduction in MMP was also observed. These observations confirm the potency of these compounds for apoptosis induction. More experimental explorations are suggested to be carried out in the future for the study of the corresponding mechanism of the lead compounds.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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