

Novel hydrazide-hydrazone derivatives containing flurbiprofen 1,2,4-triazole as anticancer agents: design, synthesis and biological evaluation

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Abstract

Hydrazone derivatives are one of the scaffolds frequently used in new drug development studies. Due to the promising pharmacological effects of the hydrazone structure, fifteen new hydrazide-hydrazone compounds containing flurbiprofen 1,2,4-triazole were synthesized in this study and their *in vitro* anticancer effects were tested. All compounds were tested for cytotoxic effects against breast cancer cell lines (MCF-7 and MDA-MB231), and glioblastoma cell line (U87) by using MTT assay. Among the synthesized compounds, compounds **7a** and **7c** exhibited the most potent cytotoxic activity with IC₅₀ values of 7.80 ± 1.20 µM and 2.40 ± 0.93 µM against MCF-7 cell line, while compound **7a** showed the highest activity with IC₅₀ value of 7.63 ± 1.05 µM against MDA-MB231 cell line. In addition, compounds **7c** and **7n** presented cytotoxic activity with IC₅₀ values of 10.31 ± 4.63 µM and 10.81 ± 6.11 µM against U87 cell line. The possible cytotoxic effects of compounds on mouse fibroblast cell line (L929) were assessed for their safety and compounds **7a**, **7c**, and **7n** were found less toxic than 5-fluorourasil. Additionally, compound **7c** was further studied to investigate its effects on apoptosis and PI3K activity, which play a role in cancer development. The results showed that compound **7c** increased apoptosis in MCF-7 cells and it displayed PI3K enzyme inhibitory activity. Our study revealed that the synthesized hydrazone compounds have the potential to be lead compounds for further studies on cancer.

Keywords: Flurbiprofen; hydrazone; 1,2,4-triazole; breast cancer; glioblastoma

1. Introduction

Despite the increasing development of today's treatment technologies, cancer maintains its vital importance in human health considering its incidence and mortality rates. Breast cancer occurs in every country in the world and is the most frequent type of cancer in women; it accounts for around 25% of all cancer cases.¹ Considering its pathophysiological features, breast cancer is a highly heterogeneous disease and is divided into two types, *in situ*

and invasive, according to its invasive ability.² One of the biggest challenges in the treatment of breast cancer is the metastasis from the breast to the liver, bone, lung, and brain, which can be frequently observed in patients. After lung cancer, breast cancer is the second most common cause of brain metastasis.³ The risk of developing brain metastasis in metastatic breast cancer patients is approximately 15–30%, which significantly limits treatment management and quality of life for breast cancer patients.⁴ The increasing development of brain metastases (BM) The increasing

number of cancer cases and the insufficient effectiveness of existing treatment strategies increase the efforts to identify new targets and develop new drug candidate compounds.

Non-steroidal anti-inflammatory drugs (NSAIDs) are a class of drugs with anti-inflammatory, analgesic, and antipyretic effects by inhibiting the cyclooxygenase (COX) enzyme responsible for the biosynthesis of prostaglandins. In addition to these effects, clinical studies have provided evidence of a relationship between COX enzyme inhibition and cancer development. It has been stated that anti-cancer activity is observed as a result of blocking the synthesis of prostaglandins, which stimulate important processes in cancer development, including angiogenesis, mutagenesis, mitogenesis, immunosuppression, antiapoptosis, and metastasis.^{5–7} In particular, it is available in the literature that inhibition of COX-2 enzyme, a subtype of the COX enzyme, prevents the development of colon, breast, lung, and prostate cancer.⁸

Flurbiprofen ((±)(*R,S*)-2-[2-fluoro-(1,1'-biphenyl)-4-yl]propanoic acid), is a non-steroidal anti-inflammatory drug that has analgesic, antipyretic, and anti-inflammatory pharmacological activity by blocking the COX enzyme. According to the literature, flurbiprofen derivatives have anticancer activity on different types of cancer such as prostate cancer by preventing metastatic formations,⁹ colon cancer by inducing 15-hydroxydehydrogenase en-

zyme,¹⁰ prostate cancer by inducing apoptosis,¹¹ breast cancer without showing toxic effects,¹² triple-negative breast cancer,¹³ thyroid cancer by inhibiting PTEN endos-tosis,¹⁴ ovarian cancer,¹⁵ and glioblastoma.¹⁶

As a result of determining the effects of non-steroidal anti-inflammatory drugs on cancer, it is aimed to develop new drug candidate compounds against macromolecular targets by adding heterocyclic rings such as triazole, oxadiazole, thiadiazole and functional groups such as thiosemicarbazide, thioether, and hydrazide-hydrazone to the structures of these active substances (Figure 1). Some drugs used in treatment today contain a 1,2,4-triazole ring in their structure: fluconazole (antifungal), estazolam (hypnotic), trapidil (antiplatelet), trazodone (antidepressant), letrozole (aromatase inhibitor), anastrozole (anti-cancer).^{17,18} Observation of anticancer activity has led to the development of promising compounds containing 1,2,4-triazole rings in cancer treatment.¹⁹ It has been reported in the literature that compounds containing 1,2,4-triazole rings are effective against prostate, breast, colon, hepatocellular carcinoma, and pancreatic cancer.^{20–25} Our previous study stated that flurbiprofen 1,2,4-triazole and thioether derivatives showed anticancer activity on the MDA-MB231 triple-negative breast cancer cell line.²⁶ Similarly, the hydrazone scaffold possessing the azomethine (-NHN=CH) group has recently become an

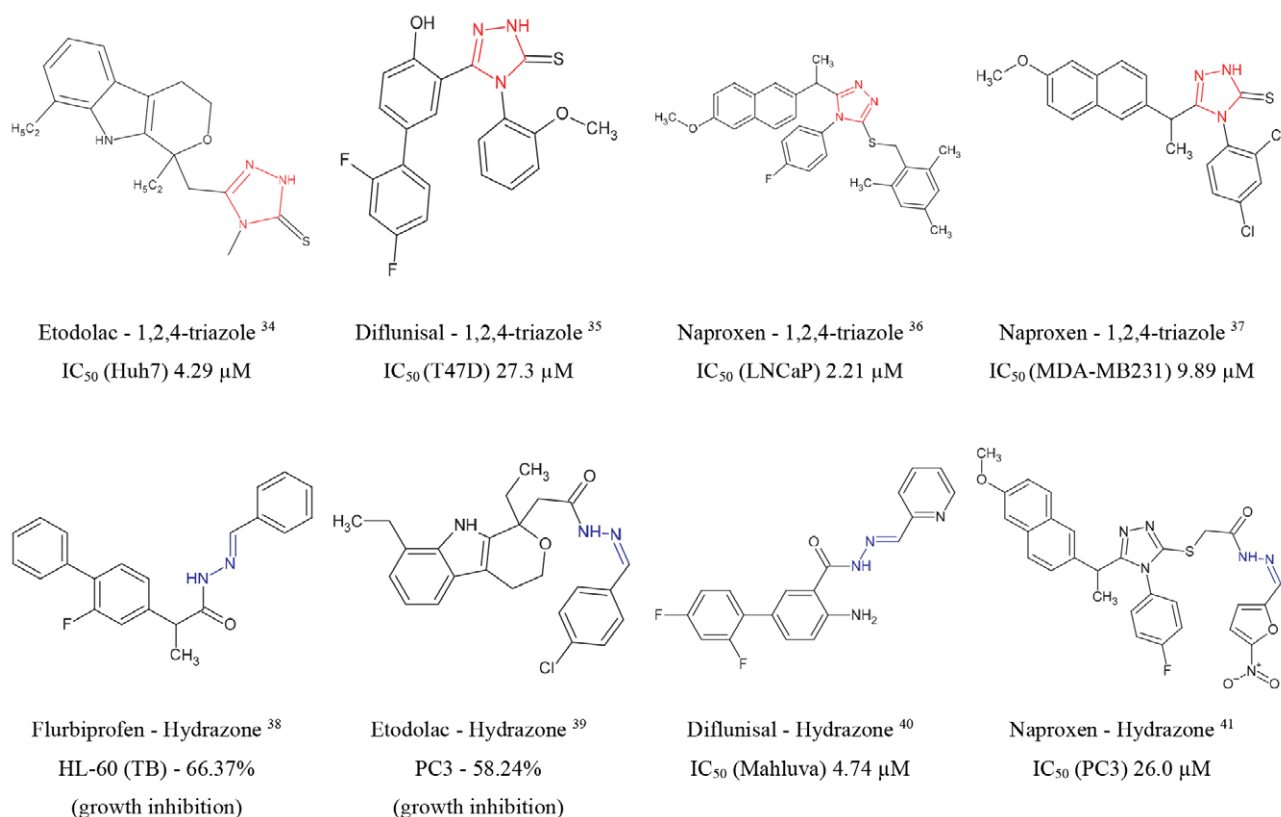


Figure 1. Some NSAID derivatives bearing 1,2,4-triazole and hydrazone scaffold with anticancer activity.

important functional group frequently used by researchers in drug discovery. Hydrazone derivatives have various pharmacological activities, including anti-inflammatory, antiplatelet, antimycobacterial, antiviral, antidepressant, and anticancer.²⁷ Among the approved drugs used in the clinic, nitrofurazone (topical antibacterial), nitrofurantoin (antibacterial), carbazochrome (hemostatic), nifuroxazide (colitis and diarrhea), dantrolene (malignant hyperthermia), zorubicin (anticancer) and bisantrene (anticancer) contain a hydrazone moiety.²⁸ It has been demonstrated in the literature that compounds bearing the hydrazone moiety show anticancer activity on various types of cancer, and this increases studies on hydrazone-based drug design in drug discovery studies for cancer.^{29–33}

Today, the synthesis of hybrid compounds containing different pharmacophore groups is an approach used in drug design to boost the biological effect and produce less toxic compounds. When compounds with anticancer activity were evaluated, taking into account the above facts, 1,2,4-triazole ring, thioether, and hydrazone structures were determined; as a result, our study focused on the synthesis of compounds containing these structures together (Figure 2).

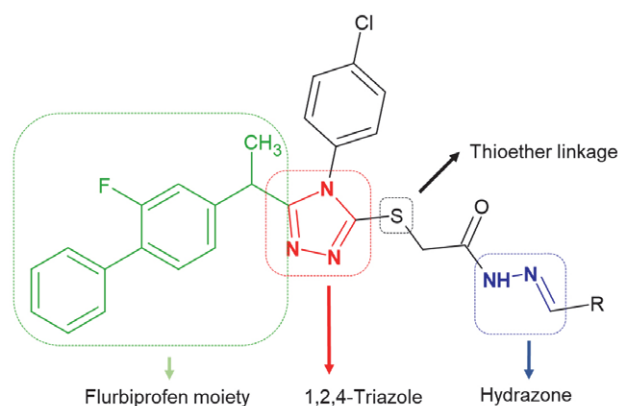


Figure 2. Design strategy by molecular hybridization approach for the synthesis of target compounds.

In light of the above information, in this study, we reported the synthesis of new derivatives bearing the 1,2,4-triazole heterocyclic ring and hydrazone moiety, based on the flurbiprofen, whose anticancer activity has been reported in the literature. The structures of the compounds were characterized by FT-IR, ¹H NMR, and ¹³C NMR spectral methods besides elemental analysis. All compounds were evaluated for their cytotoxic effects against breast cancer cell lines (MCF-7 and MDA-MB231) and glioblastoma cell line (U87) using MTT assay. Furthermore, compound **7c** (the most potent compound against MCF-7) was evaluated for Annexin-V assay to detect apoptotic cells. Additionally, it was assayed for inhibitory activity PI3K (phosphatidylinositol 3-kinase).

2. Experimental

2. 1. Chemistry

2. 1. 1. General

All the reagents and solvents were purchased from Merck and used without additional purification. The progress of all reactions was monitored by thin layer chromatography (TLC) on silica gel 60 F₂₅₄ plates (Merck) using solvent systems; M₁: petroleum ether/ethyl acetate (50/50, v/v), M₂: petroleum ether/ethyl acetate (60/40, v/v), M₃: dichloromethane/methanol (50/1, v/v). TLC spots were visualized under UV light (254 nm, *T* = 25 °C). Melting points of the synthesized compounds were determined on Kruss M3000 melting point apparatus. Elemental analyses (CHNS) were carried out on CHNS-932 (LECO). FT-IR spectra were recorded on PerkinElmer FT-IR Spectrum BX expressed in wave number ν (cm⁻¹). ¹H NMR and ¹³C NMR spectra were recorded on a Varian 600 Spectrometer. DMSO-*d*₆ was utilized as the solvent, with Me₄Si serving as the internal standard. All NMR chemical shifts are reported as δ values in parts per million (ppm). Data reported as follows: chemical shift, multiplicity (s: singlet, d: doublet, dd: double doublet, t: triplet, q: quartet, and m: multiplet), coupling constants (Hz), and integration. The numbering of carbon atoms for ¹³C NMR is given in Figure 3. The mass spectra were recorded on Agilent Technologies 6530 LC-MS QTOF instrument.

2. 1. 2. Preparation of (±)(*R,S*)-Methyl 2-[2-Fluoro-(1,1'-biphenyl)-4-yl]propanoate (**1**) (CAS Number: 66202-86-6)

Flurbiprofen (0.01 mol) was refluxed in anhydrous methanol (25 mL) for 3 h in the presence of concentrated sulfuric acid (2 mL). After cooling the reaction mixture to room temperature, it was neutralized with a sodium bicarbonate solution (NaHCO₃) (10% w/v). The resulting mixture was then extracted three times with dichloromethane, and the organic layer was evaporated.^{26,38,39,42} This process afforded compound **1** as a pure oily product in 85% yield. Oily compound, yield 85.0%; *R_f* (M₂, 25 °C): 0.9; FT-IR (ν_{max} , cm⁻¹): 3033 (aromatic C–H), 2983 (aliphatic C–H), 1738 (C=O), 1198 (C–O).

2. 1. 3. Preparation of (±)(*R,S*)-2-[2-Fluoro-(1,1'-biphenyl)-4-yl]propanehydrazide (**2**) (CAS Number: 190124-72-2)

Compound **1** (0.01 mol) and hydrazine hydrate (80%, 25 mL) were refluxed in absolute ethanol for 6 h. After cooling, the reaction mixture was diluted with distilled water. The precipitated solid was washed with water, dried and recrystallized from ethanol to give compound **2**.^{26,38,39,42} Yield 80%; m.p. 95–96 °C. This compound has been previously reported by Amir and Kumar.⁴² (Lit.: m.p.

96 °C; ^1H NMR (CDCl_3) δ 1.46 (d, $J = 7$ Hz, 3H, CH_3), 3.68 (q, $J = 7$ Hz, 1H, CH), 4.50 (s, 2H, NH_2), 7.03–7.40 (m, 8H, ArH), 8.21 (s, 1H, CONH)).⁴²

White solid, yield 80.0%; m.p. 95–96 °C; R_f (M_2 , 25 °C): 0.3; FT-IR (ν_{max} , cm^{-1}): 3311 (N–H), 3030 (aromatic C–H), 2976 (aliphatic C–H), 1634 (C=O); ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 1.33–1.34 (d, 3H, $-\text{CH}-\text{CH}_3$), 3.34–3.58 (q, 1H, $-\text{CH}-\text{CH}_3$), 4.21 (s, 2H, $-\text{NH}_2$), 7.20–7.50 (m, 8H, Ar-H), 9.22 (s, 1H, $-\text{NH}$). MS (APCI⁺) m/z calcd for $\text{C}_{15}\text{H}_{15}\text{FN}_2\text{O}$ [$\text{M}+\text{H}$]⁺, 259.1241; found 259.1258.

2. 1. 4. Preparation of ($\pm$)(R,S)-2-{2-[2-Fluoro-(1,1'-biphenyl)-4-yl]propanoyl}-*N*-methylhydrazinecarbothioamide (3) (CAS Number: 867176-57-6)

Compound 2 (0.01 mol) and equimolar amounts of 4-chlorophenyl isothiocyanate in *n*-butanol (100 mL) were stirred at room temperature for 24 hours. The reaction mixture was diluted with water and the precipitated compounds were filtered, dried, and recrystallized with ethanol. Yield 80%; m.p. 182–183 °C. This compound has been previously reported by Amir and Kumar.⁴² (Lit.: m.p. 178–180 °C; ^1H NMR (CDCl_3) δ 1.61 (d, $J = 6.6$ Hz, 3H, CH_3), 3.78 (q, $J = 6.6$ Hz, 1H, CH), 7.13–7.62 (m, 13H, 12ArH, 1NH), 8.60 (s, 1H, CSNH), 9.67 (s, 1H, CONH)).⁴²

White solid, yield 80.0%; m.p. 182–183 °C; R_f (M_3 , 25 °C): 0.4; FT-IR (ν_{max} , cm^{-1}): 3158 (N–H), 2973 (aliphatic C–H), 1683 (C=O); ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 1.41–1.42 (d, 3H, $-\text{CH}-\text{CH}_3$), 3.75 (q, 1H, $-\text{CH}-\text{CH}_3$), 7.26–7.51 (m, 12H, Ar-H), 7.13 (s, 1H, $-\text{NH}$), 9.68 (s, 1H, $-\text{NH}$), 10.15 (s, 1H, $-\text{NH}$).

2. 1. 5. Preparation of ($\pm$)(R,S)-4-(4-Chlorophenyl)-3-{1-[2-fluoro-(1,1'-biphenyl)-4-yl]ethyl}-1*H*-1,2,4-triazole-5(4*H*)-thione (4) (CAS Number: 867176-67-8)

Compound 3 (0.01 mol) was added in 4 N sodium hydroxide solution (300 mL) and the mixture was refluxed for 6 hours. At the end of the reaction, the flask content was allowed to cool, and neutralized by the addition of hydrochloric acid (10%). The resulting precipitate was filtered off, washed with water, and recrystallized from ethanol. Yield 81.6%; m.p. 172–173 °C. This compound has been previously reported by Amir and Kumar.⁴² (Lit.: 170–172 °C; ^1H NMR (CDCl_3) δ 1.73 (d, $J = 7$ Hz, 3H, CH_3), 4.34 (q, $J = 7$ Hz, 1H, CH), 7.19–7.96 (m, 12H, ArH), 11.84 (br. s, 1H, NH)).⁴²

White solid, yield 81.6%; m.p. 172–173 °C; R_f (M_1 , 25 °C): 0.8; FT-IR (ν_{max} , cm^{-1}): 3093 (triazole N–H), 3030 (aromatic C–H), 2937 (aliphatic C–H), 1624 (triazole C=N), 1129 (C=S); ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 1.50–1.51 (d, 3H, $-\text{CH}-\text{CH}_3$), 4.00–4.04 (q, 1H,

$-\text{CH}-\text{CH}_3$), 6.75–7.46 (m, 12H, Ar-H), 13.91 (s, 1H, $-\text{NH}$). MS (APCI⁺) m/z calcd for $\text{C}_{22}\text{H}_{17}\text{ClFN}_3\text{S}$ [$\text{M}+\text{H}$]⁺, 410.0889; found 410.0968.

2. 1. 6. Synthesis of ($\pm$)(R,S)-Ethyl 2-{[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio}acetate (5)

A mixture of compound 4 (0.01 mol) and ethyl bromoacetate (0.01 mol) in acetone containing anhydrous potassium carbonate (0.01 mol) was stirred at room temperature for 6 hours. After the reaction mixture was filtered, acetone was removed under reduced pressure. The resulting residue was dried and recrystallized from ethanol.

White solid, yield 80.0%; m.p. 105.5–106.5 °C; R_f (M_3 , 25 °C): 0.8; FT-IR (ν_{max} , cm^{-1}): 3094 (aromatic C–H), 2983 (aliphatic C–H), 1733 (C=O), 1623 (C=N); 1178 (C–O); ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 1.15–1.20 (t, 3H, $-\text{CH}_2-\text{CH}_3$), 1.60–1.63 (d, 3H, $-\text{CH}-\text{CH}_3$), 4.02 (s, 2H, $-\text{S}-\text{CH}_2$), 4.06–4.17 (m, 3H, $-\text{CH}_2-\text{CH}_3$, $-\text{CH}-\text{CH}_3$), 6.81–7.58 (m, 12H, Ar-H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 14.43 ($-\text{CH}_2-\text{CH}_3$), 20.98 ($-\text{CH}-\text{CH}_3$), 34.57 ($-\text{S}-\text{CH}_2$), 36.07 ($-\text{CH}-\text{CH}_3$), 61.74 ($-\text{CH}_2-\text{CH}_3$), 115.20–115.43 (C-7, $J = 23.0$ Hz), 124.02–124.05 (C-11, $J = 3.0$ Hz), 126.97 (C-17), 127.11 (C-13), 128.29 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.18 (C-9), 129.87 (C-20, C-22), 130.25 (C-19, C-23), 131.20 (C-16), 131.23 (C-14), 131.99 (C-21), 135.24 (C-18), 135.26 (C-12), 144.13–144.20 (C-6, $J = 7.0$ Hz), 150.26 (C-3), 158.00–160.45 (C-8, $J = 245.0$ Hz), 158.05 (C-5), 168.52 (C=O). Anal. calcd for $\text{C}_{26}\text{H}_{23}\text{ClFN}_3\text{O}_2\text{S}$: C, 62.96; H, 4.67; N, 8.47; S, 6.46. Found: C, 62.77; H, 5.21; N, 8.63; S, 6.52; M.W: 495.9961 g/mol; MS (APCI⁺) m/z calcd for $\text{C}_{26}\text{H}_{23}\text{ClFN}_3\text{O}_2\text{S}$ [$\text{M}+\text{H}$]⁺, 496.1256; found 496.1341.

2. 1. 7. Synthesis of ($\pm$)(R,S)-2-{[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio}acetohydrazide (6)

Compound 5 (0.01 mol) and hydrazine hydrate (80%, 25 mL) were stirred in the mixture of ethanol (100 mL) and acetone (20 mL) at room temperature. After 6 hours, the mixture was diluted with distilled water. The precipitated solid was washed with water, dried, and recrystallized from ethanol to give compound 6.

White solid, yield 87.0%; m.p. 158.9–159.9 °C; R_f (M_1 , 25 °C): 0.3; FT-IR (ν_{max} , cm^{-1}): 3231 (N–H), 3056 (aromatic C–H), 2982 (aliphatic C–H), 1655 (C=O), 1623 (C=N); ^1H NMR (600 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 1.61–1.62 (d, 3H, $-\text{CH}-\text{CH}_3$), 3.81 (s, 2H, $-\text{S}-\text{CH}_2$), 4.11–4.14 (q, 1H, $-\text{CH}-\text{CH}_3$), 4.28 (s, 2H, $-\text{NH}_2$), 6.83–7.55 (m, 12H, Ar-H), 9.31 (s, 1H, $-\text{NH}$); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$, 36 °C, TMS) δ 21.01 ($-\text{CH}-\text{CH}_3$), 34.84 ($-\text{S}-\text{CH}_2$), 36.08 ($-\text{CH}-\text{CH}_3$), 115.21–115.45 (C-7, $J = 24.0$

Hz), 124.02–124.05 (C-11, $J = 3.0$ Hz), 126.95 (C-17), 127.09 (C-13), 128.29 (C-15), 129.08–129.15 (C-10, $J = 7.0$ Hz), 129.18 (C-9), 129.96 (C-20, C-22), 130.18 (C-19, C-23), 131.20 (C-16), 131.23 (C-14), 132.08 (C-21), 135.17 (C-18), 135.26 (C-12), 144.15–144.23 (C-6, $J = 8.0$ Hz), 150.71 (C-3), 157.96–160.45 (C-8, $J = 249.0$ Hz), 158.00 (C-5), 166.49 (C=O). Anal. calcd. for $C_{24}H_{21}ClFN_5OS$: C, 59.81; H, 4.39; N, 14.53; S, 6.65. Found: C, 59.59; H, 4.51; N, 14.62; S, 6.58; M.W: 481.9728 g/mol; MS (APCI⁺) m/z calcd for $C_{24}H_{21}ClFN_5OS$ [M+H]⁺, 482.1212; found 482.1297.

2. 1. 8. N'-Substituted Benzylidene-2-{[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}acetohydrazide (7a-o)

Ethanol solution of compound **6** (0.001 mol) and equimolar amounts of appropriate aromatic aldehydes were refluxed for 6 hours. After cooling, the mixture was diluted with distilled water. The precipitated solid was washed with water, dried, and recrystallized with the appropriate solvent to give hydrazone derivatives **7a-o**.

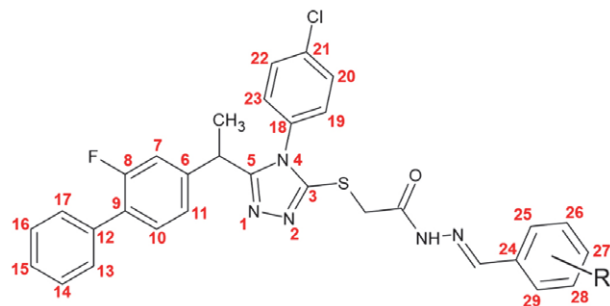


Figure 3. Number of carbon atoms of compound **7a-o**.

N'-Benzylidene-2-{[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}acetohydrazide (7a)

White solid, yield 81.2%; m.p. 104.0–105.0 °C; R_f (M_1 , 25 °C): 0.55; FT-IR ($\nu_{\max}$, cm^{-1}): 3202 (N–H), 3058 (aromatic C–H), 2979 (aliphatic C–H), 1682 (C=O), 1622 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, –CH–CH₃), 4.00 (s, 0.60H, –S–CH₂), 4.11–4.14 (q, 1H, –CH–CH₃), 4.38–4.44 (dd, 1.40H, –S–CH₂), 6.83–7.71 (m, 17H, Ar–H), 8.00 and 8.18 (s, 1H, –N=CH), 11.65–11.72 (bs, 1H, –NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.01 (–CH–CH₃), 35.40 and 35.67 (–S–CH₂), 36.10 (–CH–CH₃), 115.21–115.45 (C-7, $J = 24.0$ Hz), 124.01–124.04 (C-11, $J = 3.0$ Hz), 126.95 (C-17), 127.08 (C-13), 127.34 and 127.60 (C-26, C-28), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.18 (C-9), 129.29 and 129.31 (C-25, C-29), 129.96 (C-20, C-22), 130.17 (C-19, C-23), 130.48 and 130.65 (C-27), 131.19 (C-16),

131.21 (C-14), 132.04 and 132.16 (C-21), 134.39 and 134.51 (C-24), 135.14 and 135.20 (C-18), 135.26 (C-12), 144.19–144.26 (C-6, $J = 7.0$ Hz), 144.24 and 147.51 (–N=CH), 150.61 and 150.82 (C-3), 157.92–160.45 (C-8, $J = 253.0$ Hz), 158.00 and 158.02 (C-5), 163.78 and 169.00 (C=O). Anal. calcd for $C_{31}H_{25}ClFN_5OS \cdot 1/2H_2O$: C, 64.30; H, 4.53; N, 12.09; S, 5.54. Found: C, 64.20; H, 4.32; N, 12.27; S, 5.28; M.W: 579.0895 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{25}ClFN_5OS$ [M+H]⁺, 570.1525; found 570.1607.

N'-(4-Chlorobenzylidene)-2-{[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}acetohydrazide (7b)

White solid, yield 80.5%; m.p. 112.5–113.0 °C; R_f (M_1 , 25 °C): 0.55; FT-IR ($\nu_{\max}$, cm^{-1}): 3202 (N–H), 3057 (aromatic C–H), 2979 (aliphatic C–H), 1685 (C=O), 1623 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, –CH–CH₃), 4.00 (s, 0.65H, –S–CH₂), 4.11–4.14 (q, 1H, –CH–CH₃), 4.38–4.43 (dd, 1.35H, –S–CH₂), 6.83–7.73 (m, 16H, Ar–H), 7.99 and 8.17 (s, 1H, –N=CH), 11.72 (bs, 1H, –NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.01 (–CH–CH₃), 35.36 and 35.66 (–S–CH₂), 36.09 (–CH–CH₃), 115.21–115.44 (C-7, $J = 23.0$ Hz), 124.01–124.04 (C-11, $J = 3.0$ Hz), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 128.97 and 129.23 (C-26, C-28), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.39 (C-25, C-29), 129.96 (C-20, C-22), 130.18 (C-19, C-23), 131.17 (C-16), 131.21 (C-14), 132.02 and 132.15 (C-21), 133.35 and 133.48 (C-27), 134.90 and 135.08 (C-24), 135.15 and 135.20 (C-18), 135.26 (C-12), 142.95 and 146.19 (–N=CH), 144.18–144.25 (C-6, $J = 7.0$ Hz), 150.58 and 150.77 (C-3), 157.92–160.45 (C-8, $J = 253.0$ Hz), 158.00 and 158.02 (C-5), 163.89 and 169.07 (C=O). Anal. calcd for $C_{31}H_{24}Cl_2FN_5OS \cdot 1/2H_2O$: C, 60.69; H, 4.11; N, 11.41; S, 5.23. Found: C, 61.03; H, 4.00; N, 11.58; S, 5.30; M.W: 613.5346 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}Cl_2FN_5OS$ [M+H]⁺, 604.1135; found 604.1215

2-{[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}-N'-(4-fluorobenzylidene)acetohydrazide (7c)

White solid, yield 88.5%; m.p. 115.0–116.0 °C; R_f (M_1 , 25 °C): 0.69; FT-IR ($\nu_{\max}$, cm^{-1}): 3202 (N–H), 3057 (aromatic C–H), 2981 (aliphatic C–H), 1681 (C=O), 1614 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, –CH–CH₃), 4.00 (s, 0.65H, –S–CH₂), 4.11–4.14 (q, 1H, –CH–CH₃), 4.37–4.43 (dd, 1.35H, –S–CH₂), 6.83–7.77 (m, 16H, Ar–H), 7.99 and 8.18 (s, 1H, –N=CH), 11.66–11.73 (bs, 1H, –NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.00 (–CH–CH₃), 35.38 and 35.65 (–S–CH₂), 36.09 (–CH–CH₃), 115.21–115.44 (C-7, $J = 23.0$ Hz), 116.26–116.48 (C-26, $J = 22.0$ Hz), 124.02–124.04 (C-11, $J = 2.0$ Hz), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.47–129.56 ($J = 9$ Hz) and 129.75–129.83 ($J = 8$ Hz) (C-25), 129.96 (C-20, C-22), 130.17 (C-19, C-23),

131.02–131.05 ($J = 3$ Hz) and 131.11–131.14 ($J = 3$ Hz) (C-24), 131.18 (C-16), 131.21 (C-14), 132.03 and 132.16 (C-21), 135.14 and 135.20 (C-18), 135.26 (C-12), 143.09 and 146.39 ($-N=CH$), 144.18–144.26 (C-6, $J = 8.0$ Hz), 150.60 and 150.79 (C-3), 157.92–160.45 (C-8, $J = 253.0$ Hz), 158.00 and 158.02 (C-5), 162.27–164.73 ($J = 246.0$ Hz) and 162.39–164.86 ($J = 247$ Hz) (C-27), 163.80 and 169.00 (C=O). Anal. calcd for $C_{31}H_{24}ClF_2N_5OS \cdot H_2O$: C, 61.43; H, 4.32; N, 11.55; S, 5.29. Found: C, 61.47; H, 4.02; N, 11.69; S, 5.05; M.W: 606.0900 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}ClF_2N_5OS$ $[M+H]^+$, 588.1431; found 588.1528

***N'*-(4-Bromobenzylidene)-2-[[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio]-acetohydrazide (7d)**

White solid, yield 78.3%; m.p. 116.2–117.0 °C; R_f (M_1 , 25 °C): 0.57; FT-IR (ν_{max} , cm^{-1}): 3202 (N–H), 3056 (aromatic C–H), 2979 (aliphatic C–H), 1685 (C=O), 1623 (C=N); 1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, $-CH-CH_3$), 4.00 (s, 0.64H, $-S-CH_2$), 4.11–4.14 (q, 1H, $-CH-CH_3$), 4.38–4.43 (dd, 1.36H, $-S-CH_2$), 6.83–7.66 (m, 16H, Ar-H), 7.97 and 8.16 (s, 1H, $-N=CH$), 11.71–11.79 (bs, 1H, $-NH$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.01 ($-CH-CH_3$), 35.36 and 35.66 ($-S-CH_2$), 36.09 ($-CH-CH_3$), 115.21–115.44 (C-7, $J = 23.0$ Hz), 123.69 and 123.89 (C-27), 124.01–124.04 (C-11, $J = 3.0$ Hz), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.18 (C-9), 129.20 and 129.45 (C-25, C-29), 129.96 (C-20, C-22), 130.18 (C-19, C-23), 131.18 (C-16), 131.21 (C-14), 132.02 and 132.15 (C-21), 132.30 (C-26, C-28), 133.69 and 133.82 (C-24), 135.14 and 135.20 (C-18), 135.26 (C-12), 143.06 and 146.28 ($-N=CH$), 144.18–144.25 (C-6, $J = 7.0$ Hz), 150.58 and 150.77 (C-3), 157.92–160.45 (C-8, $J = 253.0$ Hz), 158.00 and 158.02 (C-5), 163.90 and 169.07 (C=O). Anal. calcd for $C_{31}H_{24}BrClFN_5OS$: C, 57.37; H, 3.73; N, 10.79; S, 4.94. Found: C, 56.98; H, 4.12; N, 10.86; S, 4.93; M.W: 648.9756 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}BrClFN_5OS$ $[M+H]^+$, 648.063; found 648.0710

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio]-*N'*-(4-methoxybenzylidene)acetohydrazide (7e)

White solid, yield 58.8%; m.p. 111.0–112.0 °C; R_f (M_1 , 25 °C): 0.2; FT-IR (ν_{max} , cm^{-1}): 3202 (N–H), 3056 (aromatic C–H), 2978 (aliphatic C–H), 1676 (C=O), 1605 (C=N); 1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (m, 3H, $-CH-CH_3$), 3.80 (s, 3H, $-OCH_3$), 3.98 (s, 0.60H, $-S-CH_2$), 4.11–4.14 (q, 1H, $-CH-CH_3$), 4.36–4.42 (dd, 1.40H, $-S-CH_2$), 6.83–7.65 (m, 16H, Ar-H), 7.94 and 8.11 (s, 1H, $-N=CH$), 11.52 and 11.59 (s, 1H, $-NH$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.01 ($-CH-CH_3$), 35.45 and 35.70 ($-S-CH_2$), 36.10 ($-CH-CH_3$), 55.77 ($-OCH_3$), 114.79 (C-26, C-28), 115.22–115.45 (C-7, $J = 23.0$ Hz), 124.02–124.04 (C-11, $J = 2.0$ Hz), 126.95

(C-17), 126.98 and 127.03 (C-24), 127.08 (C-13), 128.28 (C-15), 128.93 and 129.21 (C-25, C-29), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.18 (C-9), 129.97 (C-20, C-22), 130.17 (C-19, C-23), 131.18 (C-16), 131.21 (C-14), 132.04 and 132.17 (C-21), 135.13 and 135.19 (C-18), 135.26 (C-12), 144.12 and 147.40 ($-N=CH$), 144.19–144.26 (C-6, $J = 7.0$ Hz), 150.64 and 150.88 (C-3), 157.90–160.45 (C-8, $J = 255.0$ Hz), 158.00 (C-5), 161.21 and 161.36 (C-27), 163.48 and 168.72 (C=O). Anal. calcd for $C_{32}H_{27}ClFN_5O_2S \cdot 1/2H_2O$: C, 63.10; H, 4.63; N, 11.50; S, 5.26. Found: C, 63.18; H, 4.72; N, 11.59; S, 5.27; M.W: 609.1155 g/mol; MS (APCI⁺) m/z calcd for $C_{32}H_{27}ClFN_5O_2S$ $[M+H]^+$, 600.1631; found 600.1710

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio]-*N'*-(4-nitrobenzylidene)acetohydrazide (7f)

White solid, yield 69.0%; m.p. 124.0–124.7 °C; R_f (M_1 , 25 °C): 0.52; FT-IR (ν_{max} , cm^{-1}): 3203 (N–H), 3059 (aromatic C–H), 2980 (aliphatic C–H), 1692 (C=O), 1622 (C=N); 1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, $-CH-CH_3$), 4.04 (s, 0.65H, $-S-CH_2$), 4.11–4.15 (m, 1H, $-CH-CH_3$), 4.42–4.47 (dd, 1.35H, $-S-CH_2$), 6.83–8.30 (m, 16.30H, Ar-H and $-N=CH$), 8.10 (s, 0.70H, $-N=CH$), 11.97 (bs, 1H, $-NH$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 20.99 ($-CH-CH_3$), 35.24 and 35.66 ($-S-CH_2$), 36.10 ($-CH-CH_3$), 115.20–115.44 (C-7, $J = 24.0$ Hz), 124.00–124.03 (C-11, $J = 3.0$ Hz), 124.49 and 124.52 (C-26, C-28), 126.95 (C-17), 127.08 (C-13), 128.27 (C-15), 128.52 and 129.05 (C-25, C-29), 129.06–129.14 (C-10, $J = 8.0$ Hz), 129.16 (C-9), 129.95 (C-20, C-22), 130.19 (C-19, C-23), 131.17 (C-16), 131.21 (C-14), 132.00 and 132.13 (C-21), 135.16 and 135.22 (C-18), 135.24 (C-12), 140.68 and 140.86 (C-24), 141.90 and 145.06 ($-N=CH$), 144.15–144.23 (C-6, $J = 8.0$ Hz), 148.22 and 148.35 (C-27), 150.54 and 150.70 (C-3), 157.96–160.45 (C-8, $J = 249.0$ Hz), 158.00 and 158.05 (C-5), 164.32 and 169.42 (C=O). Anal. calcd for $C_{31}H_{24}ClFN_6O_3S \cdot 1/2H_2O$: C, 59.66; H, 4.04; N, 13.47; S, 5.14. Found: C, 59.81; H, 4.16; N, 13.50; S, 5.21; M.W: 624.0871 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}ClFN_6O_3S$ $[M+H]^+$, 615.1376; found 615.1443

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio]-*N'*-(4-cyanobenzylidene)acetohydrazide (7g)

White solid, yield 55.7%; m.p. 161.8–162.7 °C; R_f (M_1 , 25 °C): 0.46; FT-IR (ν_{max} , cm^{-1}): 3242 (N–H), 3058 (aromatic C–H), 2978 (aliphatic C–H), 2228 (C $\equiv$ N), 1701 (C=O), 1623 (C=N); 1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, $-CH-CH_3$), 4.02 (s, 0.65H, $-S-CH_2$), 4.11–4.14 (q, 1H, $-CH-CH_3$), 4.40–4.45 (dd, 1.35H, $-S-CH_2$), 6.83–7.92 (m, 16H, Ar-H), 8.04 and 8.24 (s, 1H, $-N=CH$), 11.88–11.97 (bs, 1H, $-NH$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.00 ($-CH-CH_3$), 35.26 and 35.63 ($-S-CH_2$), 36.09 ($-CH-CH_3$), 112.27 and

112.44 (C-27), 115.21–115.44 (C-7, $J = 23.0$ Hz), 119.11 and 119.15 (C≡N), 124.01–124.04 (C-11, $J = 3.0$ Hz), 126.95 (C-17), 127.08 (C-13), 127.92 and 128.15 (C-25, C-29), 128.29 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.95 (C-20, C-22), 130.18 (C-19, C-23), 131.18 (C-16), 131.21 (C-14), 132.01 and 132.14 (C-21), 133.20 and 133.22 (C-26, C-28), 135.15 and 135.21 (C-18), 135.25 (C-12), 138.86 and 139.02 (C-24), 142.28 and 145.55 (N=CH), 144.17–144.24 (C-6, $J = 7.0$ Hz), 150.54 and 150.69 (C-3), 157.95–160.44 (C-8, $J = 249.0$ Hz), 157.99 and 158.04 (C-5), 164.21 and 169.37 (C=O). Anal. calcd for $C_{32}H_{24}ClFN_6O_2S$: C, 64.59; H, 4.07; N, 14.12; S, 5.39. Found: C, 64.19; H, 4.20; N, 14.16; S, 5.37; M.W: 595.0890 g/mol; MS (APCI⁺) m/z calcd for $C_{32}H_{24}ClFN_6O_2S$ [M+H]⁺, 595.1478; found 595.1547

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio]-N'-(2-hydroxybenzylidene)acetohydrazide (7h)

White solid, yield 36.2%; m.p. 214.0–214.2 °C; R_f (M_1 , 25 °C): 0.57; FT-IR ($\nu_{\max}$, cm^{-1}): 3282 (N–H), 3059 (aromatic C–H), 2981 (aliphatic C–H), 1688 (C=O), 1617 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (m, 3H, -CH-CH₃), 4.00 (s, 1H, -S-CH₂), 4.11–4.16 (m, 1H, -CH-CH₃), 4.37–4.43 (dd, 1H, -S-CH₂), 6.83–7.66 (m, 16H, Ar-H), 8.30 and 8.40 (s, 1H, -N=CH), 10.06 and 10.96 (s, 1H, -OH), 11.58–11.94 (s, 1H, -NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 20.99 (-CH-CH₃), 35.38 and 35.49 (-S-CH₂), 36.10 (-CH-CH₃), 115.22–115.45 (C-7, $J = 23.0$ Hz), 116.62 and 116.82 (C-26), 119.04 and 119.84 (C-24), 119.88 and 120.47 (C-28), 124.01–124.04 (C-11, $J = 3.0$ Hz), 126.97 and 129.60 (C-29), 126.96 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.18 (C-9), 129.95 (C-20, C-22), 130.18 (C-19, C-23), 131.19 (C-16), 131.22 (C-14), 131.74 and 131.99 (C-27), 132.01 and 132.16 (C-21), 135.14 and 135.21 (C-18), 135.26 (C-12), 141.71 and 147.61 (-N=CH), 144.14–144.26 (C-6, $J = 7.0$ Hz), 150.54 and 150.84 (C-3), 156.90 and 157.74 (C-25), 157.91–160.45 (C-8, $J = 254.0$ Hz), 158.00 and 158.06 (C-5), 163.66 and 168.64 (C=O). Anal. calcd for $C_{31}H_{25}ClFN_5O_2S$: C, 63.53; H, 4.30; N, 11.95; S, 5.47. Found: C, 63.29; H, 4.27; N, 12.03; S, 5.25; M.W: 586.0789 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{25}ClFN_5O_2S$ [M+H]⁺, 586.1474; found 586.1545

N'-(3-Chlorobenzylidene)-2-[[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio]-acetohydrazide (7i)

White solid, yield 77.2%; m.p. 108.0–109.3 °C; R_f (M_1 , 25 °C): 0.43; FT-IR ($\nu_{\max}$, cm^{-1}): 3201 (N–H), 3058 (aromatic C–H), 2980 (aliphatic C–H), 1683 (C=O), 1623 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, -CH-CH₃), 4.01 (s, 0.65H, -S-CH₂), 4.11–4.14 (q, 1H, -CH-CH₃), 4.39–4.44 (dd, 1.35H, -S-CH₂), 6.83–7.76 (m, 16H, Ar-H), 7.98 and 8.17 (s, 1H, -N=CH),

11.75 and 11.86 (s, 1H, -NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.00 (-CH-CH₃), 35.32 and 35.61 (-S-CH₂), 36.10 (-CH-CH₃), 115.21–115.44 (C-7, $J = 23.0$ Hz), 124.01–124.03 (C-11, $J = 2.0$ Hz), 126.17 (C-25), 126.54 (C-29), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.95 (C-20, C-22), 130.10 (C-28), 130.17 (C-19, C-23), 130.25 (C-27), 131.19 (C-14, C-16), 132.02 and 132.16 (C-21), 134.09 and 134.15 (C-26), 135.15 and 135.20 (C-18), 135.26 (C-12), 136.64 and 136.79 (C-24), 142.61 and 145.83 (-N=CH), 144.18–144.26 (C-6, $J = 8.0$ Hz), 150.58 and 150.77 (C-3), 157.92–160.45 (C-8, $J = 253.0$ Hz), 158.00 and 158.02 (C-5), 164.02 and 169.19 (C=O). Anal. calcd for $C_{31}H_{24}Cl_2FN_5OS \cdot 1/2H_2O$: C, 60.59; H, 4.11; N, 11.41; S, 5.23. Found: C, 60.55; H, 4.13; N, 11.60; S, 5.25; M.W: 613.5346 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}Cl_2FN_5OS$ [M+H]⁺, 604.1135; found 604.1206

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio]-N'-(3-fluorobenzylidene)acetohydrazide (7j)

White solid, yield 88.2%; m.p. 114.8–116.0 °C; R_f (M_1 , 25 °C): 0.62; FT-IR ($\nu_{\max}$, cm^{-1}): 3200 (N–H), 3059 (aromatic C–H), 2979 (aliphatic C–H), 1673 (C=O), 1609 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, -CH-CH₃), 4.01 (s, 0.65H, -S-CH₂), 4.11–4.14 (q, 1H, -CH-CH₃), 4.38–4.44 (dd, 1.35H, -S-CH₂), 6.82–7.56 (m, 16H, Ar-H), 7.99 and 8.19 (s, 1H, -N=CH), 11.74 and 11.84 (s, 1H, -NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.00 (-CH-CH₃), 35.29 and 35.62 (-S-CH₂), 36.11 (-CH-CH₃), 113.19–113.41 ($J = 22$ Hz) and 113.54–113.77 ($J = 23$ Hz) (C-25), 115.21–115.44 (C-7, $J = 23.0$ Hz), 117.10–117.31 ($J = 21$ Hz) and 117.27–117.47 ($J = 20$ Hz) (C-27), 123.79–123.81 ($J = 2.0$ Hz) and 123.86–123.89 ($J = 3.0$ Hz) (C-29), 124.01–124.03 (C-11, $J = 2.0$ Hz), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.96 (C-20, C-22), 130.16 (C-19, C-23), 131.17 (C-16), 131.21 (C-14), 131.33–131.41 ($J = 8$ Hz, C-28), 132.02 and 132.16 (C-21), 135.14 and 135.20 (C-18), 135.26 (C-12), 136.94–137.02 ($J = 8$ Hz) and 137.07–137.14 ($J = 7$ Hz) (C-24), 142.79–142.82 ($J = 3$ Hz) and 146.12–146.15 ($J = 3$ Hz) (-N=CH), 144.18–144.26 (C-6, $J = 8.0$ Hz), 150.58 and 150.76 (C-3), 157.93–160.45 (C-8, $J = 252.0$ Hz), 158.00 and 158.03 (C-5), 161.63–164.05 ($J = 242$ Hz) and 161.66–164.09 ($J = 243$ Hz) (C-26), 163.99 and 169.19 (C=O). Anal. calcd for $C_{31}H_{24}ClF_2N_5OS \cdot 1/2H_2O$: C, 62.36; H, 4.22; N, 11.73; S, 5.37. Found: C, 62.48; H, 4.39; N, 11.82; S, 5.52; M.W: 597.0800 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}ClF_2N_5OS$ [M+H]⁺, 588.1431; found 588.1505

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio]-N'-(4-hydroxybenzylidene)acetohydrazide (7k)

White solid, yield 75.7%; m.p. 193.5–194.0 °C; R_f (M_1 , 25 °C): 0.13; FT-IR ($\nu_{\max}$, cm^{-1}): 3200 (N–H), 3058

(aromatic C–H), 2985 (aliphatic C–H), 1674 (C=O), 1607 (C=N); ^1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (m, 3H, $-\text{CH}-\text{CH}_3$), 3.97 (s, 0.65H, $-\text{S}-\text{CH}_2$), 4.11–4.14 (q, 1H, $-\text{CH}-\text{CH}_3$), 4.35–4.41 (dd, 1.35H, $-\text{S}-\text{CH}_2$), 6.81–7.53 (m, 16H, Ar-H), 7.89 and 8.06 (s, 1H, $-\text{N}=\text{CH}$), 9.93 (s, 1H, $-\text{OH}$), 11.45 and 11.52 (s, 1H, $-\text{NH}$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.01 ($-\text{CH}-\text{CH}_3$), 35.50 and 35.72 ($-\text{S}-\text{CH}_2$), 36.10 ($-\text{CH}-\text{CH}_3$), 115.21–115.45 (C-7, $J = 24.0$ Hz), 116.16 and 116.18 (C-26, C-28), 124.02–124.04 (C-11, $J = 2.0$ Hz), 125.40 and 125.44 (C-24), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.18 (C-9), 129.38 (C-25, C-29), 129.96 (C-20, C-22), 130.16 (C-19, C-23), 131.19 (C-16), 131.21 (C-14), 132.05 and 132.17 (C-21), 135.13 and 135.19 (C-18), 135.27 (C-12), 144.19–144.27 (C-6, $J = 8.0$ Hz), 144.57 and 147.82 ($-\text{N}=\text{CH}$), 150.65 and 150.91 (C-3), 157.89–160.45 (C-8, $J = 256.0$ Hz), 158.00 (C-5), 159.81 and 159.97 (C-27), 163.34 and 168.59 (C=O). Anal. calcd for $\text{C}_{31}\text{H}_{25}\text{ClFN}_5\text{O}_2\text{S}\cdot\text{H}_2\text{O}$: C, 61.64; H, 4.50; N, 11.59; S, 5.31. Found: C, 60.48; H, 5.08; N, 11.77; S, 5.26; M.W: 604.0889 g/mol; MS (APCI $^+$) m/z calcd for $\text{C}_{31}\text{H}_{25}\text{ClFN}_5\text{O}_2\text{S}$ $[\text{M}+\text{H}]^+$, 586.1474; found 586.1540

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}-N'-(2,4-difluorobenzylidene)acetohydrazide (7l)

White solid, yield 17.0%; m.p. 105.0–106.0 °C; R_f (M_1 , 25 °C): 0.33; FT-IR (ν_{max} , cm^{-1}): 3202 (N–H), 3062 (aromatic C–H), 2982 (aliphatic C–H), 1684 (C=O), 1615 (C=N); ^1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, $-\text{CH}-\text{CH}_3$), 3.99 (s, 0.60H, $-\text{S}-\text{CH}_2$), 4.11–4.15 (m, 1H, $-\text{CH}-\text{CH}_3$), 4.37–4.43 (dd, 1.40H, $-\text{S}-\text{CH}_2$), 6.83–7.93 (m, 15H, Ar-H), 8.14 and 8.35 (s, 1H, $-\text{N}=\text{CH}$), 11.74–11.85 (s, 1H, $-\text{NH}$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 20.99 ($-\text{CH}-\text{CH}_3$), 35.24 and 35.64 ($-\text{S}-\text{CH}_2$), 36.10 ($-\text{CH}-\text{CH}_3$), 104.73–104.98 ($J = 25$ Hz) and 104.98–105.24 ($J = 26$ Hz) (C-26), 112.96–112.99 ($J = 3$ Hz) and 113.19–113.21 ($J = 2$ Hz) (C-28), 115.20–115.44 (C-7, $J = 24.0$ Hz), 118.79–118.82 ($J = 3$ Hz) and 118.88–118.92 ($J = 4$ Hz) (C-24), 124.01–124.03 (C-11, $J = 2.0$ Hz), 126.95 (C-17), 127.09 (C-13), 128.28 (C-15), 128.40 (C-29), 129.06–129.14 (C-10, $J = 8.0$ Hz), 129.16 (C-9), 129.95 (C-20, C-22), 130.17 (C-19, C-23), 131.17 (C-16), 131.20 (C-14), 132.02 and 132.14 (C-21), 135.15 and 135.21 (C-18), 135.25 (C-12), 136.32 and 139.58 ($-\text{N}=\text{CH}$), 144.17–144.24 (C-6, $J = 7.0$ Hz), 150.53 and 150.70 (C-3), 157.95–160.45 (C-8, $J = 250.0$ Hz), 158.00 and 158.05 (C-5), 159.98–162.49 ($J = 251$ Hz) and 160.10–162.61 ($J = 251$ Hz) (C-25), 162.34–164.82 ($J = 248$ Hz) and 162.46–164.95 ($J = 249$ Hz) (C-27), 163.90 and 169.09 (C=O). Anal. calcd for $\text{C}_{31}\text{H}_{23}\text{ClF}_3\text{N}_5\text{OS}\cdot\text{H}_2\text{O}$: C, 59.66; H, 4.04; N, 11.22; S, 5.14. Found: C, 59.40; H, 4.42; N, 11.31; S, 5.12; M.W: 624.0704 g/mol; MS (APCI $^+$) m/z calcd for $\text{C}_{31}\text{H}_{23}\text{ClF}_3\text{N}_5\text{OS}$ $[\text{M}+\text{H}]^+$, 606.1337; found 606.1405

2-[[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}-N'-(2-fluorobenzylidene)acetohydrazide (7m)

White solid, yield 85.6%; m.p. 107.0–108.0 °C; R_f (M_1 , 25 °C): 0.60; FT-IR (ν_{max} , cm^{-1}): 3203 (N–H), 3058 (aromatic C–H), 2978 (aliphatic C–H), 1682 (C=O), 1614 (C=N); ^1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.05–1.07 (t, 0.5H, $-\text{CH}_3-\text{CH}_2\text{OH}$), 1.60–1.62 (t, 3H, $-\text{CH}-\text{CH}_3$), 3.42–3.47 (m, 0.5H, $-\text{CH}_3-\text{CH}_2\text{OH}$), 4.01 (s, 0.60H, $-\text{S}-\text{CH}_2$), 4.11–4.16 (m, 1H, $-\text{CH}-\text{CH}_3$), 4.36–4.37 (t, 0.25H, $-\text{CH}_3-\text{CH}_2\text{OH}$), 4.38–4.44 (dd, 1.40H, $-\text{S}-\text{CH}_2$), 6.83–7.88 (m, 16H, Ar-H), 8.21 and 8.44 (s, 1H, $-\text{N}=\text{CH}$), 11.75 and 11.87 (s, 1H, $-\text{NH}$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 19.03 ($-\text{CH}_3-\text{CH}_2\text{OH}$), 20.99 ($-\text{CH}-\text{CH}_3$), 35.27 and 35.65 ($-\text{S}-\text{CH}_2$), 36.10 ($-\text{CH}-\text{CH}_3$), 56.51 ($-\text{CH}_3-\text{CH}_2\text{OH}$), 115.21–115.44 (C-7, $J = 23.0$ Hz), 116.38–116.59 ($J = 21.0$ Hz) and 116.41–116.61 ($J = 20.0$ Hz) (C-26), 121.90–122.00 ($J = 10.0$ Hz) and 121.98–122.08 ($J = 10.0$ Hz) (C-24), 124.01–124.03 (C-11, $J = 2.0$ Hz), 125.37–125.44 ($J = 7.0$ Hz) and 125.41 (C-29), 126.72–126.74 ($J = 2.0$ Hz) and 126.84–126.86 ($J = 2.0$ Hz) (C-28), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.07–129.14 (C-10, $J = 7.0$ Hz), 129.17 (C-9), 129.95 (C-20, C-22), 130.17 (C-19, C-23), 131.17 (C-16), 131.21 (C-14), 132.01 and 132.14 (C-21), 132.36–132.45 ($J = 9.0$ Hz) and 132.57–132.65 ($J = 8$ Hz) (C-27), 135.15 and 135.21 (C-18), 135.26 (C-12), 137.06–137.10 ($J = 4$ Hz) and 140.24–140.29 ($J = 5$ Hz) ($-\text{N}=\text{CH}$), 144.17–144.25 (C-6, $J = 8.0$ Hz), 150.55 and 150.73 (C-3), 157.94–160.45 (C-8, $J = 251.0$ Hz), 158.00 and 158.05 (C-5), 159.90–162.38 ($J = 248$ Hz) and 159.98–162.46 ($J = 248$ Hz) (C-25), 163.90 and 169.11 (C=O). Anal. calcd for $\text{C}_{31}\text{H}_{24}\text{ClF}_2\text{N}_5\text{OS}\cdot 1/2\text{C}_2\text{H}_5\text{OH}$: C, 62.89; H, 4.45; N, 11.46; S, 5.25. Found: C, 61.98; H, 4.53; N, 11.52; S, 5.26; M.W: 611.1050 g/mol; MS (APCI $^+$) m/z calcd for $\text{C}_{31}\text{H}_{24}\text{ClF}_2\text{N}_5\text{OS}$ $[\text{M}+\text{H}]^+$, 588.1431; found 588.1501

N'-(2-Chlorobenzylidene)-2-[[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}-acetohydrazide (7n)

White solid, yield 95.6%; m.p. 111.0–112.0 °C; R_f (M_1 , 25 °C): 0.57; FT-IR (ν_{max} , cm^{-1}): 3201 (N–H), 3058 (aromatic C–H), 2981 (aliphatic C–H), 1686 (C=O), 1623 (C=N); ^1H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, $-\text{CH}-\text{CH}_3$), 4.00 (s, 0.60H, $-\text{S}-\text{CH}_2$), 4.11–4.16 (m, 1H, $-\text{CH}-\text{CH}_3$), 4.39–4.45 (dd, 1.40H, $-\text{S}-\text{CH}_2$), 6.83–7.94 (m, 16H, Ar-H), 8.39 and 8.57 (s, 1H, $-\text{N}=\text{CH}$), 11.82 and 11.97 (s, 1H, $-\text{NH}$); ^{13}C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.00 ($-\text{CH}-\text{CH}_3$), 35.31 and 35.65 ($-\text{S}-\text{CH}_2$), 36.10 ($-\text{CH}-\text{CH}_3$), 115.21–115.45 (C-7, $J = 24.0$ Hz), 124.01–124.04 (C-11, $J = 3.0$ Hz), 126.95 (C-17), 127.08 (C-13), 127.24 and 127.34 (C-28), 128.08 and 128.13 (C-29), 128.28 (C-15), 129.07–129.15 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.95 (C-20, C-22), 130.17 (C-19, C-23), 130.41 (C-26), 131.18 (C-16), 131.21 (C-14), 131.65 and 131.69 (C-27), 131.90 and 132.10 (C-25),

132.02 and 132.14 (C-21), 133.48 and 133.65 (C-24), 135.15 and 135.20 (C-18), 135.25 (C-12), 140.26 and 143.41 (-N=CH), 144.17–144.24 (C-6, $J = 7.0$ Hz), 150.52 and 150.71 (C-3), 157.95–160.45 (C-8, $J = 250.0$ Hz), 158.00 and 158.05 (C-5), 163.99 and 169.16 (C=O). Anal. calcd for $C_{31}H_{24}Cl_2FN_5OS \cdot 1/2H_2O$: C, 60.69; H, 4.11; N, 11.41; S, 5.23. Found: C, 60.56; H, 4.24; N, 11.51; S, 5.07; M.W: 613.5346 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{24}Cl_2FN_5OS$ [M+H]⁺, 604.1135; found 604.1206

2-{[4-(4-Chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4H-1,2,4-triazol-3-yl]thio}-N'-(2,5-dichlorobenzylidene)acetohydrazide (7o)

White solid, yield 50.4%; m.p. 135.0–136.0 °C; R_f (M_1 , 25 °C): 0.62; FT-IR (ν_{max} , cm^{-1}): 3192 (N-H), 3063 (aromatic C-H), 2981 (aliphatic C-H), 1682 (C=O), 1624 (C=N); ¹H NMR (600 MHz, DMSO- d_6 , 36 °C, TMS) δ 1.60–1.62 (t, 3H, -CH-CH₃), 4.01 (s, 0.60H, -S-CH₂), 4.10–4.16 (m, 1H, -CH-CH₃), 4.40–4.46 (dd, 1.40H, -S-CH₂), 6.83–7.92 (m, 15H, Ar-H), 8.31 and 8.51 (s, 1H, -N=CH), 11.94 (bs, 1H, -NH); ¹³C NMR (100 MHz, DMSO- d_6 , 36 °C, TMS) δ 21.01 (-CH-CH₃), 35.21 and 35.61 (-S-CH₂), 36.11 (-CH-CH₃), 115.21–115.44 (C-7, $J = 23.0$ Hz), 124.00 (C-11), 126.34 and 126.40 (C-27), 126.95 (C-17), 127.08 (C-13), 128.28 (C-15), 129.06–129.14 (C-10, $J = 8.0$ Hz), 129.17 (C-9), 129.95 (C-20, C-22), 130.16 (C-19, C-23), 131.17 (C-16), 131.21 (C-14), 131.39 and 131.57 (C-29), 132.01 and 132.15 (C-21), 132.17 and 132.22 (C-25), 132.82 and 132.87 (C-28), 133.43 and 133.51 (C-26), 135.16 and 135.21 (C-18), 135.26 (C-12), 138.89 (C-24), 142.01 and 144.13 (-N=CH), 144.18–144.25 (C-6, $J = 7.0$ Hz), 150.48 and 150.67 (C-3), 157.94–160.45 (C-8, $J = 251.0$ Hz), 158.00 and 158.06 (C-5), 164.21 and 169.35 (C=O). Anal. calcd for $C_{31}H_{23}Cl_3FN_5OS \cdot 1/2H_2O$: C, 57.46; H, 3.73; N, 10.81; S, 4.95. Found: C, 57.10; H, 3.83; N, 10.78; S, 4.86; M.W: 647.9800 g/mol; MS (APCI⁺) m/z calcd for $C_{31}H_{23}Cl_3FN_5OS$ [M+H]⁺, 638.0746; found 638.0817

2. 2. Pharmacological/biological assays

2. 2. 1. Cell culture and conditions

L929 (mouse fibroblast cell line), MCF-7 and MDA-MB231 (human breast cancer cell lines) and U87 (human glioblastoma cell line) cells were used. Cells were grown and proliferated in a medium containing 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, 2 mM L-glutamine, and 2000 mg/mL sodium bicarbonate in an incubator at 5% CO₂ and 75% humidity at 37 °C. L929, MCF-7 and MDA-MB231 cells were grown in DMEM media and U87 cells were grown in DMEM/F-12 media.

2. 2. 2. Cell Viability Assay

The effect of the synthesized compounds was determined using the MTT method on L929, MCF-7, MDA-

MB231, and U87 cells for IC₅₀ values. For this purpose, 5·10³ L929, MCF-7, MDA-MB231, and U87 cells were seeded in 100 μ L of medium in 96-well plates. After ensuring cell adhesion, the synthesized compounds were added to the cells at concentrations of 0.1, 1, 10, 100, and 1000 μ M and incubated for 24 hours. After incubation, 10 μ L MTT solution (ODC Research and Development OD-C0009D-250 Cytotoxicity assay kit) was added and incubated for 4 hours. To dissolve the formazan dye formed, the medium was aspirated and 100 μ L of solubilizing solution was added to each well. The absorbance of the resulting color was measured at 570 nm in a microplate reader. The MTT assay was performed in triplicate. The viability rate, the calculation of IC₅₀, and the statistical analyses were calculated using the program of the GraphPad Prism package.

2. 2. 3. Annexin-V/PI assay with flow cytometry

MCF-7 cells (1·10⁶ cells/well) were seeded in 6-well plates. Compound 7c (IC₅₀ values) treated with MCF-7 cells for 24 hours. After incubation, cells were harvested with trypsin-EDTA and washed with PBS. The cells were then suspended in 500 μ L of cold PBS. Commercial kit was used for Annexin-V measurement and the manufacturer's protocol was followed (BD Pharmingen, AB-2869082). 5 μ L of Annexin-V and 5 μ L of propidium iodide (PI) were added to the suspended cells, and 500 μ L of binding buffer was added and incubated for 20 minutes at room temperature in the dark. A flow cytometer (Novocyte, Agilent) was used to analyze AnnexinV/PI binding in the cells after incubation.⁴³ A total of 25,000 cells were counted in the device and their positions were determined according to the FSC/SSC method. AnnexinV/PI binding was calculated on the basis of gate coupled in control cells.

2. 2. 4. PI3K inhibition assay

PI3K (phosphatidylinositol 3-kinase) activity was quantified through the utilization of a PI3K α (p110 α /p85) Assay Kit, adhering to the established protocols set forth by the manufacturer. The measurement was conducted using the compound 7c at a concentration range of 0.1 to 40 μ M. The measurement is based on the principle that the PI3K α enzyme catalyzes the conversion of ATP to ADP while adding phosphate to the structure of the PI3K lipid substrate. A solution of 5 μ L of the compound 7c (in μ M), 5 μ L of ATP (in 12.5 μ M), and 10 μ L of the PI3K α enzyme (in 0.5 ng/ μ L) was added to a solution of 5 μ L of the PI3K lipid substrate and incubated for 40 minutes at 30 °C. Subsequently, 25 μ L of ADP-Glo reagent was added and incubated in the dark at room temperature for 45 minutes. Then, 50 μ L of kinase detection reagent was added and incubated in the dark for 30 minutes before being read on a luminescence reader (Lumate, Awareness Technology USA).

2. 2. 5. p-AKT protein levels assay

AKT phosphorylation was quantified using the Human AKT (Phospho) [pS473] ELISA Kit (Invitrogen, KHO0111), following the manufacturer's instructions. The MCF-7 cell line was seeded in 6-well plates at a density of $1 \cdot 10^6$ cells per well and incubated with compound 7c (IC_{50} values) for a period of 24 hours. Subsequently, the cells were harvested with trypsin-EDTA and disrupted with lysis buffer, after which they were centrifuged at 7500 rpm for 10 minutes. The resulting supernatant was utilized for subsequent measurement. The quantification of protein was conducted with a BCA measurement kit (ODC Research and Development), which was then employed to calculate the levels of p-AKT in mg/protein.

2. 2. 6. PI3K and AKT gene expression assay

MCF-7 cells were seeded in 6-well plates and incubated with compound 7c for 24 hours. Following this, the cells were lysed with RNA lysis buffer, and total RNA was isolated according to the manufacturer's protocol (Pure-Link, Life Technologies). The isolated RNA was converted to cDNA with the assistance of reverse transcriptase (cDNA synthesis kit, Applied Biosystem 4368814). The reaction was set up with the assistance of SYBR Green Master Mix, utilizing PI3K and AKT primers, and the cycle threshold (C_t) was analyzed in a quantitative polymerase chain reaction (qPCR) device (ABI, StepOne Plus) following 40 cycles. The primers used for the polymerase chain reaction (PCR) were as follows PI3K Forward 5'TCACATGGGTTTCCACTGTAGTT'3, Reverse 5'AAGAGTGAA-GTCTACAGTAAGGC'3, AKT Forward 5'AACGTATACACCAGGCCAGC'3, reverse 5'GCTTATGGTAAAGGCAGGCG'3, GAPDH forward, 5'-AGGGCTGCTTTTA ACTCTGGT-3' and reverse 5'-CCCACTTGATTTTG-GAGGGA-3'. GAPDH was selected as the reference gene and the $2^{-\Delta\Delta C_t}$ method was utilized for data analysis.

3. Results and discussion

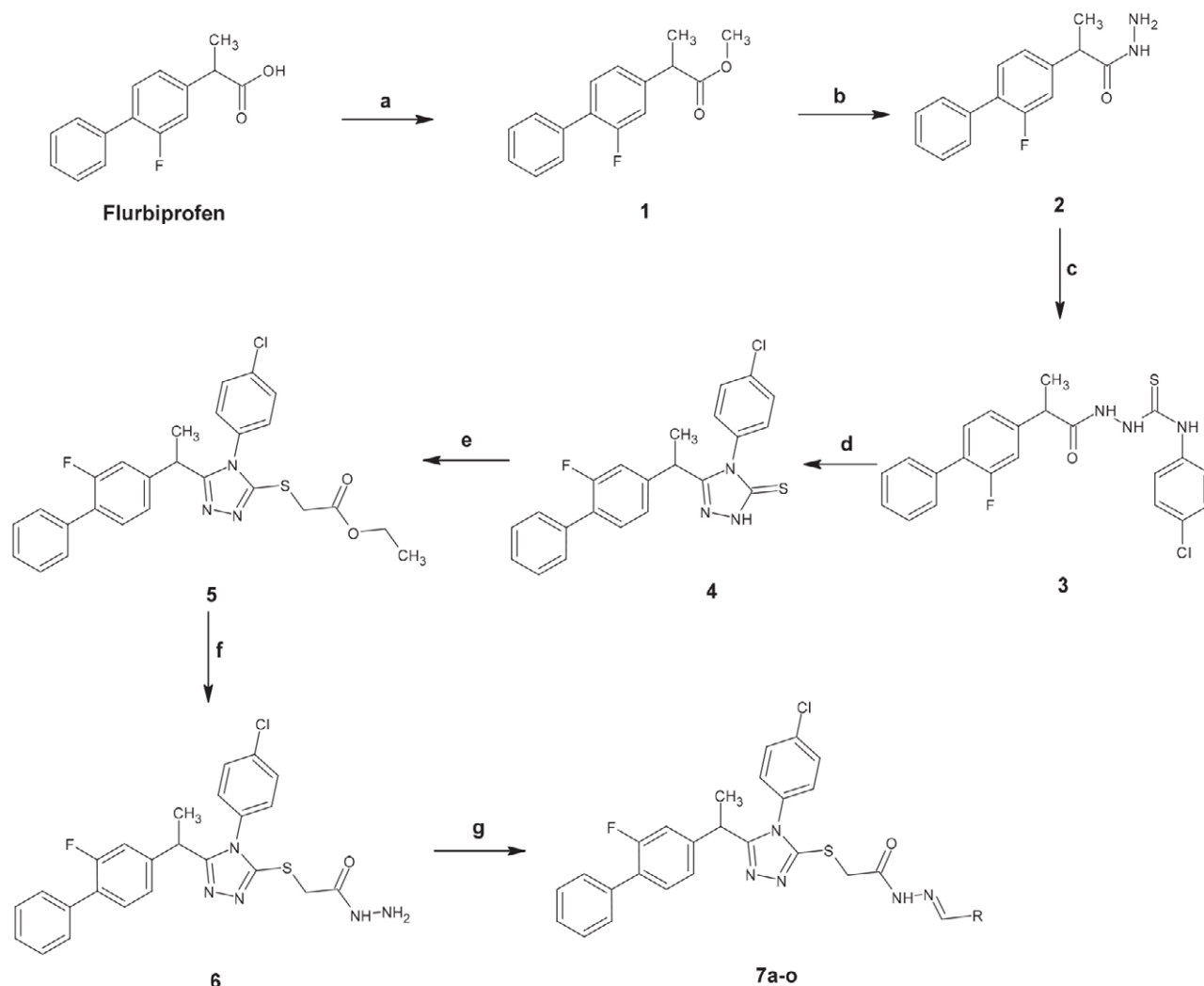
3. 1. Chemistry

In this study, fifteen new hydrazide-hydrazone derivatives were synthesized as described in Scheme 1. Flurbiprofen (($\pm$)(*R,S*)-2-[2-fluoro-(1,1'-biphenyl)-4-yl]propanoic acid) was chosen as the starting compound to synthesize the target compounds. Compounds 1 and 2 were prepared according to procedures we have previously reported.⁴² ($\pm$)(*R,S*)-2-[2-[2-Fluoro-(1,1'-biphenyl)-4-yl]propanoyl]-*N*-methylhydrazinecarbothioamide (3) was obtained by adding hydrazide derivative (compound 2) to 4-chlorophenyl isothiocyanate. ($\pm$)(*R,S*)-4-(4-chlorophenyl)-3-[1-[2-fluoro-(1,1'-biphenyl)-4-yl]ethyl]-1*H*-1,2,4-triazole-5(4*H*)-thione (4) was synthesized by cyclization of compound 3 with 4*N* NaOH. ($\pm$)(*R,S*)-Ethyl 2-[[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl]

ethyl]-4*H*-1,2,4-triazol-3-yl]thio}acetate (5) was synthesized by alkylation of compound 4 with ethyl bromoacetate in the presence of potassium carbonate using acetone as the solvent. Subsequently, ($\pm$)(*R,S*)-2-[[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio}acetohydrazide (6) was obtained as a result of the reaction of the synthesized compound with hydrazine hydrate in ethanolic solution. As the final synthesis step, compound 6 was condensed with differently substituted benzaldehydes in ethanol to obtain the target hydrazide-hydrazone compounds *N'*-substituted benzylidene-2-[[4-(4-chlorophenyl)-5-[1-(2-fluoro-[1,1'-biphenyl]-4-yl)ethyl]-4*H*-1,2,4-triazol-3-yl]thio}acetohydrazides 7a–o. Compounds 5, 6, and 7a–o are original molecules. The structures of these compounds were confirmed from elemental analysis and spectral techniques such as FT-IR, 1H NMR, and ^{13}C NMR. The spectra determined for the structure characterization of the compounds were fully consistent with the proposed structures and these spectra are provided in the Supporting Information.

In the FT-IR spectrum of compound 5, the C=O stretching band of the ester group was detected at 1733 cm^{-1} , while the C=N stretching band of the 1,2,4-triazole was detected at 1623 cm^{-1} . The transformation of flurbiprofen 1,2,4-triazoline-5-thione (4) into compound 5 was demonstrated by the disappearance of the band at 3124 cm^{-1} due to the N–H group of 1,2,4-triazole and the appearance of the C=O stretching band at 1733 cm^{-1} . The formation of compound 6 was proven by the shift of the carbonyl stretching band from 1733 cm^{-1} to 1655 cm^{-1} and by observing the N–H stretching band at 3231 cm^{-1} . In the FT-IR spectra of hydrazone derivatives 7a–o, the N–H stretching bands, C=O stretching bands, and C=N stretching bands, which the characteristic bands of the hydrazone group were observed in the range of $3192\text{--}3282\text{ cm}^{-1}$, $1673\text{--}1701\text{ cm}^{-1}$, and $1605\text{--}1624\text{ cm}^{-1}$, respectively.

1H NMR spectrum of compound 5 showed a triplet peak of methyl protons of the ester group at 1.15 ppm. A signal obtained as a three-proton multiplet in the region at 4.06–4.17 ppm was assigned to the methylene protons of ester and two protons of -S-CH₂. Aromatic protons showed multiplet peaks between 6.81–7.58 ppm. In the 1H NMR spectrum of compound 6, the absence of methyl and methylene protons belonging to the ester group and the detection of -NHNH₂ protons belonging to the hydrazide group are important evidence that the compound was obtained. A singlet peak of -NH₂ protons resonated upfield at 4.28 ppm because of the shielding effect whereas -C(O)NH proton appeared downfield at 9.31 ppm as a singlet because of the deshielding effect. In the 1H NMR spectra of compounds 7a–o, the existence of a singlet peak due to the imine (-N=CH) proton, a singlet peak for -NH proton of secondary amine, and the absence of the singlet peak for the -NH₂ protons of primary amine proved the synthesis of hydrazone compounds. Compounds 7a–o displayed a peak between 6.83–8.57 ppm due to the imine group while



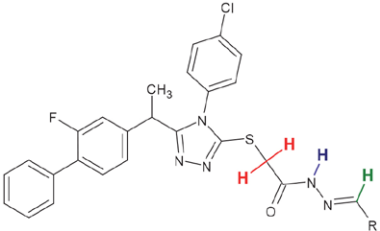
Scheme 1. Synthetic pathway of the target compounds.

a: $\text{CH}_3\text{OH}/\text{H}_2\text{SO}_4$ b: $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}/\text{C}_2\text{H}_5\text{OH}$ c: 4-Cl- C_6H_4 -NCS/*n*-butanol d: 4N NaOH/HCl e: $\text{BrCH}_2\text{COOC}_2\text{H}_5$, K_2CO_3 , acetone
f: $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}/\text{C}_2\text{H}_5\text{OH}$ g: $\text{R-CHO}/\text{C}_2\text{H}_5\text{OH}$.

Compound	R	Compound	R
7a	C_6H_5 -	7i	3-Cl- C_6H_4 -
7b	4-Cl- C_6H_4 -	7j	3-F- C_6H_4 -
7c	4-F- C_6H_4 -	7k	4-OH- C_6H_4 -
7d	4-Br- C_6H_4 -	7l	2,4-F ₂ - C_6H_3 -
7e	4-OCH ₃ - C_6H_4 -	7m	2-F- C_6H_4 -
7f	4-NO ₂ - C_6H_4 -	7n	2-Cl- C_6H_4 -
7g	4-CN- C_6H_4 -	7o	2,5-Cl ₂ - C_6H_3 -
7h	2-OH- C_6H_4 -		

they displayed 11.45–11.97 ppm due to the -NH proton. The peak between 3.97–4.47 ppm integrated for two protons was attributed to the -S-CH₂ protons. In the ¹H NMR spectra of hydrazone compounds -S-CH₂-, -N=CH and -NH protons were detected as two sets of signals. Chemical shifts (ppm) and multiplicity of these protons are given in Table 1.

According to the literature, *N*-acylhydrazones may exist in geometrical and conformational stereoisomerism due to the amide and imine groups in their structures.⁴⁴ As a result of the rotation around the C=N double bond in the imine group, two different geometric isomers, *E* and *Z*, may emerge. However, it has been pointed out that *N*-acylhydrazones do not tend to exist in the form of *Z* isomers in

Table 1. ^1H Chemical shifts (ppm) of conformers of hydrazone compounds 7a–o.


Compound	$-\text{S}-\text{CH}_2-$		$-\text{N}=\text{CH}$		$-\text{NH}$	
	-anti	-syn	-syn	-anti	-syn	-anti
7a	4.00 (s)	4.38–4.44 (dd)	8.00 (s)	8.18 (s)	11.65–11.72* (bs)	
7b	4.00 (s)	4.38–4.43 (dd)	7.99 (s)	8.17 (s)	11.72* (bs)	
7c	4.00 (s)	4.37–4.43 (dd)	7.99 (s)	8.18 (s)	11.66–11.73* (bs)	
7d	4.00 (s)	4.38–4.43 (dd)	7.97 (s)	8.16 (s)	11.71–11.79* (bs)	
7e	3.98 (s)	4.36–4.42 (dd)	7.94 (s)	8.11 (s)	11.52 (s)	11.59 (s)
7f	4.04 (s)	4.42–4.47 (dd)	6.83–8.30 (m)	8.10 (s)	11.97* (bs)	
7g	4.02 (s)	4.40–4.45 (dd)	8.04 (s)	8.24 (s)	11.88–11.97* (bs)	
7h	4.00 (s)	4.37–4.43 (dd)	8.30 (s)	8.40 (s)	11.58–11.94* (s)	
7i	4.01 (s)	4.39–4.44 (dd)	7.98 (s)	8.17 (s)	11.75 (s)	11.86 (s)
7j	4.01 (s)	4.38–4.44 (dd)	7.99 (s)	8.19 (s)	11.74 (s)	11.84 (s)
7k	3.97 (s)	4.35–4.41 (dd)	7.89 (s)	8.06 (s)	11.45 (s)	11.52 (s)
7l	3.99 (s)	4.37–4.43 (dd)	8.14 (s)	8.35 (s)	11.74–11.85* (s)	
7m	4.01 (s)	4.38–4.44 (dd)	8.21 (s)	8.44 (s)	11.75 (s)	11.87 (s)
7n	4.00 (s)	4.39–4.45 (dd)	8.39 (s)	8.57 (s)	11.82 (s)	11.97 (s)
7o	4.01 (s)	4.40–4.46 (dd)	8.31 (s)	8.51 (s)	11.94* (bs)	

* It was observed as a single signal. (s: singlet, dd: double doublet, bs: broad singlet)

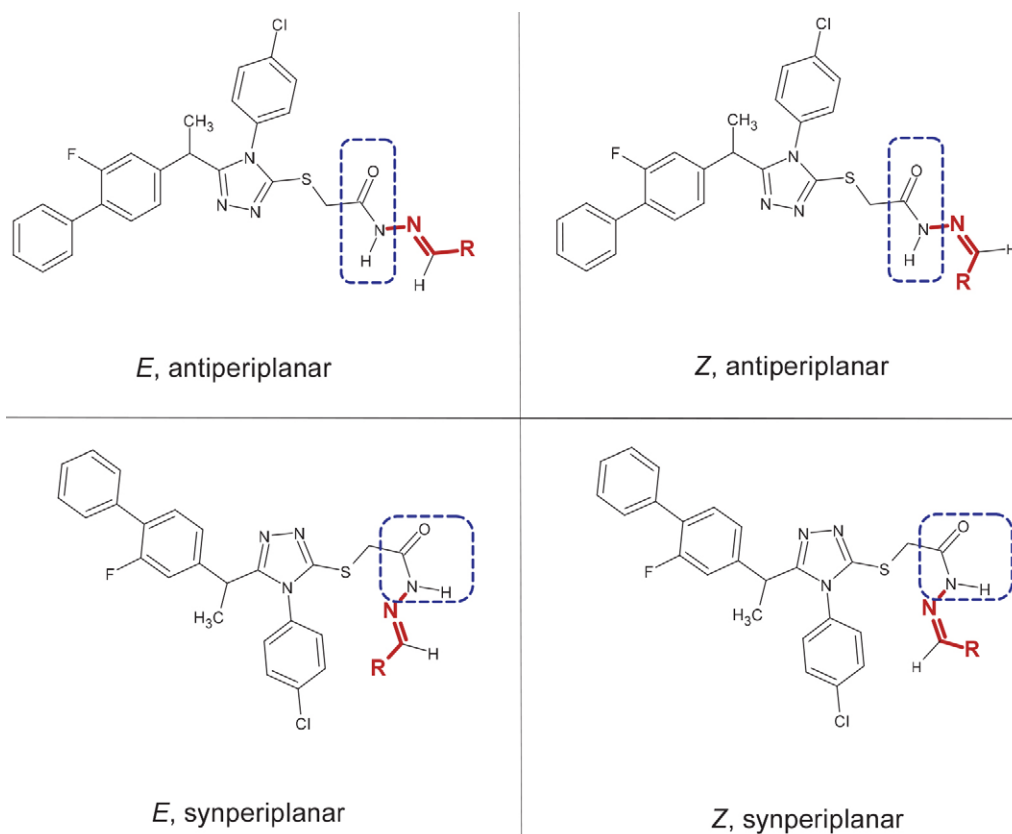


Figure 4. The structures of stereoisomers of hydrazone compounds 7a–o.

solid and liquid forms due to steric hindrance. Increased stability as a result of less steric crowding leads to a preference for the *E* geometric configuration.⁴⁵ However, in less polar solvents such as chloroform, the formation of intramolecular hydrogen bonds stabilizes the *Z* form and this form can be detected.^{46,47} On the other hand, the only isomer in DMSO-*d*₆ is the *E* isomer, and since the NMR spectra of the compounds 7a–o were performed in DMSO-*d*₆ in this study, only the *E* isomer was detected.

The second possibility is the existence of compounds 7a–o as two conformational stereoisomers; *i.e.* synperiplanar (sp) and antiperiplanar (ap). *N*-Acylhydrazones may exist as a mixture of these two conformational stereoisomers caused by the rotation of amide (C(O)-NH) bonds. According to the literature, the synperiplanar conformer is more dominant because it can make intermolecular interactions with polar solvents.⁴⁸ The ¹H NMR and ¹³C NMR spectroscopic techniques can be used to identify the described stereochemical behavior of compounds. Compounds 7a–o were detected as a mixture of two conformers in DMSO-*d*₆, *i.e.* *E*(C=N)(N–N) synperiplanar and *E*(C=N)(N–N) antiperiplanar. All of the structures of these stereoisomers are demonstrated in Figure 4.

According to the literature, the downfield signals (most deshielded) are attributed to the antiperiplanar conformer for –N=CH and –NH protons while the upfield signals are attributed to the synperiplanar conformers. In

contrast, for –S-CH₂, the proton signals assigned to the antiperiplanar conformer are upfield.^{44,45,49,50,51} In the ¹H NMR spectra of compounds 7a–o, –N=CH and –NH protons appeared as two signals around 6.83–8.39 ppm and 11.45–11.82 ppm for the synperiplanar form, while the antiperiplanar form of the same protons appeared in the region at 8.06–8.57 ppm and 11.52–11.97 ppm, respectively. The –S-CH₂ signals of the synperiplanar form resonated between 4.35–4.47 ppm, and for the antiperiplanar form between 3.97–4.04 ppm (Table 1). The peaks of the conformers observed in the ¹H NMR spectra of representative compound 7e are shown in Figure 5.

¹³C NMR spectrum of compound 5, the presence of C=O, methyl, and methylene peaks of the ester group confirmed the structure of the compound. The peaks at 14.43 and 61.74 ppm were assigned to the methyl and methylene carbons. The carbonyl carbon was detected at 168.52 ppm. In the ¹³C NMR spectrum of compound 6, the absence of methyl and methylene carbons of the ester group and the presence of carbonyl peak at 166.49 ppm proved the obtained of hydrazide. Compounds 7a–o showed a peak of methylene (–S-CH₂) carbon between 35.21–35.72 ppm and imine (–N=CH) carbon at 136.32–147.82 ppm. Carbonyl carbon exhibited a peak around 163.34–169.42 ppm. In the ¹³C NMR spectra of hydrazone compounds 7a–o, duplicated peaks were observed for certain carbon atoms, similar to the ¹H NMR spectra of the compounds. Down-

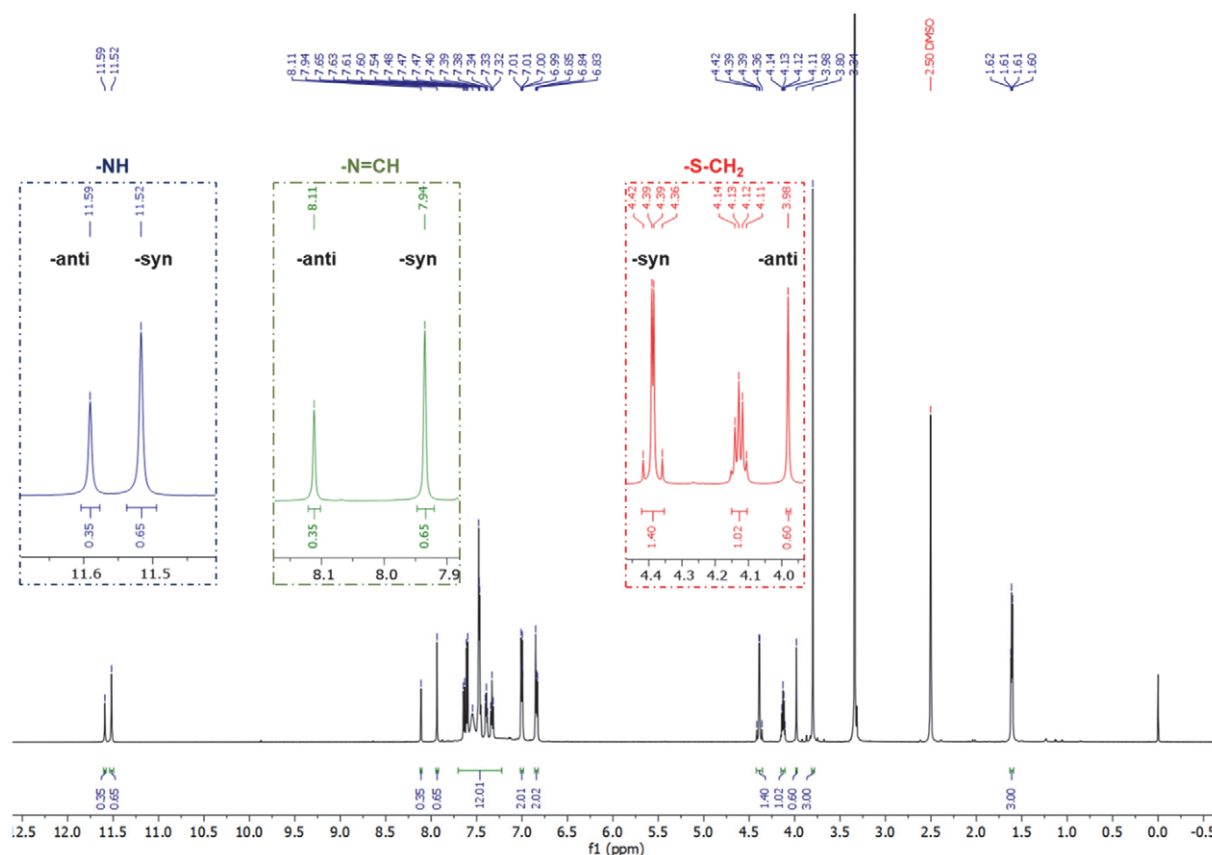
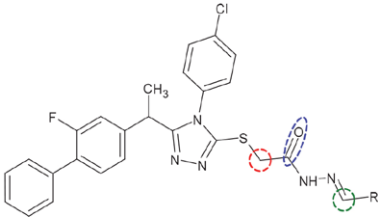


Figure 5. ¹H NMR spectra of compound 7e.

Table 2. ^{13}C Chemical shifts (ppm) of conformers of hydrazone compounds **7a–o**.


Compound	$-\text{S}-\text{CH}_2$		$-\text{N}=\text{CH}-$		$\text{C}=\text{O}$	
	-syn	-anti	-syn	-anti	-anti	-syn
7a	35.40	35.67	144.24	147.52	163.78	169.00
7b	35.36	35.66	142.95	146.19	163.89	169.07
7c	35.38	35.65	143.09	146.39	163.80	169.00
7d	35.36	35.66	143.06	146.28	163.90	169.07
7e	35.45	35.70	144.12	147.40	163.48	168.72
7f	35.24	35.66	141.90	145.06	164.32	169.42
7g	35.26	35.63	142.28	145.55	164.21	169.37
7h	35.38	35.49	141.71	147.61	163.66	168.64
7i	35.32	35.61	142.61	145.83	164.02	169.19
7j	35.29	35.62	142.79–142.82 ($J = 3 \text{ Hz}$)	146.12–146.15 ($J = 3 \text{ Hz}$)	163.99	169.19
7k	35.50	35.72	144.57	147.82	163.34	168.59
7l	35.24	35.64	136.32	139.58	163.90	169.09
7m	35.27	35.65	137.06–137.10 ($J = 4 \text{ Hz}$)	140.24–140.29 ($J = 65 \text{ Hz}$)	163.90	169.11
7n	35.31	35.65	140.26	143.41	163.99	169.16
7o	35.21	35.61	142.01	144.13	164.21	169.35

field peaks were assigned to antiperiplanar conformers for $-\text{S}-\text{CH}_2$ and $-\text{N}=\text{CH}$ carbons, while they were assigned to synperiplanar conformers for carbonyl carbon. Chemical shifts (ppm) of these carbons are given in Table 2.

3. 2. Biological activity of compounds

In this study, compounds **5**, **6**, and **7a–o** were evaluated for their in-vitro cytotoxic activity against two human breast cancer cell lines namely MCF-7 and MDA-MB231, human glioblastoma cell line U87, and mouse fibroblast cell line L929 (as control cells) by MTT assay. All the compounds were treated with different concentrations from 0.1 to 1000 μM for 24 hours. 5-Fluorouracil (5-FU), the well-known chemotherapy agent was chosen as the positive control. The results are presented in terms of IC_{50} values in Table 3 (graphs illustrating the cell viability values of the novel flurbiprofen derivatives are in the Supplementary Information).

According to the IC_{50} values presented in Table 3, the majority of the hydrazone-hydrazone compounds **7a–o** demonstrated more potent cytotoxic activity than compounds **5** and **6**. From these data, compound **7a** which contains non-substituted phenyl, and compound **7c** which contains 4-fluorophenyl exhibited strong cytotoxic activities against MCF-7 cells with IC_{50} values of 7.807 ± 1.20

μM and $2.405 \pm 0.93 \mu\text{M}$, respectively. In particular, compound **7c** was found to be the most potent compound, with even higher activity than 5-fluorouracil which was used as a positive control. In the case of MDA-MB231 cells, compound **7a** demonstrated the highest anticancer activity with an IC_{50} value of $7.631 \pm 1.05 \mu\text{M}$ superior to that of the 5-fluorouracil (IC_{50} value $10.91 \pm 0.23 \mu\text{M}$). For human glioblastoma cells (U87), compound **7c** bearing 4-fluorophenyl and compound **7n** bearing 2-chlorophenyl displayed significant cytotoxicity with IC_{50} values of $10.31 \pm 4.63 \mu\text{M}$ and $10.81 \pm 6.11 \mu\text{M}$ concerning the standard drug (IC_{50} value $18.22 \pm 3.55 \mu\text{M}$).

To evaluate the selectivity properties, the cytotoxic activities of synthesized compounds were also assessed against L929 normal mouse fibroblast cells. Most of the compounds showed selective properties against L929 cells, exhibiting significantly lower cytotoxicity than 5-fluorouracil. These results revealed, compounds **a**, **c**, and **n**, which showed cytotoxic effects against L929 cells with IC_{50} values of $118.4 \pm 11.02 \mu\text{M}$, $152.8 \pm 6.95 \mu\text{M}$, and $184.4 \pm 7.16 \mu\text{M}$, respectively, were determined as the most promising chemotherapeutic agents.

Structure–Activity Relationship (SAR)

Hydrazone derivatives were synthesized from a series of benzaldehyde derivatives bearing halogens (fluoro-

rine, chlorine, bromine), electron-donating groups (methoxy, hydroxy), and electron-withdrawing groups (nitro, cyano) substituted at the ortho, meta, or para positions. Comparison of the IC₅₀ values obtained for the MCF-7 cell line revealed that compounds bearing mono-substituted halogens, namely compound 7c (4-fluorophenyl), 7d (4-bromophenyl), 7i (3-chlorophenyl), and 7j (3-fluorophenyl), generally exhibited higher cytotoxic activity than the other derivatives. In contrast, the mono-substituted derivatives with halogens at the 2-position, such as compounds 7m and 7n, as well as the 2,4-disubstituted derivatives including compounds 7l and 7o, showed markedly reduced cytotoxic activity. In MDA-MB231 cells, electron-donating substituents, such as hydroxyl and methoxy groups, as present in compounds 7h (2-hydroxyphenyl),

7k (4-hydroxyphenyl), and additionally in compound 7e (4-methoxyphenyl), led to markedly enhanced cytotoxic activity compared to the other derivatives. In contrast, the compounds containing hydroxyl and methoxy groups exhibited low cytotoxic activity in U87 cells. Moreover compounds 7f and 7g, containing electron-withdrawing groups such as nitro and cyano, were found to exhibit low cytotoxic activity in both MDA-MB231 and U87 cell lines.

Effects of Compound 7c on Annexin-V/PI Binding

To evaluate the possible anti-apoptotic effect of compound 7c on MCF-7 cells, Annexin V/PI assay was performed. Based on the MTT assay, compound 7c was chosen as the lead compound for further analysis due to its highest in-vitro selective cytotoxic activity against MCF-7

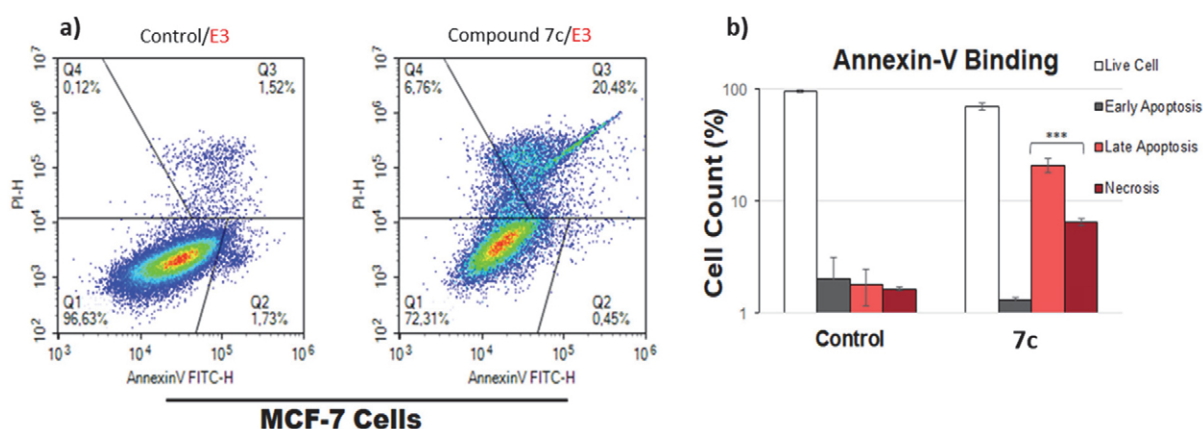


Figure 6. Annexin-V/PI binding changes of compound 7c administration to MCF-7 cells (a) Flowcytometry binding images of compound 7c. (b) Cell count of early and late apoptotic cells and necrotic cells (***p < 0.001 compared to control in MCF-7 cells).

Table 3. IC₅₀ values (μM) of novel flurbiprofen derivatives.

Compounds Cell Lines	Mouse Fibroblast Cell line	Human Glioblastoma Cell Line		Human Breast Cancer
	L929	MCF-7	MDA-MB231	U87
5	88.37 ± 2.38	163.50 ± 2.47	119.90 ± 2.45	36.73 ± 2.30
6	90.79 ± 7.65	77.42 ± 2.52	57.81 ± 2.04	125.84 ± 9.37
7a	118.40 ± 11.02	7.80 ± 1.20	7.63 ± 1.05	119.16 ± 7.08
7b	170.90 ± 12.84	157.4 ± 2.46	56.63 ± 2.03	124.79 ± 11.84
7c	152.8 ± 6.95	2.40 ± 0.93	87.68 ± 6.25	10.31 ± 4.63
7d	127.60 ± 9.06	32.12 ± 2.007	35.77 ± 1.81	157.17 ± 5.17
7e	120.20 ± 10.78	171.90 ± 5.50	28.05 ± 1.78	145.03 ± 9.42
7f	155.60 ± 6.35	54.37 ± 2.10	109.40 ± 3.54	119.53 ± 8.16
7g	125.70 ± 2.22	72.88 ± 2.14	79.52 ± 2.16	169.26 ± 7.90
7h	83.39 ± 4.59	33.14 ± 3.28	22.35 ± 1.88	125.79 ± 14.66
7i	66.71 ± 8.20	23.31 ± 1.71	91.40 ± 4.65	78.36 ± 12.05
7j	155.30 ± 13.57	27.47 ± 1.59	57.53 ± 2.05	84.18 ± 9.33
7k	143.80 ± 1.55	38.20 ± 2.03	19.71 ± 0.81	143.45 ± 12.82
7l	124.80 ± 13.09	149.90 ± 2.33	39.05 ± 1.80	169.20 ± 6.99
7m	154.70 ± 7.93	198.30 ± 12.03	41.28 ± 4.08	44.47 ± 8.71
7n	184.40 ± 7.16	164.20 ± 1.95	28.91 ± 1.95	10.81 ± 6.11
7o	162.70 ± 5.59	154.60 ± 0.83	57.30 ± 3.05	50.77 ± 9.17
5-FU (positive control)	89.18 ± 2.91	3.81 ± 0.32	10.91 ± 0.23	18.22 ± 3.55

cells. Annexin-V/PI binding is an important marker used to detect apoptosis in cells. Annexin-V binds to phosphatidylserine which is externalized on the cell membrane throughout the apoptotic process and binds to nucleic acids when the PI core structure is disrupted. Only Annexin-V binding is seen in early apoptosis and both Annexin-V and PI binding are seen in late apoptosis. In this study, MCF-7 cells were treated with compound **7c** at 2.405 μM (IC_{50} values) concentrations for 24 h, and apoptosis was quantified by flow cytometry. The graphical values of the results are given in Figure 6. As shown in Figure 6a, after the treatment of compound **7c**, the percentage of late apoptotic cells (upper right quadrant) significantly increased to 20.48% compared to control MCF-7 cells (from 1.52%). Compound **7c** did not cause any significant change in early apoptosis (lower right quadrant) in MCF-7 cells. At the same time, a considerable increase in the number of necrotic cells (upper left quadrant) was observed by 6.76%, compared to 0.12% in the control group. Taken together, the results indicate that compound **7c** is a novel potential apoptosis-inducing agent in MCF-7 cells.

Effects of Compounds **7c** on PI3K Activity and AKT Signalling

The PI3K/AKT pathway is a signaling pathway that plays a pivotal role in the pathogenesis of cancer. It regu-

lates a multitude of cellular processes, including growth, proliferation, survival, metabolism, motility, and cell cycle regulation, in cancer cells.⁵² The principal components of this pathway are PI3K (phosphatidylinositol 3-kinase) and AKT (protein kinase B). Upon phosphorylation of AKT, the pathway is activated, thereby preventing the death of cancer cells and increasing their proliferation.⁵³ Compound **7c** demonstrated a notable inhibitory effect on PI3K enzyme activity at concentrations exceeding 1 μM in MCF-7 cells, effectively halting the pathway (Figure 7a, $p < 0.001$). No alteration was observed in PI3K gene expression (Figure 7b). A notable decline was evident in AKT phosphorylation levels (p-AKT) in comparison to the control group (Figure 7c, $p < 0.001$). Additionally, there was a notable decline in AKT gene expression (Figure 7d, $p < 0.001$).

4. Conclusion

In this study, we have described the synthesis, characterization, and in-vitro cytotoxic effects of a series of new flurbiprofen hydrazone derivatives bearing 1,2,4-triazole ring **7a–o**. According to the ^1H NMR and ^{13}C NMR spectroscopic analysis results, it was determined that the synthesized compounds were a mixture of -syn and -anti

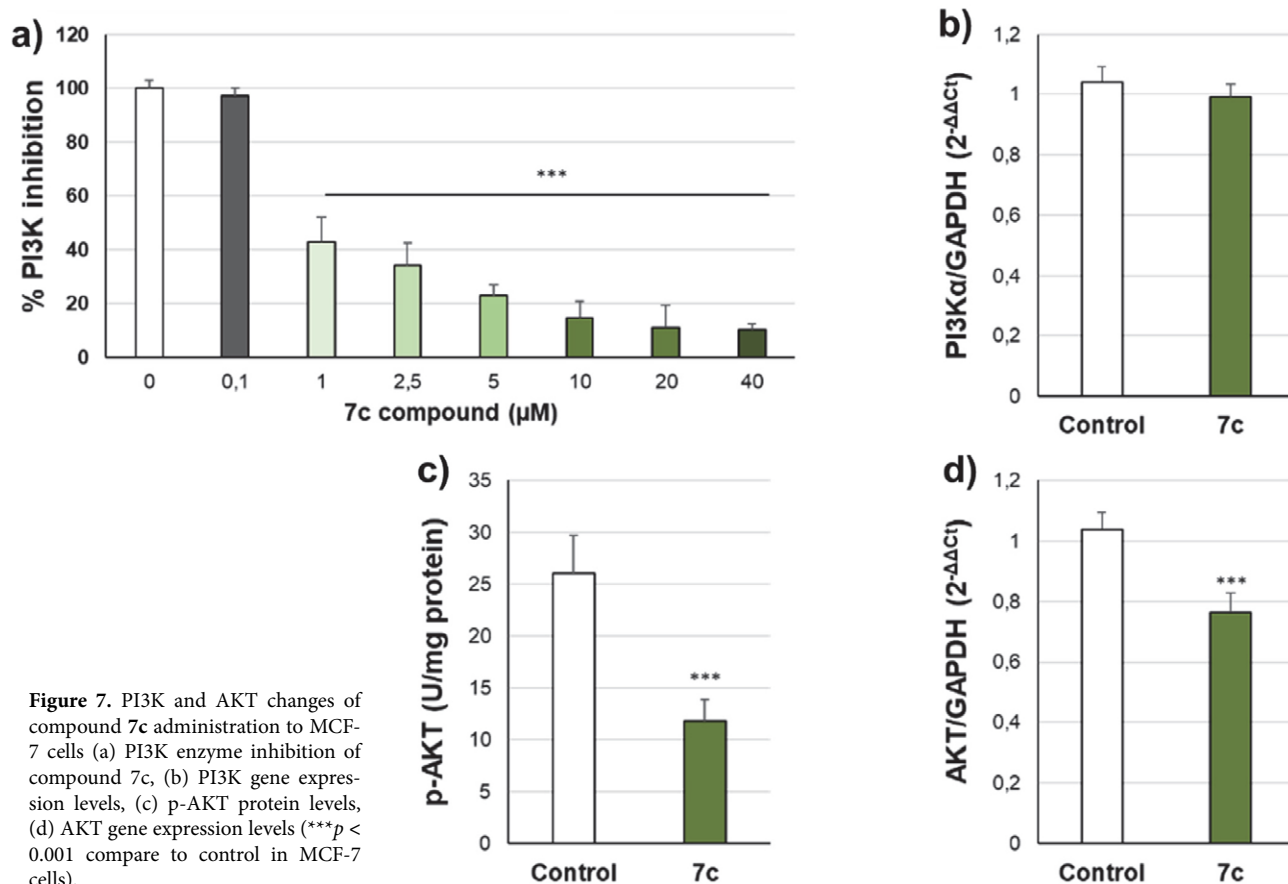


Figure 7. PI3K and AKT changes of compound **7c** administration to MCF-7 cells (a) PI3K enzyme inhibition of compound **7c**, (b) PI3K gene expression levels, (c) p-AKT protein levels, (d) AKT gene expression levels (** $p < 0.001$ compare to control in MCF-7 cells).

conformers due to the amide bond. All compounds were screened for anticancer activity against MCF-7, MDA-MB231, U87, and normal L929 fibroblast cell lines by MTT assay. Especially compounds 7a, 7c, and 7n showed remarkable cytotoxic activity against MCF-7, MDA-MB231, and U87 cell lines with a lower IC_{50} value compared to 5-fluorouracil as positive control. The majority of compounds exhibited significantly lower cytotoxicity to L929 cells than 5-fluorouracil. Compared to normal cells, the higher selectivity of compounds 7a, 7c, and 7n against cancer cells increased the safety of these compounds and allowed them to be evaluated as drug-candidate compounds. Also, compound 7c caused a significant increase in late apoptosis and necrosis compared to control MCF-7 cells. An increase in PI3K enzyme activity typically results in the overactivation of AKT in cancer cells, which in turn disrupts the growth and survival processes of the cells. In this context, PI3K inhibitors in cancer cells have the potential to impede cell growth, rendering them viable targets for new candidate compounds in cancer research. In this study, compound 7c has been identified as a novel inhibitor of PI3K enzymes, demonstrating the potential to be among the candidate compounds in the future. Its ability to suppress the growth of both breast cancer and brain cancer cells is also important for the development of metastasis that may occur during the disease process. In addition, its ability to induce apoptosis makes it a promising avenue for further investigation.

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Conflict of interest

The authors declare no conflicts of interest.

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Povzetek

Hidrazoni so pogosto uporabljena ogrodja pri razvoju in študijah novih zdravilnih učinkovin. Zaradi obetavnih farmakoloških učinkov hidrazonov, smo pripravili 15 novih hidrazidohidrazonov, ki vsebujejo flurbiprofenski 1,2,4-triazolni fragment ter preučili njihove *in vitro* aktivnosti proti rakavim celicam. Za vse spojine smo določili citotoksičnost proti celičnim linijama raka dojke (MCF-7 in MDA-MB231) in celični liniji glioblastoma (U87) z uporabo MTT analize. Izmed sintetiziranih spojin, sta **7a** in **7c** izkazali največjo citotoksično aktivnost z IC_{50} vrednostima $7.80 \pm 1.20 \mu\text{M}$ oz. $2.40 \pm 0.93 \mu\text{M}$ proti celični liniji MCF-7; spojina **7a** pa je bila najbolj aktivna proti celični liniji MDA-MB231 (IC_{50} vrednost $7.63 \pm 1.05 \mu\text{M}$). Spojini **7c** in **7n** sta pokazali citotoksično aktivnost proti celični liniji U87 (IC_{50} vrednosti $10.31 \pm 4.63 \mu\text{M}$ oz. $10.81 \pm 6.11 \mu\text{M}$). Da bi ugotovili varnost teh spojin, smo preučili njihove morebitne citotoksične učinke na celično linijo fibroblastov miši (L929) in ugotovili, da so spojine **7a**, **7c** in **7n** manj strupene kot 5-fluorourasil. Dodatno smo raziskali učinke spojine **7c** na apoptozo in PI3K aktivnost, ki igrata pomembno vlogo pri razvoju rakastih obolenj. Rezultati kažejo, da spojina **7c** poveča apoptozo v celicah MCF-7 in da izkazuje PI3K encimsko inhibitorno aktivnost. Pripravljeni hidrazoni torej kažejo potencialno možnost uporabe v vlogi spojin vodnic pri nadaljnjih študijah raka.



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