

RESEARCH ARTICLE

Design, Synthesis, and Evaluation of Heat Shock Protein 90 Inhibitors in Human Breast Cancer and Its Metastasis

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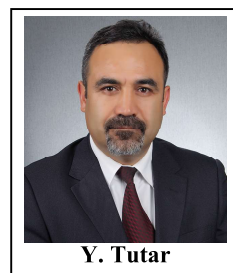
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Abstract: Background: Despite development of novel cancer drugs, invasive ductal breast carcinoma and its metastasis are still highly morbid. Therefore, new therapeutic approaches are being developed and Hsp90 is an important target for drug design. For this purpose, a series of benzodiazepine derivatives were designed and synthesized as novel Hsp90 inhibitor.

Methods: Benzodiazepine derivatives anticancer activities were determined by XTT cell proliferation assay against human breast cancer cell line (MCF-7). Effects of the compounds on endothelial function were monitored on human vascular endothelium (HUVEC) cell line as well. In order to determine the anti-proliferative mechanism of the compounds, *in silico* molecular docking studies were performed between Hsp90 ATPase domain and the benzodiazepine derivatives. Further, these compounds perturbation on Hsp90 ATPase function were tested. Fluorescence binding experiments showed that the derivatives bind Hsp90 effectively. Expression analysis of known cancer drug target genes by PCR array experiments suggest that the benzodiazepine derivatives have remarkable anticancer activity.

Results: A representative Benzodiazepine derivative D5 binds Hsp90 with Kd value of 3,93 μ M and with estimated free energy of binding -7.99 (kcal/mol). The compound decreases Hsp90 ATPase function and inhibit Hsp90 client protein folding activity. The compound inhibits expression of both Hsp90 isoforms and key proteins (cell cycle receptors; *PLK2* and *TERT*, kinases; *P13KC3* and *PRKCE*, and growth factors; *IGF1*, *IGF2*, *KDR*, and *PDGFRA*) on oncogenic pathways.

Conclusion: Benzodiazepine derivatives presented here display anticancer activity. The compounds effect on both breast cancer and endothelial cell lines show their potential as drug templates to inhibit breast cancer and its metastasis.



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ARTICLE HISTORY

Received: March 27, 2016
Revised: August 08, 2016
Accepted: October 25, 2016

DOI:
10.2174/13892010176661610311058
15

Keywords: Benzodiazepine, cancer, bone cancer, Hsp90, client proteins.

1. INTRODUCTION

Breast cancer is the second most common diagnosed cancer among women in the world. Invasive ductal carcinoma is the most prevalent type of breast cancer (about 75% of all breast cancers). Breast cancer primarily metastasizes to bone, lung, brain, liver, and lymph nodes and especially approximately 70% of all breast cancer patients have bone metastases [1, 2]. Angiogenesis is an essential physiological process for the formation of metastatic pathways in cancer cells. Angiogenesis is defined as formation of new blood vessels and

this process arise from migration, growth, and differentiation of endothelial cells. The resulting blood vessels provide oxygen and nutrients for cancer cells and therefore, tumors need angiogenesis to grow and migrate to distant healthy tissues [3, 4]. Hence, development of antiangiogenic compounds has been an important therapeutic aspect in cancer treatment.

Despite early diagnosis and the development of target specific cancer drugs, invasive ductal breast carcinoma and its metastasis are related to high mortality rate. Therefore, new therapeutic approaches are being developed for the treatment of breast cancer and its metastasis [5]. In recent years, Hsp90 has been an important biological target to develop target specific cancer drugs [6].

Hsp90 is an ATP-dependent 90 kDa molecular chaperone which is required for growth of tumor cells. Hsp90 is abundantly expressed in cancer cells and plays vital roles in stabilization, maturation, and proper folding of oncogenic client proteins for tumorigenesis [6-8]. Therefore, design of heterocyclic compounds for Hsp90 inhibition has been important

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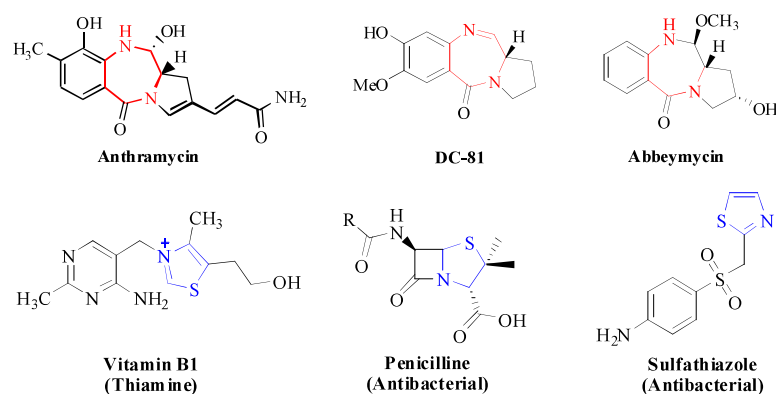


Fig. (1). Important biologically active diazepine (red) and thiazole (blue) derivatives.

therapeutic strategies in target specific cancer treatment. In eukaryotic cells, Hsp90 has four different isoforms: Hsp90 α , Hsp90 β , Trap1 and Grp94. Hsp90 α (inducible form) and Hsp90 β (constitutive form) are localized in cytosol and consist of functional and conserved N-terminal domain (NTD), middle domain (MD) and C-terminal domain (CTD) [6, 7]. NTD has a binding pocket for ATP and Hsp90 requires ATP hydrolyzed energy in protein folding processes. Therefore, NTD is target region to inhibit Hsp90 chaperone activity in cancer cells. To date, numerous compounds have been reported as Hsp90 NTD inhibitor and their pharmacological properties have been tested in cancer cells. Geldanamycin is the first natural Hsp90 NTD inhibitor and its synthetic analogs, 17-AAG and 17-DMAG, have been evaluated in clinical trials. Currently, there are more than twenty Hsp90 inhibitors under clinical trials. Modern cancer drug design efforts are underway to discover novel heterocyclic compounds that can potentially generate lead structures for further development [6-11].

Heterocyclic compounds and their derivatives are ubiquitous and play key roles in metabolism of all living cells. Activation or inhibition of biological targets can be achieved by heterocyclic compounds in treatment of many diseases. Heterocycles are common parts of FDA approved drugs and approximately 80% of drugs contain heterocyclic unit in their structures [12]. Diazepine is a seven-membered heterocyclic compound which is known to be therapeutically potent in psychiatric disorders. Diazepine skeleton possesses many different biological and pharmacological activities but especially it gained popularity with the diazepam drug in the 1960s (Fig. 1). Diazepam is a benzodiazepine derivative compound which is frequently used in the treatment of anxiety disorders, alcohol withdrawal symptoms, muscle spasms, seizures, trouble sleeping, and restless legs syndrome [13-15]. Apart from these pharmaceutical effects, natural and synthetic analogues of diazepine show anticancer activities and therefore, they have attracted the attention of synthetic drug designers in recent years due to their therapeutic activities [16-18].

In this study, we describe the synthesis, computational analysis, and antiproliferative and antiangiogenic activities of 1,5 benzodiazepine derivative of 18 compounds against MCF-7 and HUVEC cell lines. Our results indicated that 1,5 benzodiazepine derivatives are potent Hsp90 inhibitor for target specific cancer treatment.

2. MATERIALS AND METHODS

2.1. Chemistry

All chemicals used in this work were purchased from Sigma Aldrich or Merck and were used without further purification. All reactions were viewed by TLC performed on precoated 60 F254 plates (Merck, Whitehouse Station, NJ, USA). Visualization was affected with UV using Camag Thin-Layer Chromatogram Lamp (254/366 nm). Melting points were performed by Electrothermal 9200 melting point apparatus in open capillary tubes and were uncorrected. The C, H, N and S elemental analyses were performed with a Leco-932 CHNS-O Elemental Analyser Instrument (at the Faculty of Arts and Sciences, Bozok University, Yozgat, Turkey). IR spectra were measured on a Perkin Elmer Spectrum Two Model FT-IR Spectrophotometer using ATR method (at the Faculty of Arts and Sciences, Bozok University, Yozgat, Turkey). The ^1H NMR and ^{13}C NMR spectra were recorded at room temperature on a Bruker Avance III 400 MHz with DMSO- d_6 as the solvent. The chemical shifts (δ) were reported in parts per million (ppm) and were relative to the central peak of the solvent, which was DMSO- d_6 .

MCF-7 and HUVEC cell lines were obtained from ATCC (American Type Culture Collection, USA). Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies. Heat-inactivated fetal bovine serum, Trypsin-EDTA and phosphate buffer saline (PBS) solution were obtained from Biological Industries Ltd. L-glutamine-penicillin-streptomycin solution was obtained from Sigma-Aldrich. XTT cell proliferation kit was obtained from Applied Chem.

2.2. General Procedure for 4-[alkyl]-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one compounds (D)

To solution of 1 mmol of thiazolyl β -ketoester (C) in 40 mL dioxane, 1 mmol orthophenyldiamine or 4,5-dimethylbenzene-1,2-diamine was added. Then, the mixture of reaction stirred at reflux conditions for 24 h and the progress of the reaction was monitored by TLC. After the solvent was removed under reduced pressure, the residue was treated with the isopropyl alcohol to give crude product as a yellow solid. After filtration of mixture, the remaining residue was purified by recrystallization from different solvents to obtain compounds D (Yields 68-86%).

2.2.1. 4-[2-(methylamino)-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D1)

Recrystallized from acetonitrile, Yield 0.220 g, 81%, mp 271 °C, FT-IR (ATR, cm^{-1}): 3204 (NH); 3118-2804 (aliphatic and Ar-H); 1665 (C=O); 1610-1474 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 10.41 (s, 1H, CONH); 8.32 (q, $^3J_{\text{H-H}}=4.2$ Hz, 1H, CH_3NH); 7.87 (s, CH, thiazole); 7.25-7.10 (m, 4H, Ar-H); 3.37 (s, 2H, CH_2); 2.88 (d, $^3J_{\text{H-H}}=4.7$ Hz, 3H, CH_3NH). ^{13}C NMR (DMSO- d_6 , ppm): δ 174.1 (C=N, thiazole); 166.5 (C=O); 152.8 (C=N, diazepine); 146.1; 139.9; 130.4; 127.8; 126.7; 125.6; 124.6; 122.4 (C=C); 31.4 (CH_3); in DMSO (CH_2). Calcd. for $\text{C}_{13}\text{H}_{12}\text{N}_4\text{OS}$ (272.33): C, 57.34; H, 4.44; N, 20.57; S, 11.77. Found: C, 56.93; H 4.10; N, 20.26; S, 11.40 %.

2.2.2. 4-[2-(ethylamino)-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D2)

Recrystallized from acetonitrile, Yield 0.226 g, 79%, mp 264-265 °C, FT-IR (ATR, cm^{-1}): 3192 (NH); 3063-2848 (aliphatic and Ar-H); 1669 (C=O); 1595-1446 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 10.40 (s, 1H, CONH); 8.38 (t, $^3J_{\text{H-H}}=5.3$ Hz, 1H, $\text{CH}_3\text{CH}_2\text{NH}$); 7.86 (s, CH, thiazole); 7.25-7.10 (m, 4H, Ar-H); 3.36 (s, 2H, diazepine); in DMSO (m, 2H, $\text{CH}_3\text{CH}_2\text{NH}$); 1.17 (t, $^3J_{\text{H-H}}=7.2$ Hz, 3H, $\text{CH}_3\text{CH}_2\text{NH}$). ^{13}C NMR (DMSO- d_6 , ppm): δ 172.9 (C=N, thiazole); 166.5 (C=O); 152.8 (C=N, diazepine); 146.0; 139.9; 130.4; 127.8; 126.5; 125.6; 124.6; 122.4 (C=C); 14.6 ($\text{CH}_3\text{CH}_2\text{NH}$); in DMSO (CH_2CO); in DMSO (CH_2NH). Calcd. for $\text{C}_{14}\text{H}_{14}\text{N}_4\text{OS}$ (286.35): C, 58.72; H, 4.93; N, 19.57; S, 11.20. Found: C, 58.90; H, 4.93; N, 19.21; S, 10.87 %.

2.2.3. 4-[2-(allylamino)-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D3)

Recrystallized from acetonitrile, Yield 0.248 g, 83%, mp 256-257 °C, FT-IR (ATR, cm^{-1}): 3184 (NH); 3114-2832 (aliphatic and Ar-H); 1665 (C=O); 1606-1450 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 10.41 (s, 1H, CONH); 8.53 (t, $^3J_{\text{H-H}}=5.6$ Hz, 1H, CH_2NH); 7.86 (s, CH, thiazole); 7.25-7.13 (m, 4H, Ar-H); 5.96-5.83 (ddt, $^3J_{\text{trans(H-H)}}=17.1$ Hz, $^3J_{\text{cis(H-H)}}=10.4$ Hz, $^3J_{\text{H-H}}=5.3$ Hz, 1H, $\text{CH}_2=\text{CHCH}_2$); 5.28-5.22 (dd, $^3J_{\text{trans(H-H)}}=17.2$ Hz; $^2J_{\text{gem(H-H)}}=1.6$ Hz, 1H, $\text{CH}_2=\text{CHCH}_2\text{NH}$); 5.16-5.13 (dd, $^3J_{\text{cis(H-H)}}=10.3$ Hz; $^2J_{\text{gem(H-H)}}=1.5$ Hz, 1H, $\text{CH}_2=\text{CHCH}_2\text{NH}$); 3.94 (t, $^3J_{\text{H-H}}=5.3$ Hz, 2H, $\text{CH}_2=\text{CHCH}_2\text{NH}$); 3.36 (s, 2H, CH_2CO). ^{13}C NMR (DMSO- d_6 , ppm) δ 172.4 (C=N, thiazole); 166.0 (C=O); 152.3 (C=N, diazepine); 145.3; 139.3; 134.1; 129.9; 127.3; 126.41; 125.2; 124.1; 121.9; 116.2 (C=C); 46.40 (CH_2NH); in DMSO (CH_2CO). Calcd. for $\text{C}_{15}\text{H}_{14}\text{N}_4\text{OS}$ (298.36): C, 60.38; H, 4.73; N, 18.78; S, 10.75. Found: C, 60.71; H 4.74; N, 18.64; S, 10.55 %.

2.2.4. 4-(2-anilino-1,3-thiazole-5-yl)-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D4)

Recrystallized from dioxane, Yield 0.268 g, 80%, mp 287 °C, FT-IR (ATR, cm^{-1}): 3196 (NH); 3122-2868 (aliphatic and Ar-H); 1665 (C=O); 1610-1454 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 10.70 (s, 1H, NH); 10.47 (s, 1H, NH); 8.06 (s, CH, thiazole); 7.66-7.02 (m, 9H, Ar-H); 3.45 (s, 2H, CH_2). ^{13}C NMR (DMSO- d_6 , ppm): δ 168.0 (C=N, thiazole); 166.4 (C=O); 152.8 (C=N, diazepine);

145.0; 140.7; 139.6; 130.5; 129.6; 128.4; 128.0; 126.1; 124.7; 123.0; 122.4; 118.4 (C=C); in DMSO (CH_2). Calcd. for $\text{C}_{18}\text{H}_{14}\text{N}_4\text{OS}$ (334.39): C, 64.65; H, 4.22; N, 16.75; S, 9.59. Found: C, 64.34; H 4.23; N, 16.51; S, 9.32 %.

2.2.5. 4-[2-[(4-fluorophenyl)amino]-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D5)

Recrystallized from acetic acid, Yield 0.264 g, 75%, mp 298 °C, FT-IR (ATR, cm^{-1}): 3200 (NH); 3157-2840 (aliphatic and Ar-H); 1673 (C=O); 1614-1482 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 10.72 (s, 1H, NH); 10.47 (s, 1H, NH); 8.05 (s, CH, thiazole); 7.70- 7.16 (m, 8H, Ar-H); 3.45 (s, 2H, CH_2). ^{13}C NMR (DMSO- d_6 , ppm): δ 168.1 (C=N, thiazole); 166.4 (C=O); 152.8 (C=N, diazepine); 158.0 (d, $^1J_{\text{C-F}}=239$ Hz, ipso C); 144.9; 139.6; 137.2; 130.5; 128.4; 128.0; 126.1; 124.7; 122.4; 120.3; 116.2 (d, $^2J_{\text{C-F}}=22$ Hz) (C=C); in DMSO (CH_2). Calcd. for $\text{C}_{18}\text{H}_{13}\text{FN}_4\text{OS}$ (352.39): C, 61.35; H, 3.72; N, 15.90; S, 9.10. Found: C, 61.70; H 3.52; N, 15.71; S, 8.85 %.

2.2.6. 4-[2-[(4-bromophenyl)amino]-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D6)

Recrystallized from acetic acid, Yield 0.355 g, 86%, mp 290-292 °C, FT-IR (ATR, cm^{-1}): 3180 (NH); 3118-2829 (aliphatic and Ar-H); 1673 (C=O); 1606-1478 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm): δ 10.84 (s, 1H, NH); 10.49 (s, 1H, NH); 8.09 (s, CH, thiazole); 7.67-7.64 (d, part A of the system AA'BB', $^3J_{\text{H-H}}=9.0$ Hz, 2H, H-3); 7.55-7.52 (d, part B of the system AA'BB', $^3J_{\text{H-H}}=9.0$ Hz; 2H, H-2); 7.28-7.16 (m, 4H, Ar-H); 3.46 (s, 2H, CH_2). ^{13}C NMR (DMSO- d_6 , ppm): δ 167.4 (C=N, thiazole); 166.4 (C=O); 152.8 (C=N, diazepine); 144.8; 140.0; 139.5; 132.3; 130.5; 129.0; 128.0; 126.2; 124.7; 122.4; 120.2; 114.2 (C=C); in DMSO (CH_2). Calcd. for $\text{C}_{18}\text{H}_{13}\text{BrN}_4\text{OS}$ (413.29): C, 52.31; H, 3.17; N, 13.56; S, 7.76. Found: C, 51.94; H 3.14; N, 13.29; S, 7.42 %.

2.2.7. 4-[2-[2-(1-phenylethylidene)hydrazino]-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D7)

Recrystallized from acetic acid, Yield 0.319 g, 70%, mp 285 °C, IR (ATR, cm^{-1}): 3180 (NH); 3086-2856 (aliphatic and Ar-H); 1669 (C=O); 1610-1478 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 11.76 (s, 1H, NH); 10.44 (s, 1H, NH); 8.08 (s, CH, thiazole); 7.84-7.16 (m, 9H, Ar-H); 3.41 (s, 2H, CH_2); 2.35 (s, 3H, CH_3). ^{13}C NMR (DMSO- d_6 , ppm): δ 166.5 (C=N, thiazole); 165.9 (C=O); 152.8 (C=N, diazepine); 146.9; 139.7; 138.2; 130.5; 129.5; 128.9; 128.0; 127.9; 126.4; 126.0; 124.8; 124.6; 122.4 (C=C and C=N); 14.7 (CH_3); in DMSO (CH_2). Calcd. for $\text{C}_{20}\text{H}_{19}\text{N}_5\text{OS}$ (375.45): C, 63.98; H, 4.56; N, 18.65; S, 8.54. Found: C, 63.65; H 4.29; N, 18.48; S, 8.27 %.

2.2.8. 4-(2-[2-[1-(4-methylphenyl)ethylidene]hydrazino]-1,3-thiazole-5-yl)-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D8)

Recrystallized from acetic acid, Yield 0.273 g, 85%, mp 265-268 °C, FT-IR (ATR, cm^{-1}): 3192 (NH); 3114-2914 (aliphatic and Ar-H); 1665 (C=O); 1610-1474 (C=N and C=C). ^1H NMR (DMSO- d_6 , ppm) δ 11.83 (s, 1H, NH); 10.45 (s, 1H, NH); 8.08 (s, CH, thiazole); 7.74-7.71 (d, part A of the system AA'BB', $^3J_{\text{H-H}}=8.2$ Hz, 2H, H-2); 7.26-7.23 (d, part

B of the system AA'BB', $^3J_{H-H}=8.0$ Hz; 2H, H-3); 7.32-7.13 (m, 4H, Ar-H); 3.42 (s, 2H, CH₂); 2.33 (s, 6H, 2 x CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 172.7 (C=N, thiazole); 166.5 (C=O); 152.8 (C=N, diazepine); 151.0; 139.8; 139.2; 135.4; 130.5; 129.5; 127.9; 126.3; 125.9; 125.8; 124.6; 122.6; 122.4 (C=C ve C=N); 39.6 (CH₂); 21.3 (Ar-CH₃); 14.7 (CH₃). Calcd. for C₂₁H₁₉N₅OS (389.47): C, 64.76; H, 4.92; N, 17.98; S, 8.23. Found: C, 64.40; H 5.23; N, 17.67; S, 7.88 %.

2.2.9. 4-(2-{2-[1-(4-methoxyphenyl)ethylidene]hydrazino}-1,3-thiazole-5-yl)-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D9)

Recrystallized from acetic acid, Yield 0.276 g, 68%, mp 275-276 °C, FT-IR (ATR, cm⁻¹): 3317 (NH); 3176-2840 (aliphatic and Ar-H); 1669 (C=O); 1610-1478 (C=N and C=C). ¹H NMR (DMSO-*d*₆, ppm): δ 11.81 (s, 1H, NH); 10.44 (s, 1H, NH); 8.07 (s, CH, thiazole); 7.79-7.76 (d, part A of the system AA'BB', $^3J_{H-H}=8.9$ Hz; 2H, H-2); 7.31-7.13 (m, 4H, Ar-H); 7.01-6.98 (d, part B of the system AA'BB', $^3J_{H-H}=9.0$ Hz; 2H, H-3); 3.79 (s, 3H, CH₃O); 3.41 (s, 2H, CH₂); 2.32 (s, 3H, CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 172.5 (C=N, thiazole); 166.5 (C=O); 152.8 (C=N, diazepine); 160.6; 150.8; 144.0; 139.8; 130.7; 130.4; 127.9; 126.0; 125.9; 124.6; 122.5; 122.4; 114.3 (C=C and C=N); 55.7 (OCH₃); 14.6 (CH₃); in DMSO (CH₂). Calcd. for C₂₁H₁₉N₅O₂S (405.47): C, 62.21; H, 4.72; N, 17.27; S, 7.91. Found: C, 61.88; H 4.81; N, 16.93; S, 7.59 %.

2.2.10. 7,8-dimethyl-4-[2-(methylamino)-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D10)

Recrystallized from acetonitrile, Yield 0.234 g, 78%, mp 284 °C, FT-IR (ATR, cm⁻¹): 3340 (NH); 3219-2856 (aliphatic and Ar-H); 1669 (C=O); 1610-1442 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 10.24 (s, 1H, NH); 8.25 (q, $^3J_{H-H}=4.5$ Hz, 1H, CH₃NH); 7.81 (s, CH, thiazole); 7.02 and 6.88 (s, 2H, Ar-H); 2.88 (d, $^3J_{H-H}=4.6$ Hz, 3H, CH₃NH); 2.20 (s, 6H, 2 x Ar-CH₃); in DMSO (CH₂). ¹³C NMR (DMSO-*d*₆, ppm): δ 173.6 (C=N, thiazole); 166.2 (C=O); 151.9 (C=N, diazepine); 145.4; 137.8; 134.1; 132.8; 128.3; 128.0; 126.9; 122.8 (C=C); 31.3 (CH₃NH); 19.4; 19.1 (2 x Ar-CH₃); in DMSO (CH₂). Calcd. for C₁₅H₁₆N₄OS (300.38): C, 59.98; H, 5.37; N, 18.65; S, 10.67. Found: C, 59.64; H 5.00; N, 18.31; S, 10.43 %.

2.2.11. 4-[2-(ethylamino)-1,3-thiazole-5-yl]-7,8-dimethyl-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D11)

Recrystallized from acetonitrile, Yield 0.252 g, 80%, mp 280-281 °C, FT-IR (ATR, cm⁻¹): 3196 (NH); 3082-2860 (aliphatic and Ar-H); 1665 (C=O); 1622-1439 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 10.24 (s, 1H, CONH); 8.33 (t, $^3J_{H-H}=4.3$ Hz; 1H, CH₃CH₂NH); 7.80 (s, CH, thiazole); 7.02 and 6.87 (s, 2H, Ar-H); 2.20 (s, 6H, Ar-CH₃); 1.17 (t, $^3J_{H-H}=7.2$ Hz; 3H, CH₃CH₂NH), in DMSO (CH₂CO); in DMSO (CH₃CH₂NH). ¹³C NMR (DMSO-*d*₆, ppm): δ 172.6 (C=N, thiazole); 166.2 (C=O); 151.9 (C=N, diazepine); 145.3; 137.8; 134.1; 132.8; 128.3; 128.0; 126.6; 122.8 (C=C); 19.4; 19.1 (2 x Ar-CH₃); 14.6 (CH₃CH₂NH); in DMSO (CH₂CO); in DMSO (CH₃CH₂NH). Calcd. for C₁₆H₁₈N₄OS (314.41): C, 61.12; H, 5.77; N, 17.82; S, 10.20. Found: C, 60.79; H 5.92; N, 17.84; S, 9.89 %.

2.2.12. 4-[2-(allylamino)-1,3-thiazole-5-yl]-7,8-dimethyl-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D12)

Recrystallized from acetonitrile, Yield 0.277 g, 85%, mp 286-287 °C, FT-IR (ATR, cm⁻¹): 3188 (NH); 3082-2836 (aliphatic and Ar-H); 1669 (C=O); 1622-1442 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 10.25 (s, 1H, CONH); 8.49 (t, $^3J_{H-H}=5.7$ Hz, 1H, CH₂NH); 7.80 (s, CH, thiazole); 7.02 and 6.87 (s, 2H, Ar-H); 5.95-5.83 (m, 1H, CH₂=CHCH₂); 5.27-5.21 (dd, $^3J_{trans(H-H)}=17.2$ Hz, 1H, $^2J_{gem(H-H)}=1.6$ Hz, CH₂=CHCH₂); 5.16-5.12 (dd, $^3J_{cis(H-H)}=10.3$ Hz; 1H, $^2J_{gem(H-H)}=1.5$ Hz, CH₂=CHCH₂); 3.93 (t, $^3J_{H-H}=5.3$ Hz, 2H, CH₂=CH-CH₂NH); 2.20 (s, 6H, 2 x Ar-CH₃); in DMSO (CH₂CO). ¹³C NMR (DMSO-*d*₆, ppm): δ 172.2 (C=N, thiazole); 165.7 (C=O); 151.4 (C=N, diazepine); 144.6; 137.3; 134.2; 133.6; 132.3; 127.8; 127.5; 126.6; 122.3; 116.2 (C=C); 46.4 (CH₂NH); 18.9; 18.6 (2 x Ar-CH₃); in DMSO (CH₂CO). Calcd. for C₁₇H₁₈N₄OS (326.42): C, 62.55; H, 5.56; N, 17.16; S, 9.82. Found: C, 62.85; H 5.64; N, 16.93; S, 9.55 %.

2.2.13. 4-(2-anilino-1,3-thiazole-5-yl)-7,8-dimethyl-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D13)

Recrystallized from dimethylformamide-water (20:1), Yield 0.268 g, 74%, mp 316 °C, FT-IR (ATR, cm⁻¹): 3188 (NH); 3090-2860 (aliphatic and Ar-H); 1669 (C=O); 1614-1454 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 10.66 (s, 1H, NH); 10.31 (s, 1H, NH); 8.01 (s, CH, thiazole); 7.66-6.90 (m, 7H, Ar-H); 3.40 (s, 2H, CH₂); 2.21 (s, 6H, 2 x Ar-CH₃). ¹³C NMR (DMSO-*d*₆, ppm) δ 167.7 (C=N, thiazole); 166.1 (C=O); 151.9 (C=N, diazepine); 144.4; 140.7; 137.6; 134.6; 132.9; 129.6; 128.7; 128.4; 128.1; 122.9; 122.8; 118.4 (C=C); 19.4; 19.1 (2 x Ar-CH₃); in DMSO (CH₂). Calcd. for C₂₀H₁₈N₄OS (362.45): C, 66.28; H, 5.01; N, 15.46; S, 8.85. Found: C, 65.89; H 4.66; N, 15.12; S, 8.66 %.

2.2.14. 4-[2-[(4-fluorophenyl)amino]-1,3-thiazole-5-yl]-7,8-dimethyl-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D14)

Recrystallized from dioxane, Yield 0.274 g, 72%, mp 305 °C, FT-IR (ATR, cm⁻¹): 3180 (NH); 3082-2852 (aliphatic and Ar-H); 1665 (C=O); 1618-1486 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 10.67 (s, 1H, NH); 10.30 (s, 1H, NH); 7.99 (s, CH, thiazole); 7.70-7.16 (m, 4H, Ar-H); 7.07 and 6.90 (s, 2H, Ar-H); 3.39 (s, 2H, CH₂); 2.21 (s, 6H, 2 x Ar-CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 167.7 (C=N, thiazole); 166.1 (C=O); 151.8 (C=N, diazepine); 159.4 (d, $^1J_{C-F}=245$ Hz, ipso C); 144.3; 137.5; 137.2; 134.6; 132.9; 128.4; 128.1; 122.8; 120.2; 120.1; 116.1 (d, $^2J_{C-F}=22$ Hz) (C=C); 19.4; 19.1 (2 x Ar-CH₃); in DMSO (CH₂). Calcd. for C₂₀H₁₇FN₄OS (380.44): C, 63.14; H, 4.50; N, 14.73; S, 8.43. Found: C, 62.94; H 4.27; N, 14.68; S, 8.42 %.

2.2.15. 4-[2-[(4-bromophenyl)amino]-1,3-thiazole-5-yl]-7,8-dimethyl-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D15)

Recrystallized from dimethylformamide, Yield 0.371 g, 84%, mp 327-329 °C FT-IR (ATR, cm⁻¹): 3176 (NH); 3082-2844 (aliphatic and Ar-H); 1665 (C=O); 1614-1482 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 10.80 (s, 1H, NH); 10.33 (s, 1H, NH); 8.03 (s, CH, thiazole); 7.67-7.64 (d, part A of the system AA'BB', $^3J_{H-H}=8.9$ Hz, 2H, H-3); 7.54-7.51 (d, part B of the system AA'BB', $^3J_{H-H}=9.0$ Hz; 2H, H-2); 7.08

and 6.90 (s, 2H, Ar-H); 3.41 (s, 2H, CH₂); 2.22 (s, 6H, 2 x Ar-CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 167.1 (C=N, thiazole); 166.1 (C=O); 151.8 (C=N, diazepine); 144.2; 140.0; 137.5; 134.7; 132.9; 132.3; 129.3; 128.4; 128.1; 122.8; 120.1; 114.0 (C=C); 19.4; 19.1 (2 x Ar-CH₃); in DMSO (CH₂). Calcd. for C₂₀H₁₇BrN₄OS (441.34): C, 54.43; H, 3.38; N, 12.69; S, 7.27. Found: C, 54.10; H 3.91; N, 12.69; S, 6.98 %.

2.2.16. 7,8-dimethyl-4-[(2-[1-(phenylethylidene)hydrazino]-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D16)

Recrystallized from acetic acid, Yield 0.308 g, 76%, mp 318 °C, FT-IR (ATR, cm⁻¹): 3176 (NH); 3090-2860 (aliphatic and Ar-H); 1665 (C=O); 1618-1439 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 11.72 (s, 1H, NH); 10.28 (s, 1H, NH); 8.02 (s, CH, thiazole); 7.84-7.41 (m, 5H, Ar-H); 7.09 and 6.90 (s, 2H, Ar-H); 3.36 (s, 2H, CH₂); 2.35 (s, 3H, CH₃); 2.22 (s, 6H, 2 x Ar-CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 166.5 (C=N, thiazole); 166.2 (C=O); 151.8 (C=N, diazepine); 146.5; 138.2; 137.7; 137.7; 134.5; 132.9; 129.5; 129.1; 128.9; 128.4; 128.1; 126.4; 122.8 (C=C and C=N); 19.4; 19.1 (2 x Ar-CH₃); 14.6 (CH₃); in DMSO (CH₂). Calcd. for C₂₂H₂₃N₅OS (405.52): C, 65.49; H, 5.25; N, 17.36; S, 7.95. Found: C, 65.62; H 4.93; N, 17.01; S, 7.93 %.

2.2.17. 7,8-dimethyl-4-(2-[(2-[1-(4-methylphenyl)ethylidene]hydrazino)-1,3-thiazole-5-yl]-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D17)

Recrystallized from dimethylformamide-water (20:1), Yield 0,305 g, 73%, mp 325-326 °C, FT-IR (ATR, cm⁻¹): 3153 (NH); 3090-2856 (aliphatic and Ar-H); 1669 (C=O); 1618-1439 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 11.69 (s, 1H, NH); 10.29 (s, 1H, NH); 8.02 (s, CH, thiazole); 7.73-7.71 (d, part A of the system AA'BB', ³J_{H-H}=7.9 Hz, 2H, H-2); 7.25-7.23 (d, part B of the system AA'BB', ³J_{H-H}=7.7 Hz; 2H, H-3); 7.09 and 6.90 (s, 2H, Ar-H); 3.36 (s, 2H, CH₂); 2.33 (s, 3H, Ar-CH₃); 2.32 (s, 3H, CH₃-C=N); 2.22 (s, 6H, 2 x Ar-CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 166,6 (C=N, thiazole); 166,2 (C=O); 151,8 (C=N, diazepine); 146,6; 139,1; 137,7; 135,5; 135,4; 134,4; 132,8; 132,8; 129,5; 128,4; 128,2; 128,1; 126,3; 122,8 (C=C ve C=N); 19,4; 19,1 (Ar-CH₃); 14,6 (CH₃); in DMSO (CH₂). Calcd. for C₂₃H₂₃N₅OS (417.53): C, 66.16; H, 5.55; N, 16.77; S, 7.68. Found: C, 65.93; H 5.57; N, 16.49; S, 7.41 %.

2.2.18. 4-(2-[(2-[1-(4-methoxyphenyl)ethylidene]hydrazino)-1,3-thiazole-5-yl]-7,8-dimethyl-1,3-dihydro-2H-1,5-benzodiazepine-2-one (D18)

Recrystallized from acetic acid, Yield 0.312 g, 72%, mp 317-318 °C, FT-IR (ATR, cm⁻¹): 3149 (NH); 3090-2856 (aliphatic and Ar-H); 1669 (C=O); 1618-1442 (C=N, C=C). ¹H NMR (DMSO-*d*₆, ppm) δ 11.64 (s, 1H, NH); 10.29 (s, 1H, NH); 8.01 (s, CH, thiazole); 7.79-7.76 (d, part A of the system AA'BB', ³J_{H-H}=8.9 Hz, 2H, H-2); 7.01-6.98 (d, part B of the system AA'BB', ³J_{H-H}=8.9 Hz; 2H, H-3); 7.09 and 6.90 (s, 2H, Ar-H); 3.79 (s, 3H, CH₃O); 3.36 (s, 2H, CH₂); 2.32 (s, 3H, CH₃-CN); 2.22 (s, 6H, Ar-CH₃). ¹³C NMR (DMSO-*d*₆, ppm): δ 172.9 (C=N, thiazole); 166.2 (C=O); 151.8 (C=N, diazepine); 160.6; 148.6; 137.7; 134.4; 132.9; 130.7; 128.4; 128.1; 128.1; 127.8; 127.1; 122.8; 114.3 (C=C and

C=N); 55.7 (OCH₃); 19.4; 19.1 (Ar-CH₃); 14.6 (CH₃); in DMSO (CH₂). Calcd. for C₂₃H₂₃N₅O₂S (433.53): C, 63.72; H, 5.35; N, 16.15; S, 7.40. Found: C, 63.90; H 5.18; N, 15.98; S, 7.22 %.

2.3. XTT Assay

MCF-7 (human invasive ductal breast carcinoma cells) and HUVEC (human umbilical vein endothelial cells) were cultured in DMEM with 10% heat-inactivated fetal bovine serum, 1% l-glutamine, 100 IU/mL penicillin and 10 mg/mL streptomycin in 75 cm² polystyrene flasks. Cells were cultivated at 37°C in a humidified atmosphere with 5% CO₂.

Antiproliferative activity of eighteen 1,5 benzodiazepine derivative compounds were tested by colorimetric 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) cell proliferation assay on MCF-7 and HUVEC cell lines. The yellow-colored tetrazolium salt of XTT was reduced to orange-colored formazan dye by dehydrogenase enzymes in metabolically active cells. Approximately 10⁴ cells were seeded in a final volume of 200 μl in 96-well flat-bottom plates. After overnight incubation, compounds were added by serial dilution (200 μM- 100 μM – 50 μM – 25 μM- 12,5 μM- 6,25 μM), and plates were incubated at 37°C in a 5% CO₂ incubator for 24 hours. 50 μl of XTT reagent was added to each well and plates were incubated at 37°C for four hours. Absorbance was measured at 450 nm and IC₅₀ values of compounds were calculated using GrapPad Prism 5 software.

2.4. Molecular Docking Studies

Crystal structure of Hsp90α NTD was obtained from Protein Data Bank (pdb code: 1UYM). The molecular structures of all the compounds were drawn using MarvinSketch software (Chemaxon®). Binding energy, inhibition constant and intermolecular interactions were calculated using Docking Server web tool (<http://www.dockingserver.com/web>) [19]. Docked structures were visualized and edited using PyMOL software.

2.5. Expression and Purification of Recombinant Human Hsp90α

Human Hsp90α plasmid DNA was purchased commercially from Applied Biological Materials (ABM) Inc. (Catalog No: PV020433). Plasmid DNA was transformed into BL21(DE3) competent *E. coli* cells and they were spread on LB-Agar plates which contain 50 μM kanamycin. Then, single colony was inoculated in 10 ml of LB containing 50 μM kanamycin and the cells were grown in mini cultures overnight (150 rpm, 37 °C). After overnight incubation, growing *E. coli* cells were transferred into 1 L of LB containing 50 μM kanamycin. Protein expression was induced by 0,05 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 hours. Harvested cells were lysed by bead beater using silica beads in buffer (pH:8, 50 mM sodium phosphate, 10 mM NaCl, 15 mM BME).

For purification of Hsp90α, ion-exchanged chromatography was applied using DEAE Sephadex A-50 weak anion exchange resin. Hsp90α was eluted with 200 mM NaCl solu-

tion and purified Hsp90 α was separated on 10% SDS polyacrylamide gel.

2.6. Binding Assay

The titrations were performed in 20 mM HEPES, 20 mM KCl, 5 mM MgCl₂, pH:7.4 at room temperature. Fluorescence of the protein with ANS probe was measured in Shimadzu Spectrofluorometer with excitation and emission wavelength at 340 nm and at 520 nm, respectively. Compound D5 and geldanamycin were added in sequential increments to protein solution. Then, K_d values of the compound D5 and geldanamycin were calculated.

2.7. ATP Hydrolysis Experiments

ATP hydrolysis experiment was carried out according to our previous study [19]. Coupling assay was performed to measure ATP hydrolysis rate. Briefly, 10 μ g/ μ l purified Hsp90 protein was incubated at 37 °C for 5 minutes in 500 μ l, pH 7.4 reaction mixture (50 mM HEPES, 50 mM NaCl, 4 mM MgCl₂, 0,2 mM NADH, 0,5 mM PEP, 18 unit LDH, 24-unit pyruvate kinase/1 ml). Different concentrations of geldanamycin and D5 compound (5, 10, 25, 50, 75 and 100 μ M) and 0.5 mM ATP were used in the reaction mixture. ADP formation was measured by NADH optical density at 340 nm (Fig. 5).

2.8. Luciferase Aggregation Assay

Luciferase aggregation assay was performed according to our previous work [19]. Basically, luciferase was treated with urea to denature completely and diluted in 25 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 2 mM ATP, and 5 mM dithiothreitol pH 7.4 solution. MCF-7 cell lysate (CL) was added to the reaction mixture. CL contains Hsp90 and the assay measures efficiency of D5 inhibitor against Hsp90. The mixture was incubated with geldanamycin as well (10 μ M and 100 μ M). Same experimental procedure was applied in the presence of non-hydrolysable ATP (PMP-AMP). The level of aggregation prevention was calculated spectrometrically at 320 nm (Fig. 6).

2.9. PCR Array Experiments

To determine the alteration of breast cancer gene profiling with compound D5 and geldanamycin, PCR array experiments were carried out on MCF-7 cells. Same experiment without inhibitors was used as negative control and all experiments were performed twice. Compound D5 and geldanamycin were incubated with MCF-7 cells for 24 h and their IC₅₀ 4.16 and 7.63 μ M values were used respectively. Both compounds were dissolved in 0.1% DMSO and same amount of DMSO was also used in untreated cells. Once MCF-7 cells reach approximately 80% confluence on plates, both inhibitors were applied on the cells for 24 h. At the end of the incubation time, total RNA (Fermentas[®]) was isolated and first strand cDNA (Roche[®]) was synthesized by a commercially available kit. QIAGEN[®] Human Cancer Drug Targets PCR Array kit was used in this experiment. Experiments were performed in Roche 480 Light Cycler RT-qPCR and data analysis was performed with the built-in software. Gene expression changes were calculated and the results are represented in Figs. (7 and 8).

3. RESULTS AND DISCUSSION

3.1. Chemistry

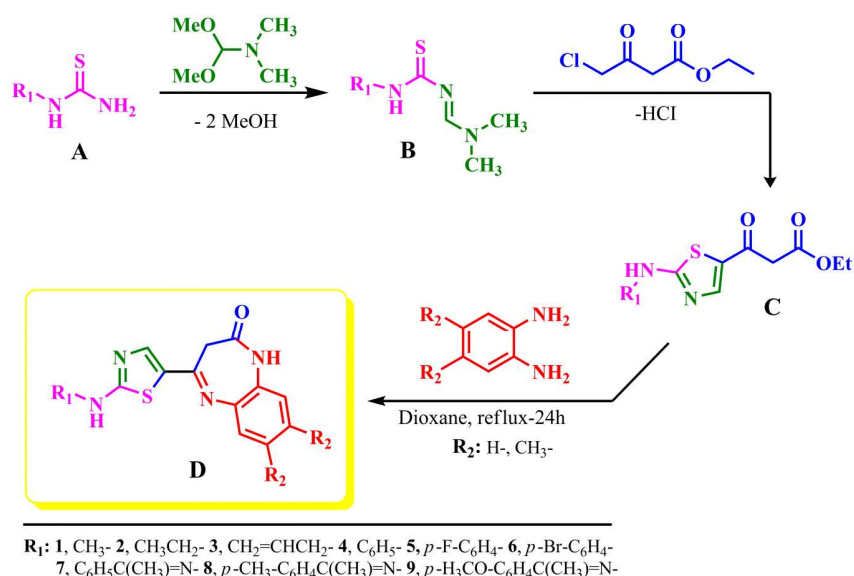
In this paper, we described the synthesis of novel diazepine derivatives bearing thiazolyl moiety according to our previously reported method [19]. In the first step, we synthesized the starting material thiazolyl β -ketoester compounds C. We extended our synthetic program to the synthesis of heterocyclic ring systems utilizing thiazolyl β -ketoesters as starting material. In the second step, eighteen thiazolyl diazepine derivatives E were prepared from the reaction of thiazolyl β -ketoesters C with orthophenyldiamine and 4,5-dimethyl orthophenyldiamine in dioxane at reflux conditions in good yields (68-81%) and were purified with different solvents by crystallization (Tables 1, 2). The synthetic route of the thiazolyl diazepine compounds (D) is shown in Scheme 1.

The synthesized novel products (D1-D18) were characterized by elemental analyses, IR, ¹H NMR and ¹³C NMR methods. As an example, the IR spectrum of D3 exhibited a characteristic amide carbonyl at 1665 cm⁻¹, NH at 3184 cm⁻¹, C=C and C=N at 1606–1450 cm⁻¹ as stretching vibrations. Its ¹H NMR spectrum in DMSO-d₆ showed a singlet sharp signal at 10.41 ppm (CONH), a singlet signal at 7.86 ppm (CH, thiazole), a singlet signal at 3.36 ppm (CH₂, benzodiazepine) and multiplet signals belonging to four phenyl protons at 7.25–7.13 ppm. In the ¹H NMR spectra of D1, CH₂NH proton signals were observed as a triplet signals in the range of 8.57–8.53 ppm due to neighboring group effect of the methyl protons (3JH-H=5,6 Hz, CH₂NH). Its ¹H NMR spectrum showed as a triplet signal in the range of 3.95-3.92 ppm (CH₂NH, 3JH-H=5,3 Hz), two doublet of doublet signal in the range of 5.28–5.13 ppm (CH₂ protons of the allyl group; 3Jtrans(H-H)=17,2 Hz; 2JH-H=1,6 Hz; 3Jtrans(H-H)=10,3 Hz; 2JH-H=1,5 Hz) and a triplet of doublet of doublet signal in the range of 5.96–5.83 ppm (CH₂CHCH protons of the allyl group; 3Jtrans(H-H)=17.2; 3Jtrans(H-H)=10.3; 3JH-H=5.3 Hz). As a result, in ¹H NMR spectrum of D3, alignment of the coupling constant values (J) confirmed the effect of neighboring groups (Fig. 2). In the ¹H NMR spectra, it was observed that there were similar splittings owing to existing of aliphatic groups attached to NH groups in the compounds **D1**, **D2**, **D10**, **D11** and **D12**. The detailed results of ¹H NMR spectra are presented in the experimental part.

¹³C NMR spectrum of the compound D3 showed five signals at around δ ppm 172.4 (C=N, thiazole), 166.0 (CO, amide carbonyl), 152.3 (C=N, diazepine), 145.3-116.2 (C=C, aromatic and aliphatic) and 46.4 (the CH₂ carbon which was attributed to the NH group), respectively. Its ¹³C NMR spectrum in DMSO-d₆ did not show a signal corresponding to the CH₂ carbon of the diazepine ring. We assume that we could not see this signal because of signal of the CH₂ carbon shifted within DMSO signals. The triplet signal of CH₂ protons which were observed in the range of 3.95-3.92 ppm in the ¹H NMR spectra of D3 support the spectroscopic analysis of structure. The ¹H and ¹³C NMR spectra of D1–D18 are similar to those of D3 except for groups which are attached to the thiazole and diazepine rings. The detailed results of ¹³C NMR spectra are presented in the experimental part. The

Table 1. Some properties of synthesized compounds D1-D9.

Entry	R ₁ -	R ₂ -	Mp (°C)	Mol. Formula Mol. wt.	Yields	Elemental analyses		
							Calcd %	Found %
D1	H ₃ C—	H-	271	C ₁₃ H ₁₂ N ₄ OS 272.33	81	C	57.34	56.98
						H	4.44	4.10
						N	20.57	20.26
						S	11.77	11.40
D2	CH ₃ CH ₂ —	H-	264-265	C ₁₄ H ₁₄ N ₄ OS 286.35	79	C	58.72	58.90
						H	4.93	4.93
						N	19.57	19.21
						S	11.20	10.87
D3	CH ₃ CHCH-	H-	256-257	C ₁₅ H ₁₄ N ₄ OS 298.36	83	C	60.38	60.71
						H	4.73	4.74
						N	18.78	18.64
						S	10.75	10.55
D4		H-	287	C ₁₈ H ₁₄ N ₄ OS 334.39	80	C	64.65	64.34
						H	4.22	4.23
						N	16.75	16.51
						S	9.59	9.32
D5		H-	298	C ₁₈ H ₁₃ FN ₄ OS 352.39	75	C	61.35	61.70
						H	3.72	3.52
						N	15.90	15.71
						S	9.10	8.85
D6		H-	290-292	C ₁₈ H ₁₃ BrN ₄ OS 413.29	86	C	52.31	51.94
						H	3.17	3.14
						N	13.56	13.29
						S	7.76	7.42
D7		H-	285	C ₂₀ H ₁₉ N ₅ OS 375.45	70	C	63.98	63.65
						H	4.56	4.29
						N	18.65	18.48
						S	8.54	8.27
D8		H-	265-268	C ₂₁ H ₁₉ N ₅ OS 389.47	85	C	64.76	64.30
						H	4.92	5.24
						N	17.98	17.68
						S	8.23	7.97
D9		H-	275-276	C ₂₁ H ₁₉ N ₅ O ₂ S 405.47	68	C	62.21	61.88
						H	4.72	4.81
						N	17.27	16.93
						S	7.91	7.59



Scheme (1). The synthesis of novel thiazolyl diazepine (**D**) compounds.

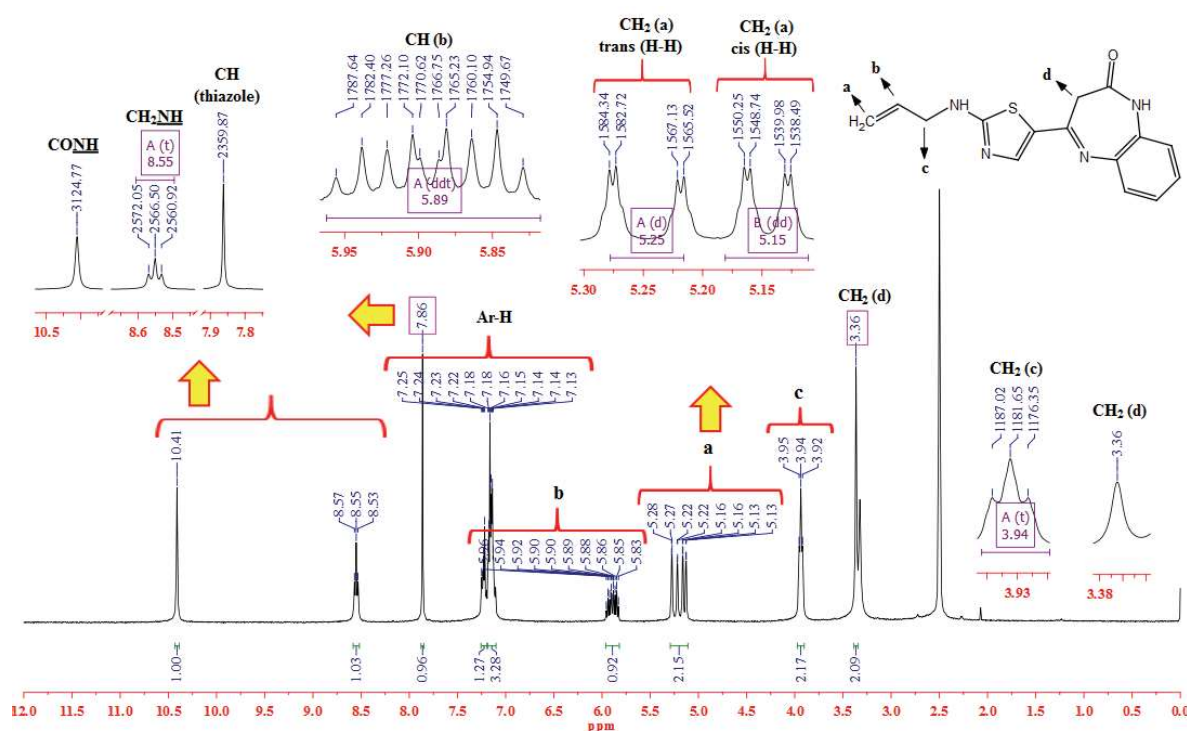


Fig. (2). ¹H NMR spectrum of compound **D3**.

structures of the compounds **D1-D18** were supported additionally by the elemental analyses of CHNS, which showed the correct elemental composition.

3.2. Cell Proliferation Assay

Antiproliferative activities of all the synthesized target compounds (**D1-D18**) were evaluated against MCF-7 and HUVEC cell lines by XTT assay. Compounds possessed significant anticancer activities and IC₅₀ values of the compounds are summarized in Table 3. Compounds were dis-

solved in DMSO and diluted in culture medium before treatment. The control cells were treated with culture medium containing 0.1% DMSO to determine cytotoxicity of the compounds.

Among the tested compounds, two of them (**D5** and **D14**) demonstrated potent antiproliferative activity against MCF-7 and HUVEC cell lines, respectively. **D5** and **D14** have fluorine side group which is essential in cancer drug discovery. Fluorine atom can act as a hydrogen bond acceptor in protein-ligand interactions. High electronegative and lipophilic

Table 2. Some properties of synthesized compounds D10-D18.

Entry	R ₁ -	R ₂ -	Mp (°C)	Mol. Formula Mol. wt.	Yields	Elemental analyses					
						Calcd %		Found %			
						C	H	N			
D10	H ₃ C—	H ₃ C—	284	C ₁₅ H ₁₆ N ₄ OS 300.38	78	59.98	5.37	18.65	59.64	5.00	18.31
D11	CH ₃ CH ₂ —	H ₃ C—	280-281	C ₁₆ H ₁₈ N ₄ OS 314.41	80	61.12	5.77	17.82	60.79	5.92	17.84
D12	CH ₃ CHCH-	H ₃ C—	286-287	C ₁₇ H ₁₈ N ₄ OS 326.42	85	62.55	5.56	17.16	62.85	5.64	16.93
D13		H ₃ C—	316	C ₂₀ H ₁₈ N ₄ OS 362.45	74	66.28	5.01	15.46	65.89	4.76	15.12
D14		H ₃ C—	305	C ₂₀ H ₁₇ FN ₄ OS 380.44	72	63.14	4.50	14.73	62.94	4.27	14.68
D15		H ₃ C—	327-329	C ₂₀ H ₁₇ BrN ₄ OS 441.34	84	54.43	3.88	12.69	54.10	3.91	12.69
D16		H ₃ C—	318	C ₂₂ H ₂₃ N ₅ OS 405.52	76	65.49	5.25	17.36	65.62	4.93	17.01
D17		H ₃ C—	325-326	C ₂₃ H ₂₃ N ₅ OS 417.53	73	66.16	5.55	16.77	65.93	5.57	16.49
D18		H ₃ C—	317-318	C ₂₃ H ₂₃ N ₅ O ₂ S 433.53	72	63.72	5.35	16.15	63.90	5.18	15.98

features of fluorine are the most important parameters for anticancer activity. Fluorine is much more lipophilic than hydrogen, and therefore drugs can easily penetrate into cell membranes and serve high bioavailability [20].

Table 3. IC₅₀ values of the compounds in MCF-7 and HUVEC cell lines.

	IC ₅₀ (μ M)	
	MCF-7	HUVEC
D1	17,11	44,21
D2	54,46	>100
D3	>100	>100
D4	>100	68,16
D5	4,16	12,36
D6	9,98	18,33
D7	7,25	8,12
D8	28,75	9,05
D9	15,76	13,65
D10	38,15	44,58
D11	>100	>100
D12	>100	>100
D13	65,48	36,45
D14	12,36	6,60
D15	16,87	11,44
D16	8,52	>100
D17	12,44	15,58
D18	10,59	21,78

The *in vitro* results indicated that addition of methyl groups were decreased cytotoxicity in MCF-7 and HUVEC cell lines. **D1**, **D2**, **D3**, **D10**, **D11** and **D12** have methyl groups on thiazol ring. Compared to **D1**, **D2** and **D3** displayed the lowest anticancer activity for two cell lines. Similarly, **D11** and **D12** have lower cytotoxic effect as compared to **D10** compounds both for MCF-7 and HUVEC cell lines. These extra methyl groups increase steric interference, and therefore they affect the anticancer activity of the compounds negatively.

Methoxy (-OCH₃) group containing compounds, **D9** and **D18**, have higher anticancer activity than compound **D7**, **D8**, **D16** and **D17** in MCF-7 and HUVEC cell lines. Methoxy is an important group for ROS-activated anticancer pro-drugs that target cancer cells. The high levels of ROS induce DNA damage, apoptosis and autophagy pathways in cancer cells [21].

3.3. Molecular Docking

Molecular docking was used to predict binding conformation of the compounds on Hsp90 NTD to determine the differences of their anticancer activities. Human Hsp90 NTD (pdb code-1UYM) was selected as the docking model and molecular docking studies were carried out by using docking server web tool (www.dockingserver.com/). The binding parameters of the compounds are shown in Table 4 and the interaction between Hsp90 NTD and compounds is depicted in Fig. (3).

Table 4. Estimated free energy of binding and estimated inhibition constant values of the compounds in Hsp90 NTD.

	Est. Free Energy of Binding (kcal/mol)	Est. Inhibition Constant, K _i (μ M)
D1	-6.57	15.25
D2	-6.87	9.19
D3	-7.16	5.69
D4	-7.85	1.75
D5	-7.99	1.40
D6	-7.67	2.38
D7	-7.16	5.62
D8	-7.52	3.06
D9	-6.96	7.89
D10	-7.00	7.41
D11	-7.22	5.13
D12	-6.65	13.38
D13	-7.91	1.60
D14	-7.67	2.38
D15	-7.33	4.23
D16	-7.58	2.77
D17	-7.70	2.26
D18	-7.50	3.20

Interaction analysis of the compounds with ATP binding site of the Hsp90 NTD demonstrated that all compounds bind to the conserved ATP binding pocket with binding energies ranging from -6.57 to -7.99 kcal/mol. Based on the *in vitro* cell proliferation assay results, our compounds displayed significant anticancer activities in MCF-7 and HUVEC cell lines. Since 1,5 benzodiazepine compounds are effective on HUVEC cells and these cells provide a model to study endothelial function - tumor associated angiogenesis. These compounds have potential as scaffold for antiangiogenic drugs. Compounds **D1-D18** block ATP binding region of the Hsp90 NTD and inhibit Hsp90 chaperone activity.

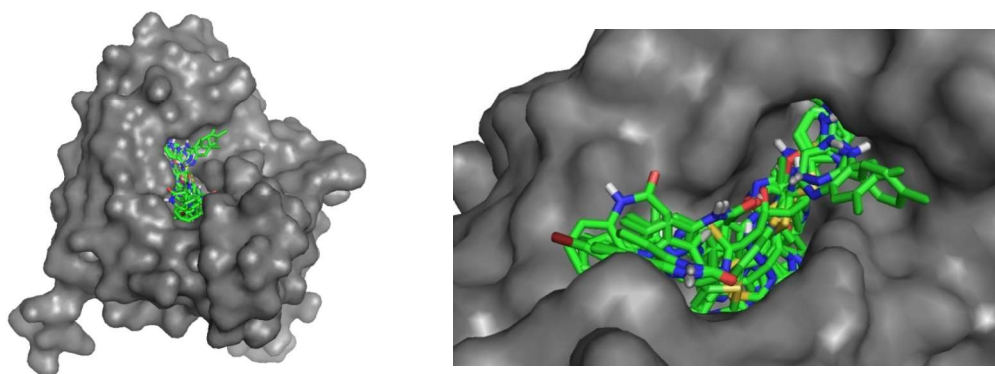


Fig. (3). Binding regions of the compounds (D1-D18) on human Hsp90 NTD. The proteins are displayed by grey color and compounds are displayed by green sticks.

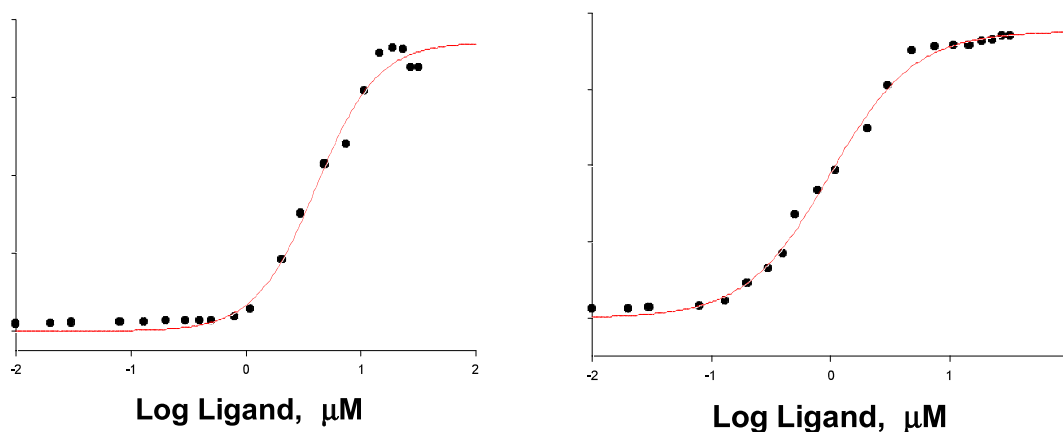


Fig. (4). D5 (Left) and Geldanamycin (Right) binding curves to Hsp90.

Thus, breast cancer and angiogenesis-related oncogenic proteins cannot gain proper three dimensional structures and tumorigenesis is inhibited.

Structure-activity relationships of 1,5 benzodiazepine derivatives as N terminal Hsp90 inhibitor provide a starting template for innovative drug design. These sets of benzodiazepines have lower IC_{50} values and inhibition constants, thus making the compounds unique in effectiveness.

The compounds further bind to a cavity that blocks Hsp90 N terminal allosteric transition. Allosteric transitions are important conformational changes that may affect the function, and perturbation of the allostery enhances the function adversely. Since Hsp90 is one of the abundantly expressed proteins in human, especially at cancer cells, inhibitory effects of 1,5 benzodiazepine derivatives greatly benefit cancer treatment and drug design researches.

3.4. Binding Assay

To understand the binding efficiency of the compounds, D5 was selected for binding studies and geldanamycin was used as positive control since geldanamycin is a well-known

Hsp90 inhibitor. ANS (8-Anilino-1-naphthalenesulfonic acid) extrinsic probe was used to measure ligand binding. K_d for (D5) and geldanamycin were found to be 3,93 μM and 2,06 μM respectively. The result indicates that benzodiazepine derivatives may have similar binding affinities as geldanamycin.

3.5. ATP Hydrolysis Experiment

Fluorometric binding data and docking studies indicate that D5 binds Hsp90 effectively and perturbs allosteric function of Hsp90. To further, support inhibition effect, ATP hydrolysis experiments were performed. Inhibition of Hsp90 function either by D5 compounds or geldanamycin decreases ATP hydrolysis rate in a concentration dependent way (Fig. 5). ATP hydrolysis is an indirect measure of Hsp90 function, since Hsp90 folds substrate proteins by ATP hydrolysis energy. The set of experiments indicate effectiveness of D5 inhibitor.

3.6. Luciferase Aggregation Assay

Cell lysate contains Hsp90 coordinating and cooperating heat shock proteins along with ATP molecules. Luciferase

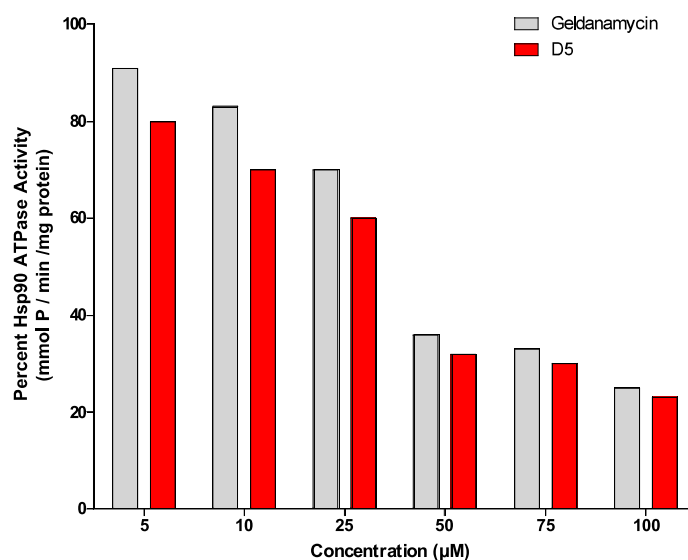


Fig. (5). ATPase activity is an essential process to perform chaperone activity of Hsp90. ATP hydrolysis assay in the presence of geldanamycin and compound D5 effectively inhibited ATP hydrolysis of Hsp90.

peptide was denatured and refolding of luciferase by Hsp90 was monitored at two different concentrations. To understand D5 inhibitory effects, geldanamycin was used as a positive control, and cell lysate (CL) and non-hydrolysable ATP (PNP-AMP or nhATP) were used as negative control. CL has weak refolding activity and addition of Hsp90 in the presence of nhATP to CL does not improve refolding of luciferase. But addition of ATP increases luciferase refolding activity of Hsp90. The activity was inhibited by D5 and geldanamycin even in the presence of ATP (Fig. 6). The experiments support that D5 is an effective Hsp90 inhibitor by interfering its ATP hydrolysis function.

3.7. PCR Array Experiment

According to binding assays, *in vitro* and *in silico* results, compound D5 inhibits Hsp90 α chaperone function and shows effective anticancer activity in MCF-7 cell lines. In the array experiment (QIAGEN[®] Human Cancer Drug Targets PCR Array kit), alteration of breast cancer related gene expressions were determined and altered expression profile is shown in Figs. (7 and 8). Geldanamycin was used as positive control and untreated MCF-7 cell gene expression was used as negative control. Fold expression change of the transcripts was calculated by comparing D5 inhibitor with negative control. A duplicate set of array experiments was performed and calculations were made by average numerical values.

Compound D5 suppresses *HSP90AA1* (Hsp90 α) and *HSP90B1* (Grp94) gene expression in MCF-7 cell line. In cell, Hsp90 α and Grp94 are localized in cytosol and endoplasmic reticulum, respectively. Two isoforms of the Hsp90 are overexpressed in cancer cells and they are involved in all phases of tumorigenesis. PCR array data indicated that compound D5 has a big potential for inhibition of Hsp90 α and Grp94 chaperone activities in cancer cells. Inhibition of Hsp90 drives the cells to apoptosis, and therefore benzodi-

azepine derivatives are proper starting templates for Hsp90 based drug design studies.

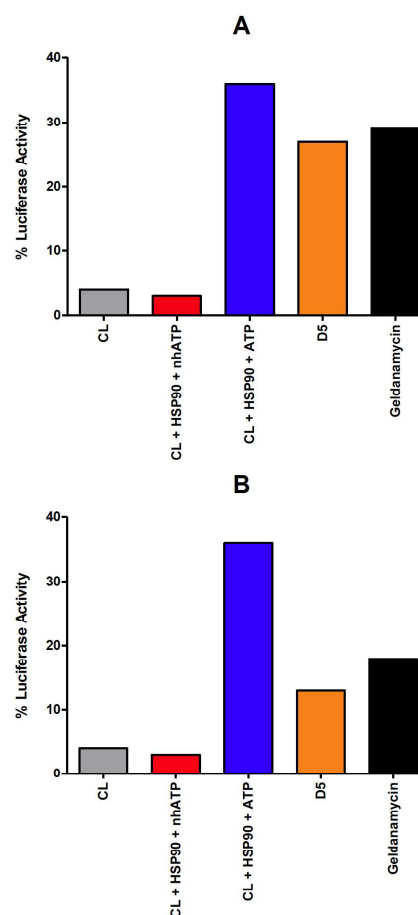


Fig. (6). Hsp luciferase activity at A: 10 µM in CL, B: 100 µM in CL. CL; cell lysate, nhATP; non-hydrolysable ATP (PNP-AMP).

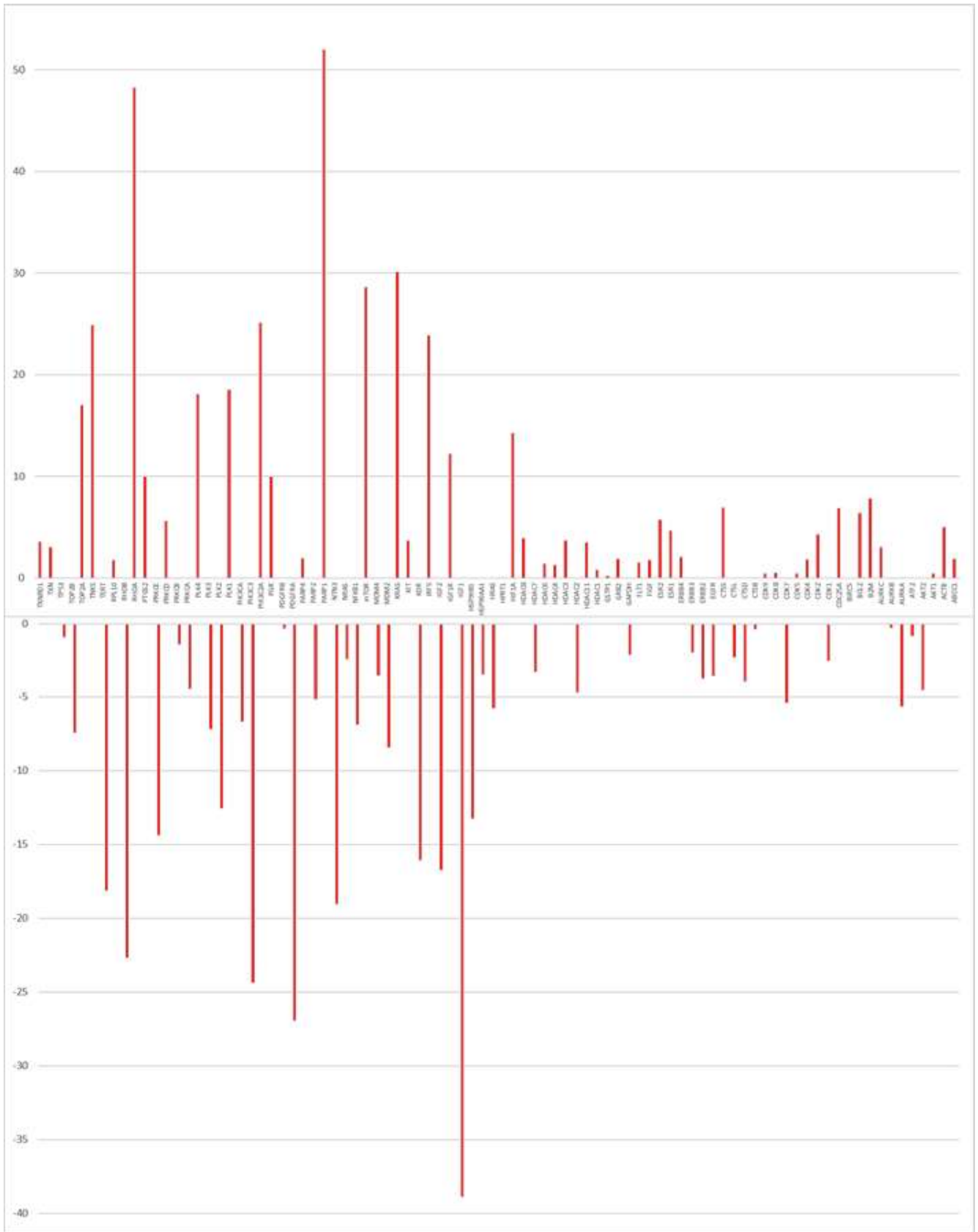


Fig. (7). Fold change of gene expression in the presence of D5.

portation to lysosomes [23]. In addition, decrease in PRKCE kinase transcription may affect apoptosis and heat shock response [24]. D5 further inhibits growth factors *IGF1*, *IGF2*, *KDR*, and *PDGFRA*. The inhibitor also decreases the expression of *PLK2* and *TERT* gene and these are involved in cell division and telomerase formation, respectively [25, 26]. *PLK1* is involved in tumor suppressor p53 related biochemical pathways. It was reported in the literature that *PLK1* may inhibit transactivation pro-apoptotic functions of p53 either by interaction or phosphorylation. Interestingly D5 increases the transcription of *PLK1* [27].

However, presence of both D5 and geldanamycin increase the transcripts of *mTOR* and PI-3 kinases and these genes are involved in cell proliferation and cell growth [28,29]. In a similar fashion, expression of *TOP2A* topoisomerase, *HIF1A* and *IRF5* transcription factors, *KRAS* signaling molecule, *PLK1* and *PLK4* kinase and *PARP1* and *TNKS* polymerase transcripts increase with D5 as well as geldanamycin treatment. Contribution of the increased transcription levels on overall effect needs to be elucidated but D5 dependent expression changes have cytotoxic effect on cancer cells and inhibit cell growth-proliferation as shown by cell cytotoxicity experiments. Inhibition effect of D5 on Hsp90 genes, cell cycle receptor *TERT* gene, kinases, and growth factor genes contribute to inhibition of MCF-7 cells and contribute to cell cytotoxicity either directly or by off-target effects.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was funded through TUBITAK (The Scientific and Technological Research Council of Turkey) grant (#114Z365) and a seed grant from Bozok University.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

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