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ORIGINAL PAPER

Synthesis, characterization and cytotoxic studies of novel 1,2,4-triazole-azomethine conjugates

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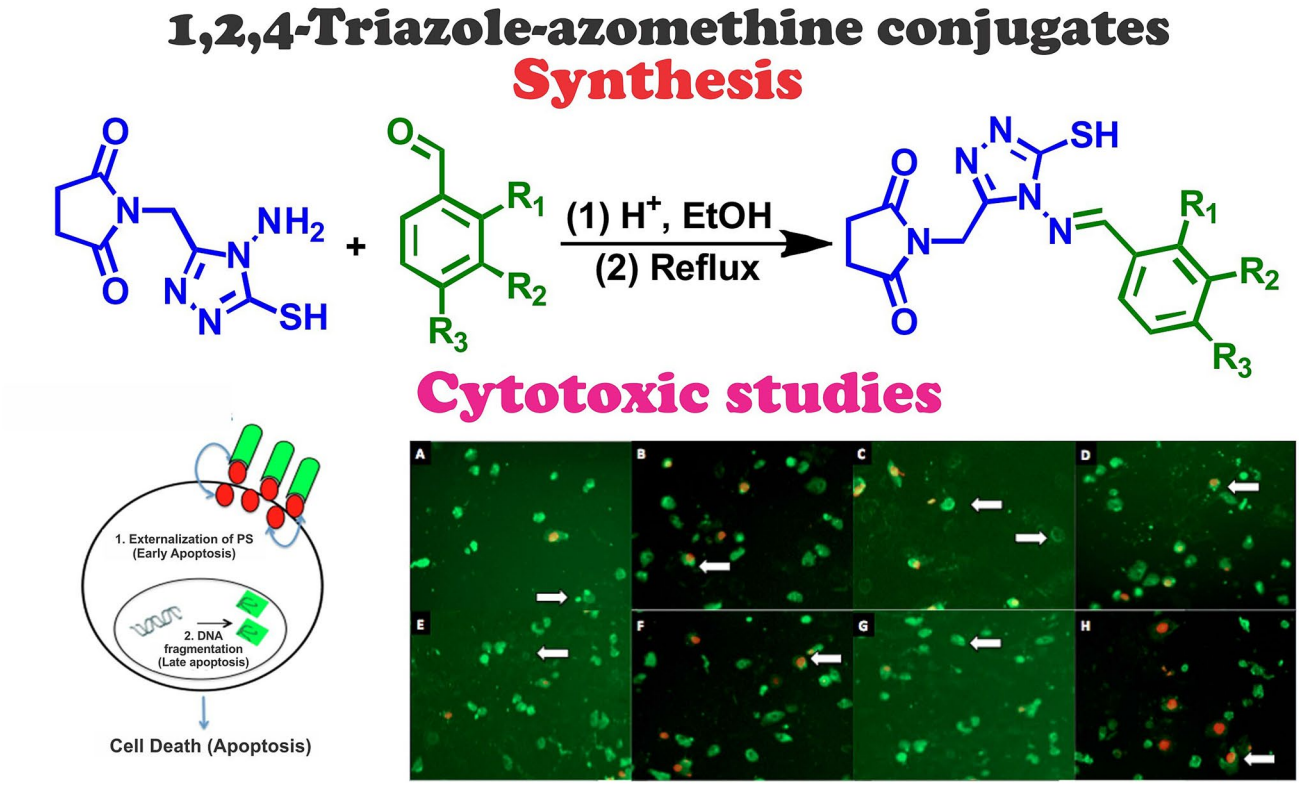
Abstract

A series of 1,2,4-triazole-schiff hybrids were synthesized and characterized by mass spectrometry, FTIR and NMR spectroscopy. The compounds were screened for anticancer activity against human breast cancer cell line (MCF-7 and T47D) and human cervical cancer cell line (HeLa). The result indicates that newly synthesized compounds exhibit cytotoxicity to all cell lines studied. In particular, MCF-7 cells were shown to be more sensitive with $EC_{50} < 50$ µM/ml with the exception of compound T2. Interestingly, T6 produced more high cytotoxicity on MCF-7 cells (< 10 µM/ml), which is comparable to cisplatin EC_{50} 13.10 µM/ml. To further investigate the mode of cell death, early and late apoptosis studies were done on MCF-7 cells. The externalization of phosphatidylserine and DNA fragmentation supports the apoptosis as the major mode of cell death induced by derivatives on MCF-7 cells.

Graphic abstract



Keywords 1,2,4-Triazole · Azomethine · Aza apoptosis · Cytotoxicity · MCF-7 · T47D · HeLa

Extended author information available on the last page of the article

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Introduction

Cancer can be defined as malignant neoplasia encompassing a wide group of ailments all involving unregulated cell growth in which cells grow and divide uncontrollably developing tumors that may metastasize to invade other body parts. These neoplastic cells regress highly specialized cells forming more primitive stages which, unlike the normal parent cells, divide unceasingly although inefficiently [1–3]. The chief characteristics of neoplastic tissue contain escaping immune destruction, reprogramming energy metabolism, activating invasion and metastasis, inducing angiogenesis, enabling replication, evading growth suppressors, resisting cell death and sustained proliferative signaling [4, 5].

Almost 12.7 million cancers were identified worldwide (excluding noninvasive cancers and non-melanoma skin cancers), and 7.6 million persons died of cancer all over the world in 2008. This figure keeps increasing with time [6, 7]. The World Health Organization has reported that cancers cause thirteen percent of all deaths per year with the most common being: breast cancer (460,000 deaths), colorectal cancer (610,000 deaths), liver cancer (700,000 deaths), stomach cancer (740,000 deaths) and lung cancer (1.4 million deaths) [8, 9]. Age has been cited as the most common risk factor, with those over 65 years being most affected [10, 11]. Kenyans expire daily from various forms of cancers with around 80,000 cases of cancer diagnosed per year [12].

In the past 40 years, there were promising progresses in the cancer diagnosis as well as in the area of molecular oncology. But, the curing rate of most cancers remains small. Numerous approaches have been employed to treat cancer amongst which the most popular is immunotherapy, radiotherapy, chemotherapy and surgery [13–15]. Chemotherapy with cytotoxic drugs is the main treatment modality for certain types of cancer [16, 17]. Chemotherapy of cancer uses drugs to destroy the cancerous cells. This works by destroying or slowing the growth of cancerous cells or stopping the cancerous cells from spreading [18–20]. It is therefore important to discover novel cytotoxic agents with diverse activity and toxicity. During the past two decades, small organic compounds have delivered a number of valuable cancer chemotherapeutic drugs [21–24]. The main class of anticancer drugs that exist today is alkylating agents, antitumor antibiotics, azole-mediated agents, antimetabolites, spindle poisons and kinase inhibitors. However, most anticancer agents cause damage to normal tissues and lack tumor specificity, leading to side effects. Amongst the most effective chemotherapeutic drugs are docetaxel, mitomycin and cisplatin [25, 26]. All of these drugs cause long-term complication or serious side effects. These side effects include blood vessel damage, nerve damage, liver damage, lower blood count, hearing loose and kidney damage [27–29]. Therefore, significant attention is still required for development of highly efficient drugs for cancer treatment.

Amongst the different classes of cytotoxic agents for cancer treatment, azole-mediated agents are gaining tremendous attention in the last decade owing to promising properties in terms of tumor specificity [30–35]. In particular, investigation reveals that 5-functionalized 4-amino-1,2,4-triazole-3-thiol derivatives are highly efficient for cancer treatment and products fewer side effects as compared to other cytotoxic agents [36–38]. Further, cytotoxic drugs based on 5-functionalized 4-amino-1,2,4-triazole-3-thiol derivatives do not cause vomiting, immunosuppression, nausea, hair loss and anamia as well as the long-term cardiac, neurological, renal and reproductive consequences [23, 36–40]. These literature results motived us to develop new and more potent analogous of 4-amino-1,2,4-triazole-3-thiols [41, 42]. Moreover, pyrrolidine-2,5-dione derivatives are also observed as part of the highly popular cytotoxic agents, for example, 3-amino-N-functionalized-pyrrolidine-2,5-dione-*N*-mustard hydrochloride [43], 1-(3-bromo-1-phenylsulfonyl-2-indolylmethyl) pyrrolidine-2,5-diones [44], 3-amino-pyrrolidine-dione-nitrogen mustard analogous [45] and 3-(9-fluorenyl) pyrrolidine-2, 5-dione analogous [46], etc. Therefore, in this work, we decided to use pyrrolidine-2,5-dione moiety for derivatizing 4-amino-1,2,4-triazole-3-thiols.

In continuation of our ongoing investigation for the development of highly effective cytotoxic agents for cancer treatment, [47–49] herein, we have developed 1,2,4-triazole-azomethine conjugates in appropriate yield by coupling substituted 4-amino-1,2,4-triazole-3-thiol derivatives with pyrrolidine-2,5-dione and screened for anticancer activity against human breast cancer cell line (MCF-7 and T47D) and human cervical cancer cell line (HeLa). Hopefully, the results of our research would provide a strong inspiration for further research progress in the area of design and development of cytotoxic drugs.

Materials and methods

Materials

All required chemicals and cell culture reagents were obtained from Sigma-Aldrich, USA. Culture wares and other plastic consumables were purchased from Nunc, Denmark. Cancer cell lines were purchased from American Type Cell Culture, ATCC, USA. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent from thermofisher scientific, USA, ApoAlertTM Annexin V, Clontech, USA, DeadEnd™ Fluorometric TUNEL Promega USA.

Experimental

Synthesis of 2-chloro-*N*′-(hydrazinecarbonothioyl) acetohydrazide (c)

In chloroform solvent (25 mL), thiocarbohydrazide **b** (6.4 g, 60.0 mmol) and potassium carbonate (8.3 g, 60.0 mmol) were mixed and then, dropwise addition of chloroacetyl chloride (6.8 g, 4.8 mL, 56.5 mmol) was done. The mixture was stirred at 50 °C for 8 h (Scheme 1). After completion of reaction, mixture was cooled at room temperature and diluted with distilled water for extraction of required product with ethyl acetate. Organic portion was dried over anhydrous sodium sulfate filtered and dried by rotary evaporator. Light yellow colored purified product was obtained.

m.p. 179–181 °C

Synthesis of succinic imide (e)

In a reaction flask, succinic acid (12.0 g, 120.0 mmol) was fused with ammonium carbonate (12.48 g, 130 mmol) with continuous stirring at 300 °C. For 2 h till completion, then reaction was cooled to room temperature, product was washed with distilled water and purified product was dried in desiccator.

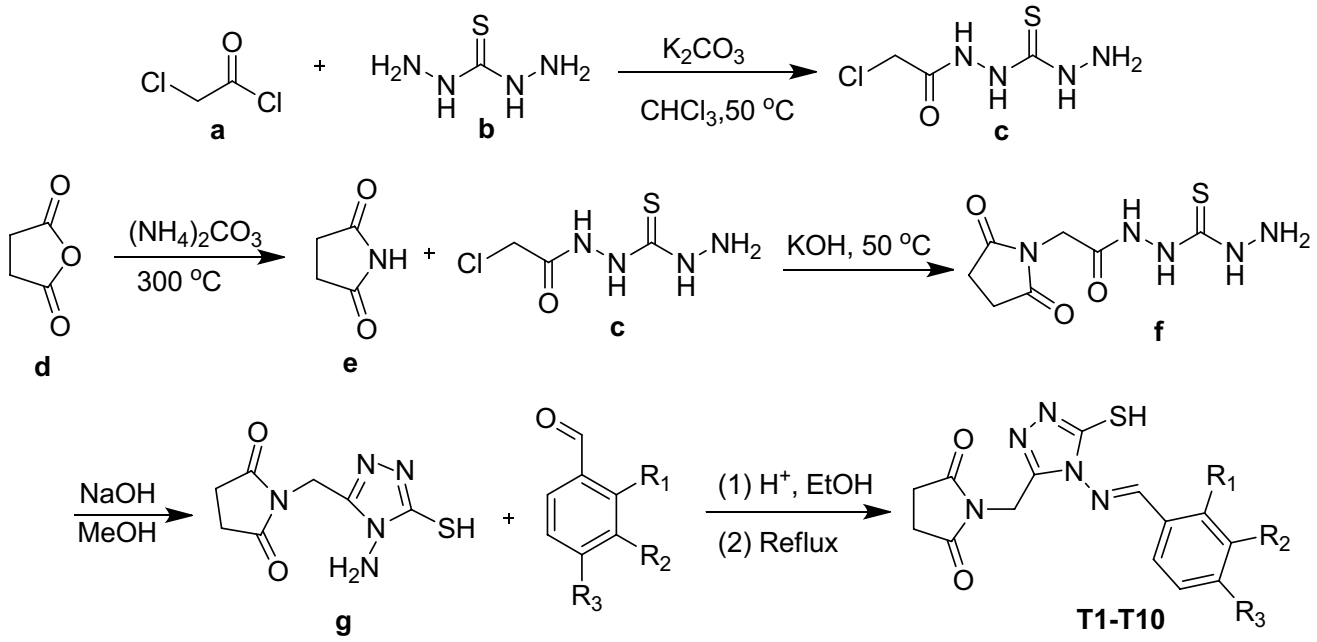
m.p. 121–124 °C

Synthesis of 2-(2,5-dioxopyrrolidin-1-yl)-*N*′-(hydrazinecarb onothioyl)acetohydrazide (f)

Compound f was synthesized by reaction of **c** (8 g, 45 mmol) with **e** (4.46 g, 45 mmol) in chloroform (25 mL) using powdered potassium hydroxide (0.06 g, 45 mmol) as base, and mixture was stirred for 3.5 h at 50 °C and monitored by TLC. After completion, reaction mixture was cooled to room temperature, extracted with DCM and dried by vacuum evaporator.

Synthesis of 1-((4-amino-5-mercapto-4*H*-1,2,4-triazol-3-yl) methyl)pyrrolidine-2,5-dione (g)

A solution of compound f (9 g, 37 mmol) in methanol (25 mL) using sodium hydroxide (1.6 g, 40 mmol) was refluxed for 4 h. The mixture was cooled to room temperature, then diluted with distilled water and acidified at 3–4 pH with HCl in order to afford precipitated product, and



T 1 R_1 = H, R_2 = H, R_3 = H
T 2 R_1 = H, R_2 = H, R_3 = $N(CH_3)_2$
T 3 R_1 = H, R_2 = H, R_3 = $N(C_2H_5)_2$
T 4 R_1 = H, R_2 = H, R_3 = OCH_3
T 5 R_1 = OH, R_2 = OCH_3, R_3 = H
T 6 R_1 = H, R_2 = NO_2, R_3 = H
T 7 R_1 = H, R_2 = H, R_3 = NO_2
T 8 R_1 = H, R_2 = NO_2, R_3 = Cl
T 9 R_1 = H, R_2 = H, R_3 = Cl
T 10 R_1 = Cl, R_2 = H, R_3 = Cl

Scheme 1 (*E*)-1-((4-(arylylideneamino)-5-mercapto-4*H*-1,2,4-triazol-3-yl)methyl)pyrrolidine-2,5-dione

obtained product was filtered and washed with excess of distilled water and recrystallized with ethanol.

Yellow powder, yield 87%, m.p. 195–197 °C, ATR (cm^{-1}) 3418–3309 (NH_2, NH), 2933 (C–Haliph.), 2550 (SH), 1780, 1726 (C=O imide), 1684 (C=O), 1621, 1611 (C=N), 1450 (CH_2), 1212 (C–S) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 14.11 (s, 1H), 5.80 (s, 2H), 5.03 (s, 2H), 4.18 (s, 4H); ^{13}CNMR($CDCl_3$) δ 176.7, 167.5, 148.7, 40.1, 33.1.

General Procedure for the Synthesis of *(E)*-1-((4-(aryliden eamino)-5-mercapto-*4H*-1,2,4-triazol-3-yl)methyl)pyrrolidine-2,5-diones (T1–T10)

1-((4-amino-5-mercapto-4*H*-1,2,4-triazol-3-yl)methyl)pyrrolidine-2,5-dione (0.34 g, 1.5 mmol) and acetic acid (4–5 drops) were added to a solution of aryl aldehydes (1.5 mmol) in dry ethanol (10 mL). The mixture was refluxed for 3–4 h and was monitored by TLC until the completion of reaction of reaction obtained. Solvent evaporated to dryness to give a residue, which was purified by recrystallization. This procedure was applied for all the compounds [50].

(*E*)-1-((4-(benzylideneamino)-5-mercapto-4*H*-1,2,4-triazol-3-yl)methyl)pyrrolidine-2,5-dione (T1) Yield 84%, R_f 0.8, m.p. 163–165 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3117 (C=C–H), 2958, 2847 (C–H), 2594 (SH), 1843, 1780 (C–N, imide), 1603 (Aromatic, $C{=}C_{str}$), 1633 (C=N), 1618 (C=N), 1455 (CH_2), 946(C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.13 (s, 1H), 8.33 (d, J = 8.5 Hz, 1H), 8.10 (d, J = 8.5 Hz, 1H),7.94–85 (m, 3H), 5.03 (s, 2H), 2.92 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 167.5, 162.5, 160.4, 150.0, 146.8, 138.4, 135.9, 131.8, 130.2, 129.8, 129.1, 124.6, 123.9, 33.1, 28.4, MS for $C_{14}H_{13}N_5O_2S$ (m/z, APCI): calcd 315.08 (M^+), found 316.16 $[M+2H]^+$.

(*E*)-1-((4-((4-(dimethylamino)benzylidene)amino)-5-mercapto-4*H*-1,2,4-triazol-3-yl) methyl)pyrrolidine-2,5-dione (T2) Yield 87%, R_f 0.73, m.p. 172–173 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3121 (C=C–H), 2960, 2851 (C–H), 2550 (SH), 1845, 1782 (C–N, imide), 1605 (Aromatic, $C{=}C_{str}$), 1637 (C=N), 1620 (C=N), 1455 (CH_2), 1367 (CH_3), 946 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.10 (s, 1H), 7.29 (d, J = 7.5 Hz, 2H), 6.82 (d, J = 7.5 Hz, 2H), 5.03 (s, 2H), 2.92 (s, 6H), 2.27 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 154.5, 149.8, 143.2, 128.9, 121.4, 111.3, 41.9, 32.6, 28.4; MS for $C_{16}H_{18}N_6O_2S$ (m/z, APCI): calcd 358.15 (M^+), found 359.09 $[M+H]^+$.

(*E*)-1-((4-((4-(diethylamino)benzylidene)amino)-5-mercapto-4*H*-1,2,4-triazol-3-yl) methyl)pyrrolidine-2,5-dione (T3) Yield 89%, R_f 0.65, m.p. 177–179 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3123 (C=C–H), 2961, 2853 (C–H), 2551(SH), 1844, 1781 (C–N, imide), 1607 (Aromatic, $C{=}C_{str}$), 1637(C=N), 1619 (C=N), 1456 (CH_2), 1365 (CH_3), 946 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.12 (s, 1H), 7.25 (d, J=7.5 Hz, 2H), 6.91 (d, J=7.5 Hz, 2H), 5.03 (s, 2H), 3.60 (q, J=6.0 Hz, 2H), 2.92 (s, 4H), 2.32 (s, 1H), 1.22 (t, J=6.0 Hz, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 152.2, 149.8, 143.2, 130.3, 121.8, 110.1, 46.3, 32.6, 28.4, 13.0; MS for $C_{18}H_{22}N_6O_2S$ (m/z, APCI): calcd 386.15 (M^+), found 387.21 $[M+H]^+$.

(*E*)-1-((5-mercapto-4-((4-methoxybenzylidene) amino)-4*H*-1,2,4-triazol-3-yl)methyl) pyrolidine-2,5-dione (T4) Yield 88%, R_f 0.59, m.p. 181–183 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3127 (C=C–H), 2963, 2858 (C–H), 2555(SH), 1845, 1783 (C–N, imide), 1605 (Aromatic, $C{=}C_{str}$), 1635(C=N), 1620 (C=N), 1455 (CH_2), 1212 (C–O), 1369 (CH_3), 946 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.08 (s, 1H), 7.37 (d, J = 7.5 Hz, 2H), 7.01 (d, J = 7.5 Hz, 2H), 5.03 (s, 2H), 3.81 (s, 3H), 2.92 (s, 4H), 2.27 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 161.0, 149.8, 143.2, 129.6, 127.1, 114.5, 56.0, 32.6, 28.4; MS for $C_{15}H_{15}N_5O_3S$ (m/z, APCI): calcd 354.09 (M^+), found 348.09 $[M+H]^+$.

(*E*)-1-((4-((2-hydroxy-3-methoxybenzylidene)amino)-5-mercapto-4*H*-1,2,4-triazol-3-yl) methyl)pyrrolidine-2,5-dione (T5) Yield 85%, R_f 0.49, m.p. 192–195 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3123 (C=C–H), 2957, 2843 (C–H), 2549 (SH), 1842, 1779 (C–N, imide), 1601 (Aromatic, $C{=}C_{str}$), 1629 (C=N), 1616 (C=N), 1451 (CH_2), 1215 (C–O), 1371 (CH_3), 946 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.10 (s, 1H), 6.99–6.75 (m, 3H), 5.03 (s, 2H), 4.44 (s, 1H), 3.81 (s, 3H), 2.92 (s, 4H), 2.26 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 148.6, 148.2, 143.2, 140.0, 123.2, 120.6, 119.5, 116.4, 56.8, 32.6, 28.4; MS for $C_{15}H_{15}N_5O_4S$ (m/z, APCI): calcd 361.08 (M^+), found 362.05 $[M+2H]^+$.

(*E*)-1-((5-mercapto-4-((3-nitrobenzylidene) amino)-4*H*-1,2,4-triazol-3-yl)methyl) pyrolidine-2,5-dione (T6) Yield 83%, R_f 0.67, m.p. 167–168 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3119 (C=C–H), 2948, 2839 (C–H), 2547 (SH), 1841, 1780 (C–N, imide), 1598 (Aromatic, $C{=}C_{str}$), 1630 (C=N), 1616 (C=N), 1553, 1351 (NO_2), 1455 (CH_2), 946 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.11 (s, 1H), 10.21 (s, 1H), 8.63 (s, 1H), 8.41 (d, J = 7.5 Hz, 1H), 8.25 (d, J = 7.0 Hz, 1H), 7.87 (dd, J = 8, 7.5 Hz, 1H), 5.03 (s, 2H), 4.18 (s, 4H), ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 167.5, 162.5, 161.6, 148.7, 146.7, 135.3, 135.2, 134.1, 131.8, 127.3, 123.9, 123.0, 33.1, 28.4; MS for $C_{14}H_{12}N_6O_4S$ (m/z, APCI): calcd 360.06 (M^+), found 361.05 $[M+H]^+$.

(*E*)-1-((5-mercapto-4-((4-nitrobenzylidene) amino)-4*H*-1,2,4-triazol-3-yl)methyl) pyrolidine-2,5-dione (T7) Yield 81%, R_f 0.69, m.p. 174–176 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3067 (C=C–H), 2915, 2847 (C–H), 2545(SH), 1596 (Aromatic, $C{=}C_{str}$), 1621 (C=N), 1617 (C=N), 1551,

1349 (NO_2), 947 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.12 (s, 1H), 8.14 (d, J = 7.5 Hz, 2H), 7.72 (d, J = 7.5 Hz, 2H), 4.95 (s, 2H), 3.10 (s, 4H), 0.14 (d, J = 5.5 Hz, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 149.8, 148.5, 143.2, 140.1, 128.5, 128.5, 124.6, 32.6, 28.4; MS for $C_{14}H_{12}N_6O_4S$ (m/z, APCI): calcd 360.06 (M^+), found 361.09 $[M+H]^+$.

(*E*)-1-((4-((4-chloro-3-nitrobenzylidene)amino)-5-mercapto-4*H*-1,2,4-triazol-3-yl) methyl)pyrrolidine-2,5-dione (T8) Yield 73%, R_f 0.67, m.p. 185–187 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3049(C=C–H), 2913, 2827 (C–H), 2541(SH), 1593 (Aromatic, C=C_{str}), 1629 (C=N), 1618 (C=N), 1549, 1345 (NO_2), 947(C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.10 (s, 1H), 8.14 (s, 1H), 7.70 (d, J = 7.5 Hz, 1H), 7.60 (d, J = 7.5 Hz, 1H), 5.03 (s, 2H), 2.91 (s, 4H), 2.29 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 149.3, 147.4, 143.2, 137.9, 133.6, 130.3, 126.8, 125.1, 32.6, 28.4; MS for $C_{14}H_{11}ClN_6O_4S$ (m/z, APCI): calcd 394.03 (M^+), found 395.02 $[M+H]^+$.

(*E*)-1-((4-((4-chlorobenzylidene)amino)-5-mercapto-4*H*-1,2,4-triazol-3-yl)methyl)pyrolidine-2,5-dione (T9) Yield 77%, R_f 0.73, m.p. 177–179 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3047(C=C–H), 2911, 2825 (C–H), 2542(SH), 1591 (Aromatic, C=C_{str}), 1627 (C=N), 1618 (C=N), 947(C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.56 (s, 1H), 7.43 (d, J = 7.5 Hz, 2H), 7.35 (d, J = 7.5 Hz, 2H), 4.95 (s, 2H), 2.91 (s, 4H), 2.27 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 149.8, 143.2, 136.0, 133.9, 129.7, 129.4, 32.6, 28.4; MS for $C_{14}H_{12}ClN_5O_2S$ (m/z, APCI): calcd 349.04 (M^+), found 351.03 $[M+2H]^+$.

(*E*)-1-((4-((2,4-dichlorobenzylidene)amino)-5-mercapto-4*H*-1,2,4-triazol-3-yl) methyl)pyrolidine-2,5-dione (T10) Yield 69%, R_f 0.71, m.p. 181–183 °C, ATR (Neat, cm^{-1}) $\tilde{\upsilon}_{max}$: 3043 (C=C–H), 2909, 2823 (C–H), 2540 (SH), 1589 (Aromatic, C=C_{str}), 1620 (C=N), 1616 (C=N), 947 (C–S); ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.06 (s, 1H), 7.63 (d, J = 1.5 Hz, 1H), 7.49 (d, J = 7.5 Hz, 1H), 7.38 (dd, J = 7.5, 1.4 Hz, 1H), 5.03 (s, 2H), 2.92 (s, 4H), 2.27 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 177.8, 165.1, 145.5, 143.2, 135.4, 135.2, 130.9, 130.1, 129.7, 128.4, 32.6, 28.4; MS for $C_{14}H_{11}C_{l2}N_5O_2S$ (m/z, APCI): calcd 383.00 (M^+), found 384.01 $[M+H]^+$.

Cell culturing

The human cell lines MCF-7, T47D (human breast adenocarcinoma) and HeLa (cervical cancer) were cultured and incubated in RPMI 1640 medium (Gibco Life Technologies) supplemented with 10% fetal calf serum (FCS; Gibco) 1% antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) using humidified atmosphere with 5% CO_2 at 37 °C.

Cytotoxicity studies

Cell viability in treated cell lines (MTT Assay)

Cytotoxicity study was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) MTT assay [51]. The cell viability was determined by measurement of purple formazan, i.e., metabolized product of tetrazolium bromide by living cells. The cells were plated in 96 well plate at a density of 6000 cells/well and incubated at 37 °C in a CO_2 incubator overnight. Next, the old medium was replaced with fresh media containing treatments at concentration of 0, 1.562, 3.125, 6.25, 12.50, 25, 50 and 100 µM/ml. After 72 h, 20 µl of MTT (5 mg/ml) was added to each well and incubated for 4 h. The 100 µl of DMSO was added to each well after removing media containing treatments, and absorbance was recorded at 570 nm using ELISA reader (Multiskan, Thermo fisher Scientific USA). Treatment was carried out in three replicates, and the results were calculated as percent growth inhibition values with the mean of two independent values (± SEM). Effective concentration or cytotoxicity was expressed as EC_{50} (half effective inhibitory concentration) and was derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable) using Graphpad Prism 7 (GraphPad, San Diego, California, USA).

Mode of cell death study

Determination of early apoptosis in treated cells

The apoptotic effect of the three potent derivatives (EC_{50} < 15 µM) on the MCF-7 was determined according to the previously reported method [52]. The MCF-7 cells was plated at a cell density of 10,000 cells/well and incubated for 24 h at 37 °C. Next, the medium was removed and fresh media containing individual treatment of **T4**, **T6** and **T10** and control (without treatment) at concentration of EC_{50}. The cells were incubated in 200 µl of binding buffer (5 µl of Annexin V-FITC and 10 µl of PI) at 37 °C for 10 min and then observed under Image Xpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA), for images.

Determination of late apoptosis in treated cells

For in situ DNA fragmentation, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)

assay was carried out using DeadEndTM Fluorometric TUNEL system (Promega, USA). The assay was done according to the manufacturer's instructions. Briefly, the MCF-7 cell line was seeded in Labtek Chamber Slides (Nunc, Denmark) with density of 2×10^4 and incubated at 37 °C for 24 h. Next, treated and control (without treatment) cells were fixed by 4% paraformaldehyde solution in PBS (pH 7.4) for 25 min at 4 °C followed by washing with phosphate buffer saline (PBS) for 5 min. After this, fixed cells were permeabilizing for 5 min by immersing the slide in 0.2% triton X-100 in PBS. The permeabilized cells were washed with PBS. Each step of washing was done thrice for 5 min. The nick ends of DNA were labeled by reaction mixture, containing equilibration buffer, nucleotide mix and enzyme rTdT (ratio 45:5:1). Reaction mixture (50 µl) was added and incubated in dark at 37 °C. Dilute 20X SSC in ratio 1:10 with deionized water and add enough to fill a standard chamber. Furthermore, propidium iodide (PI) solution was freshly prepared (1 µg/ml in PBS) and added to stain the nuclear DNA for 15 min at room temperature in the dark. Image Xpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA) was used to detect the green fluorescence of FITC-labeled apoptotic cells.

Results and discussion

All the compounds were synthesized in good to excellent yield, and preliminarily syntheses of the products were confirmed by using thin-layer chromatography technique (Scheme 1). Pre-coated TLC plates of silica gel were loaded with respect to starting material when consumption of reactants in mixture was confirmed, then the products were freed from solvent and dry products were recrystallized from ethanol. The purified compounds were further subjected for determining of melting point temperature which also confirmed the formation of new products. For further confirmation of the compounds, the mass spectrometry, FTIR and NMR spectroscopic techniques were used in FTIR and all the products showed disappearance of peak from 3400 to 3600 cm^{-1} which confirms the absence of NH_2 group in the product. Besides, the appearance of a new peak in FTIR peak at 1625–1640 cm^{-1} confirms the formation of Schiff Base. Compounds TS2, TS3, TS4 and TS5 exhibited prominent peak of CH_3 at 1367, 1365, 1369 and 1371 cm^{-1}, respectively. The presence of nitro group in TS6, TS7 and TS8 was confirmed from their representative peaks at (1553, 1351), (1552, 1351) and (1551, 1349) cm^{-1}, respectively.

All the compounds were justified by their NMR spectra; ^{1}HNMR of compound T1 showed that the imine proton absorbs at δ 9.57 ppm and rest of phenyl protons absorbs as multiplet from δ 7.81–6.83 as multiplet, and T2 showed singlet of Schiff base at δ 9.47 ppm with two doublets of para-substituted aromatic moiety at δ 7.29 and 6.82, respectively, and singlet of six protons at δ 2.92 ppm is due to dimethyl groups directly attached to nitrogen atom. Splitting pattern of T3 is same like T2 with slight change in values at δ 3.60 ppm quartet and at δ 1.22 ppm triplet; both these signals are due to the presence of ethyl group on nitrogen atom of aromatic ring. T4 displayed two doublets for para-substituted aromatic ring at δ 7.37 and 7.01 ppm with singlet of three protons at 3.81 due to methoxy protons at para position Schiff base proton at δ 9.52 ppm. Derivative T5 tri-substituted has characteristic peaks δ 9.49 ppm singlet for imine proton of Schiff base multiplet at δ 6.99–6.75 for three aromatic protons. Compound T6 showed main characteristic doublet with coupling constant value of 1.2 at δ 8.33 showed the presence of *meta*-substituted aromatic ring. Substituted pattern of compound T7 was confirmed by the presence of two doublets at δ 8.14 and 7.72 ppm. Compound two displayed a distinguished singlet at 8.14 ppm for isolated proton present at position adjacent to imine substituent on aromatic ring with two doublets of single integration at δ 8.14 and 7.70 ppm. Compound T9 showed same pattern as T4 and T7 displayed, and T10 has its characteristic singlet at δ 7.63 ppm.

Cytotoxicity in treated cell lines

The cytotoxic effects of **T1–10** on human breast carcinoma MCF-7, T47D and cervical cancer HeLa cell lines were investigated. MCF-7, T47D and HeLa showed the $EC_{50} < 50$ µM/ml except for **T2** on MCF-7 and **T8** and **T10** on T47D when treated with as **T1–10**. A cytotoxicity produced by **T4** and **T10** on MCF-7 cell line, **T4** and **T9** on T47D and **T6**, **T7** and **T9** was less than 20 µM/ml. Interestingly, **T6** produced more effective cytotoxicity on MCF-7 cells (< 10 µM/ml), which was comparable to cisplatin EC_{50} 13.10 µM/ml. The effective concentrations (EC_{50}) given in Table 1 showed that the cytotoxicity efficiencies of the derivatives under study following the order: T6 > T4 > T10 > T7 > T9 > T8 > T5 > T1 > T3 > T2 for MCF-7 cells [53]. From the results, it is evident that the MCF-7 was more sensitive toward **T4**, **T6** and **T10** as compared to T47D and HeLa cells in our studies. Therefore, these three were selected for MCF cells to further investigation for mode of cell death (apoptosis). The cytotoxicity of derivatives is depending on their death inducing ability via apoptosis or necrotic mode of cell death.

Apoptosis study (early apoptosis)

In order to determine the mode of cell death, Annexin V-FITC was used. MCF-7 cells were treated with **T4**, **T6** and **T10** at concentration of EC_{50} at 72 h for 9 h and 18 h along with positive (cisplatin). The derivatives were shown

Table 1 EC_{50} values of **T1–10** and cisplatin (μM/ml)

Samples	EC_{50}		
	MCF-7	T47D	HeLa
T1	40.10 ± 0.10	20.05 ± 0.21	25.10 ± 0.37
T2	> 100	25.22 ± 0.07	26.31 ± 0.08
T3	40.11 ± 0.05	22.20 ± 0.32	37.18 ± 0.05
T4	12.00 ± 0.20	18.10 ± 0.08	25.16 ± 0.64
T5	33.19 ± 0.40	50.12 ± 0.39	29.18 ± 0.29
T6	6.10 ± 0.32	40.00 ± 0.32	19.23 ± 0.99
T7	20.15 ± 0.12	24.20 ± 0.91	15.12 ± 0.03
T8	28.00 ± 0.32	> 100	21.16 ± 0.04
T9	20.17 ± 0.19	15.12 ± 0.16	15.00 ± 1.08
T10	12.50 ± 0.09	> 100	45.21 ± 1.20
Cisplatin	13.10 ± 0.30	21.40 ± 0.15	15.60 ± 0.02

to induce the exposure of phosphoserine (PS), which was detectable in cells by green stain of Annexin-FITC confirmed the presence of early apoptosis after 9 h incubation. Interestingly, induction of apoptosis both early (V^+) and late apoptosis (PI^+) was visible by after 18 h. Moreover, the most of cell death occurred due to apoptosis ($AnnexinV^+/PI^+$) with very less cells undergoing necrosis ($Annexin\ V^-/PI^+$) as shown in Fig. 1. Apoptosis is highly regulated mechanism of cell death quite visible by morphological feature of cell death such as permeabilization of plasma membrane, cell shrinkage and nuclear condensation. The early step in induction of apoptosis is externalization of phosphoserine protein (PS) to cell membrane [54]. The PS that is externalized contributes to the signal of recognition and engulfment of apoptotic cells by phagocytes. Interestingly, the results revealed the cytotoxic effects of **T4**, **T6** and **T10** by induction of early apoptosis in MCF-7 cell line.

Apoptosis study (late apoptosis)

To further validate the cytotoxicity by induction of apoptosis in MCF-7 cell line fluorescence, TUNEL detection system was used. The terminal transferase mediated DNA nick end labeling (TUNEL) assay, a fluorometric sensitive and accurate detection of apoptosis in cancer cells. Figure 2 rows A–D presents that the cells treated with **T4**, **T6**, **T10** and cisplatin alone, respectively, at the concentrations of EC_{50} 72 h. The green nuclei of treated were morphologically detectible due to polymerization of labeled nucleotides at the site of fragmented DNA of MCF-7 cells at 36 h. Thus, the presence of fragmentation in genomic DNA considered as hallmark of apoptosis has major role in the detection of apoptosis [55]. Furthermore, green stained nuclei was also observed in cisplatin (positive control)-treated cells. However, nuclei of negative control (without

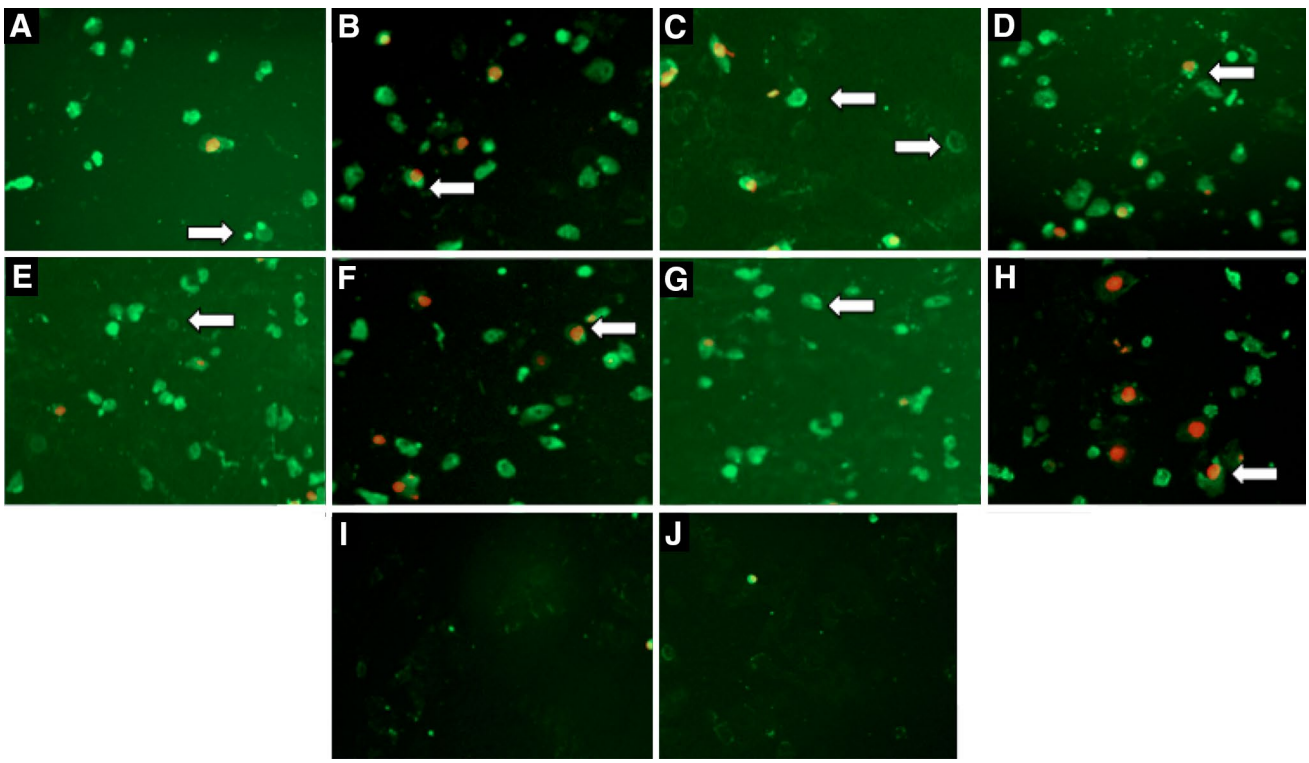


Fig. 1 The green stain (annexin-FITC) signifies induction of early apoptosis and red (PI) late apoptosis in MCF-7 cells treated with; **a**, **b** T4 for 9 h and 18 h; **c**, **d** T6 for 9 h and 18 h; **e**, **f** T10 for 9 h and 18 h; **g**, **h** cisplatin for 9 h and 18 h; **i**, **j** negative control (untreated) for 9 h and 18 h

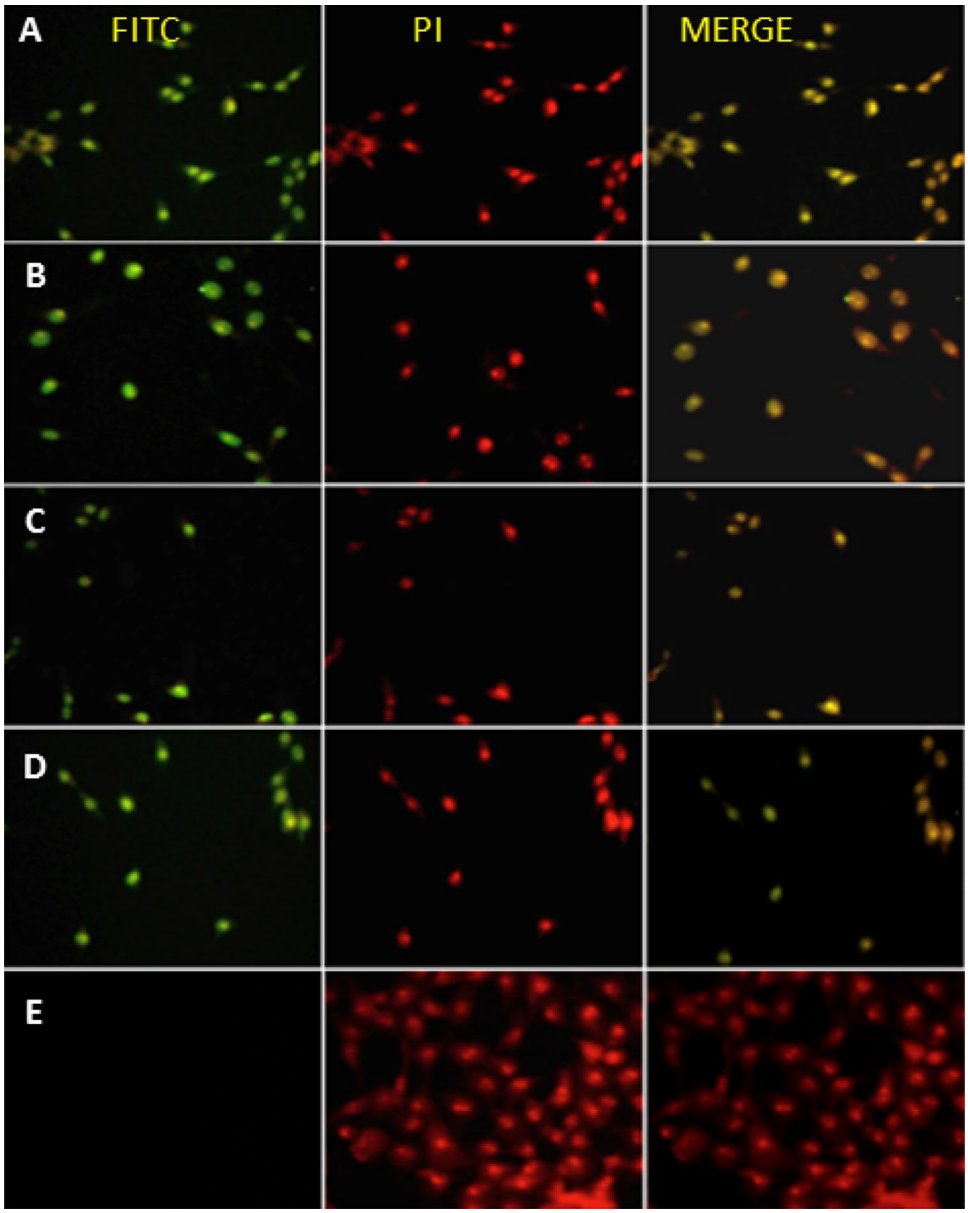


Fig. 2 The presence of green stain (FITC), which indicates DNA fragmentation in MCF-7 cells treated with row; **a** T4, **b** T6, **c** T10, **d** cisplatin, **e** negative control (untreated) for 36 (×20 magnification)

treatment) MCF-7 cells remain intact and lack green stain when observed under Image Xpress Micro XLS Widefield High-Content Analysis System (HCS) (Sunnyvale, USA). Thus, the results evidence that **T6** produces effective cytotoxicity that could act as lead compound for future studies in cancer drug discovery and development.

Conclusions

Results of cytotoxic assay (MTT) reveal that all cell lines (MCF-7, T47D and HeLa) were sensitive toward derivatives. The T6 derivative containing substituted aromatic ring with nitro group at *meta*-position was shown to be more sensitive toward MCF cells amongst other derivatives. The apoptosis was major mode of cell death in MCF-7 cells evidence by externalization of phosphatidylserine and induction of DNA fragmentation. The mode of cell death was helpful to speculate on the mechanism of action of these derivatives. However, the most active derivative T6 with EC_{50} values 6.10 μM may serve as a suitable lead compound in the development of future chemotherapeutic agents.

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