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RESEARCH ARTICLE

Cytotoxic activities of some benzothiazole-piperazine derivatives

Enise Ece Gurdal[1], Irem Durmaz[2], Rengul Cetin-Atalay[2], and Mine Yarim[1]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Yeditepe University, Kayisdagi, Istanbul, Turkey and* [2]*Department of Molecular Biology and Genetics, BilGen, Genetics and Biotechnology Research Center, Faculty of Science, Bilkent University, Bilkent, Ankara, Turkey*

Abstract

Synthesis, characterization and cytotoxic activities of ten benzothiazole-piperazine derivatives were reported. *In vitro* cytotoxic activities of compounds were screened against hepatocellular (HUH-7), breast (MCF-7) and colorectal (HCT-116) cancer cell lines by sulphorhodamine B assay. Based on the GI_{50} values of the compounds, most of the benzothiazole-piperazine derivatives are active against HUH-7, MCF-7 and HCT-116 cancer cell lines. Compound **1d** is highly cytotoxic against all tested cancer cell lines. Further investigation of compound **1d** by Hoechst Staining and Fluorescence-Activated Cell Sorting Analysis (FACS) revealed that this compound causes apoptosis by cell cycle arrest at $subG_1$ phase.

Keywords

Anticancer, benzothiazole, cytotoxicity, piperazine, sulphorodamine B

History

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Introduction

Cancer, remaining to be a major health problem today, is caused by abnormal cell division without control. Cancer cells are able to spread into other parts of the body through blood and lymph system. Although there are many advances in treatment of cancer, most of anticancer drugs still need to be improved because of various limitations like emergence of drug resistance, low therapeutic index and lack of selectivity. Thus, cancer studies continue to develop new anticancer medicines, targeted therapies, monoclonal antibodies, etc.

Benzothiazole derivatives were reported to be highly cytotoxic against HCT-116 (colorectal cancer cell line)[1], MCF-7 (breast cancer cell line)[2], U937 and THP-1 (leukemia cancer cell lines)[3]. Isatin Mannich bases with benzothiazole moiety were reported as potential anti-breast cancer drugs. Flow cell cytometry showed that most active compound in series resulted in cell cycle arrest at G2/M phase[4]. Thiourea derivatives bearing benzothiazole moiety were reported to be cytotoxic against MCF-7 and HeLa breast cancer cell lines. Most active derivatives of the series were proven to act by damaging DNA with alkaline Comet assay[5]. 2-(4-Aminophenyl)benzothiazoles with cytotoxic activity against MCF-7 (breast) and IGROV-1 (ovarian) cancer cell lines caused generation of DNA adducts which induce apoptosis[6].

Piperazine ring is another value for anticancer drug candidates. Piperazinobenzopyranones were reported as breast cancer resistance protein inhibitors which are usually responsible with demolished effect of anticancer medication[7]. Piperazine ring carrying nucleoside analogues were evaluated for their cytotoxic activities on various cancer cell lines and most active compounds were further analyzed to evaluate mechanism of action. Agents caused induction of senescence-associated cell death through the inhibition of some kinase proteins[8]. In addition, Yarim and her co-workers previously reported various piperazine derivatives with high cytotoxic activity against liver (HUH-7, FOCUS, MAHLAVU, HepG-2, Hep-3B), breast (MCF-7, BT20 and T47D), colon (HCT-116), gastric (KATO-3), cervix (HeLa) and endometrial (MFE-296) cancer cell lines[9–11].

According to anticancer activity studies of benzothiazole-piperazine backbone, arylsulphonamides and arylthiol derivatives have potent cytotoxicity against a large scale of cancer cell lines such as breast (MCF-7), hepatocellular (HepG-2), prostate (DU-145) cancers and $CD4^+$ human acute T-lymphoblastic leukaemia (CCRF-CEM)[12,13].

In this study, with the aid of aforementioned studies, we reported the synthesis, purification and characterization of novel compounds which contain benzothiazole-piperazine backbone in their molecular structure. These compounds were tested for their cytotoxic activities against hepatocellular (HUH-7), breast (MCF-7) and colorectal (HCT-116) cancer cell lines with sulphorhodamine B assay. The advanced analysis, Hoechst staining and fluorescence-activated cell sorting analysis were also done for compound **1d** to understand the mechanism of cytotoxicity.

Methods and materials

Chemistry

All chemicals and reagents used in current study were of analytical grade. The reactions were monitored by thin layer chromatography (TLC) on Merck pre-coated silica GF254 plates. Melting points (°C) of the compounds were determined by using a Mettler Toledo FP62 capillary melting point apparatus (Mettler-Toledo, Greifensee, Switzerland) and are uncorrected. Infrared spectra were recorded on a Perkin-Elmer Spectrum One series FT-IR apparatus Version 5.0.1 (Perkin Elmer, Norwalk, CT), using

Address for correspondence: Enise Ece Gurdal, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Yeditepe University, 34755 Kayisdagi, Istanbul, Turkey. Tel: +90 216 5780000 (ext. 1592). Fax: +90 216 5780068. E-mail: egurdal@yeditepe.edu.tr

potassium bromide pellets, the frequencies were expressed in cm^{-1}. Elemental analyses were performed on LECO 932 CHNS (LECO-932, St. Joseph, MI) instrument. UV spectra were recorded on Evolution™ 201/220 UV-visible spectrophotometer and ethanol was used as solvent. The ^{1}H-NMR spectra were recorded with a Varian Mercury-400 FT-NMR spectrometer (Varian Inc., Palo Alto, CA), using tetramethylsilane (TMS) as the internal reference, with chloroform ($CDCl_3$) as solvent, the chemical shifts were reported in parts per million (ppm). Coupling constants were recorded in Hertz (Hz).

*General procedure for synthesis of 2-chloro-N-(6-methylbenzothiazol-2-yl)acetamide (**1**)*

2-Amino-6-methylbenzothiazole (0.012 mol (2 g)) was dissolved in 42 ml benzene: triethlyamine mixture (20:1)[14]. Later, acetylation was performed with 0.011 mol (0.87 ml) chloroacetyl chloride in room temperature. Because of the highly reactant nature of chloroacetyl chloride, it was added slowly in small amounts. Reaction was monitored by TLC with silica gel plate and benzene:methanol (9:1) mobile phase mixture. Reaction completed in four days at room temperature. Precipitated crude product was filtered, washed with benzene and dried. Ethanol crystallization gave pure product.

*General procedure for synthesis of N-(6-methylbenzothiazol-2-yl)-2-(4-substitued piperazinyl)acetamide derivatives (**1a–f**)*

N-(6-methylbenzothiazol-2-yl)-2-(4-substituedpiperazinyl)acetamides were synthesized in acetone by the reaction of 0.0025 mol (0.602 g) 2-chloro-*N*-(6-methylbenzothiazol-2-yl)acetamide and 0.0025 mol of suitable piperazine, in presence of 0.0025 mol (0.345 g) anhydrous K_2CO_3. Reactions were monitored by TLC with silica gel plate and benzene:methanol (9:1) mobile phase mixture. Reactions completed in two days at room temperature. Potassium carbonate was removed by filtration. After evaporation of acetone, precipitated products were recrystallized from absolute ethanol or acetone:distilled water mixture.

*General procedure for synthesis of 2-chloro-N-(6-ethoxybenzothiazol-2-yl)acetamide (**2**)*

2-Amino-6-ethoxybenzothiazole (0.012 mol (2.22 g)) was dissolved in 42 ml benzene: triethylamine mixture (20:1)[14]. Later, acetylation was performed with 0.013 mol (0.78 ml) chloroacetyl chloride at room temperature. Because of the highly reactant nature of chloroacetyl chloride, it was added slowly in small amounts. Reaction was monitored by TLC with silica gel plate and benzene:methanol (9:1) mobile phase mixture. Reaction completed in four days at room temperature. Precipitated crude product was filtered, washed with benzene and dried. Ethanol crystallization gave pure product.

*General procedure for synthesis of N-(6-ethoxybenzothiazol-2-yl)-2-(4-substitued-piperazinyl)acetamides (**2a–d**)*

N-(6-ethoxybenzothiazol-2-yl)-2-(4-substituedpiperazinyl)acetamides were synthesized in acetone by the reaction of 0.0025 mol (0.677 g) 2-chloro-*N*-(6-ethoxybenzothiazol-2-yl)acetamide and 0.0025 mol of suitable piperazine, in presence of 0.0025 mol (0.345 g) anhydrous K_2CO_3. Reactions were monitored by TLC with silica gel plate and benzene:methanol (9:1) mobile phase mixture. Reactions completed in two days at room temperature. Potassium carbonate was removed by filtration. After evaporation of acetone, precipitated products were recrystallized from absolute ethanol or acetone:distilled water mixture.

Cytotoxicity studies

The cytotoxic activity of the synthesized compounds was investigated on liver (HUH-7), breast (MCF-7) and colon (HCT-116) cancer cell lines, by means of sulphorhodamine B (SRB) assays in triplicate. Serial dilutions from 100 μM to 2.5 μM were used, 5-fluorouracil (5-FU) was the reference compound.

Cell culture

The human cancer cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin. Each cell line was maintained in an incubator at 37 °C supplied with 5% CO_2 and 95% air.

NCI-60 sulphorhodamine B (SRB) assay

Cancer cells (range of 2000 cell/well to 5000 cell/well) were inoculated into 96-well plates in 200 μl of media and incubated in 37 °C incubators containing 5% CO_2 and 95% air. After a 24 h incubation period, one plate for each cell line was fixed with 100 μl 10% ice-cold trichloroacetic acid (TCA). This plate represents the behaviour of the cells just prior to drug treatment and is accepted as the time-zero plate. The compounds to be tested were solubilized in DMSO to a final concentration of 40 mM and stored at +4 °C. While treating the cells with the compounds, the corresponding volume of the compound was applied to the cell to achieve the desired drug concentration and diluted through serial dilution. After drug treatment, the cells were incubated in 37 °C incubators containing 5% CO_2 and 95% air for 72 h. Following the termination of the incubation period after drug treatment, the cells were fixed with 100 μl 10% ice-cold TCA and incubated in the dark at +4 °C for 1 h. Then the TCA was washed away with ddH_2O five times and the plates were left to air dry. For the final step, the plates were stained with 100 μl of 0.4% sulphorhodamine B (SRB) solution in 1% acetic acid solution. Following staining, the plates were incubated in dark for 10 min at room temperature. The unbound dye was washed away using 1% acetic acid and the plates were left to air dry. To measure the absorbance results, the bound stain was then solubilized using 200 μl of 10 mM Tris-Base. The OD values were obtained at 515 nm.

Hoechst staining analysis

Cells were seeded on coverslips in 6-well plates. After overnight culture, cells were exposed to compounds at a concentration of their GI_{50} values for 72 h. To determine nuclear condensation by Hoechst 33258 (Sigma-Aldrich) staining, coverslips were washed twice with icecold PBS, fixed in 1 ml of cold methanol for 10 min, and then incubated with 3 Ig/ml of Hoechst 33258 for 5 min in darkness. The coverslips were then rinsed with distilled water, mounted on glass microscopic slides using 50% glycerol, and examined under fluorescent microscopy (40×).

Fluorescence-activated cell sorting analysis

Human cancer cell line (HUH-7) of interest were inoculated into 100-mm culture dishes (300 000 cells/dish). Twenty-four hours later, cells were then treated with the desired compounds according to their GI_{50} values and incubated for 72 h. Cells were then collected by trypsinization and the pellets were fixed in ice-cold 70% ethanol and stored at −20 °C. Before the analysis, the samples were stained with MUSE cell cycle reagent (contains propidium iodide solution) according to the manufacturer's protocol. Cell cycle analysis was conducted with MUSE cell cycle analyzer.

Results

Chemistry

*N-(6-methylbenzothiazol-2-yl)-2-[4-(2-methoxyphenyl)piperazinyl]acetamide (**1a**)*

White powder, 48% (0.417 g), m.p. 121.9 °C (reported: 122–124 °C). UV (MeOH, λ_{max}, nm); 291 (log ε: 4.21). FT-IR (KBr, cm^{-1}); 3333 (N–H), 3058 (C–H, aromatic), 2908 (C–H, aliphatic), 1707 (C=O, amide), 1605 (C=C, aromatic), 1242 (C–N), 1056 (C–O)[15]. ^{1}H-NMR ($CDCl_3$, ppm); 2.48 (s, 3H, Ar–CH_3), 2.86 (t, 4H, piperazine $H_{2,6}$, $J = 4.8$ Hz), 3.18 (bs, 4H, $H_{3,5}$), 3.36 (s, 2H, –COCH_2N–), 3.88 (s, 3H, –OCH_3), 6.89 (d, 1H, phenyl H_2, $J = 9.2$ Hz), 6.94–6.98 (m, 2H, phenyl $H_{3,5}$), 7.02–7.06 (m, 1H, phenyl H_4), 7.25–7.28 (m, 1H, benzothiazole H_5), 7.62 (s, 1H, benzothiazole H_7), 7.68 (d, 1H, benzothiazole H_4, $J = 8$ Hz), 10.47 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{21}H_{24}N_4O_2S$ (396.506): C, 63.61; H, 6.10; N, 14.13; S, 8.09. Found: C, 63.52; H, 6.14; N, 14.25; S, 8.04.

*N-(6-methylbenzothiazol-2-yl)-2-[4-(2-methoxyethyl)piperazinyl]acetamide (**1b**)*

Cream colored, shiny powder. 12% (0.099 g), m.p. 81.1 °C. UV (MeOH, λ_{max}, nm); 292 (log ε: 4.25). FT-IR (KBr, cm^{-1}); 3120 (N–H), 3050 (C–H, aromatic), 2946 (C–H, aliphatic), 1697 (C=O, amide) 1607 (C=C, aromatic), 1259 (C–N), 1067 (C–O). ^{1}H-NMR ($CDCl_3$, ppm); 2.46 (s, 3H, Ar-CH_3), 2.62 (t, 2H, –N$CH_2$$CH_2$–, $J = 5.2$ Hz), 2.69 (bs, 8H, piperazine), 3.27 (s, 2H, –COCH_2N–), 3.36 (s, 3H, –OCH_3), 3.51 (t, 2H, –CH_2OCH_3, $J = 5.2$ Hz), 7.23–7.26 (m, 1H, benzothiazole H_5), 7.61 (s, 1H, benzothiazole H_7), 7.67 (d, 1H, benzothiazole H_4, $J = 8$ Hz), 10.39 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{17}H_{24}N_4O_2S$ (348.463): C, 58.26; H, 7.48; N, 15.99; S, 9.15. Found: C, 58.22; H, 7.44; N, 15.97; S, 9.18.

*N-(6-methylbenzothiazol-2-yl)-2-(4-cyclohexylpiperazinyl)acetamide (**1c**)*

White powder. 2.8% (0.026 g), m.p. 181.3 °C. UV (MeOH, λ_{max}, nm); 289 (log ε: 4.25). FT-IR (KBr, cm^{-1}); 3261 (N–H), 2932 (C–H, aromatic), 2853 (C–H, aliphatic), 1703 (C=O, amide), 1605 (C=C, aromatic), 1262 (C=N). ^{1}H-NMR ($CDCl_3$, ppm); 1.09–1.27 (m, 6H, cyclohexyl), 1.63–1.66 (m, 1H, cyclohexyl), 1.80–1.88 (m, 4H, cyclohexyl), 2.48 (s, 4H, piperazine $H_{2,6}$), 2.48 (s, 3H, Ar–CH_3), 2.67 (s, 4H, piperazine $H_{3,5}$), 3.26 (s, 2H, –COCH_2N–), 7.25–7.27 (m, 1H, benzothiazole H_5), 7.62 (s, 1H, benzothiazole H_7), 7.68 (d, 1H, benzothiazole H_4, $J = 8$ Hz), 10.41 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{20}H_{28}N_4OS$ (372.528): C, 64.14; H, 8.07; N, 14.96; S, 8.56. Found: C, 64.12; H, 8.08; N, 14.97; S, 8.58.

*N-(6-methylbenzothiazol-2-yl)-2-[4-(pyridin-4-yl)piperazinyl]acetamide (**1d**)*

White powder. 22% (0.195 g), m.p. 100.8 °C. UV (MeOH, λ_{max}, nm); 293 (log ε: 4.13). FT-IR (KBr, cm^{-1}); 3192 (N–H), 3050 (C–H, aromatic), 2842 (C–H, aliphatic), 1703 (C=O, amide), 1603 (C=C, aromatic), 1212 (C–N). ^{1}H-NMR ($CDCl_3$, ppm); 2.48 (s, 3H, Ar-CH_3), 2.78 (t, 4H, piperazine $H_{2,6}$, $J = 5.4$ Hz), 3.45 (t, 4H, piperazine $H_{3,5}$, $J = 4.8$ Hz), 3.60 (s, 2H, –COCH_2N–), 6.68–6.70 (dd, 2H, pyridine $H_{3,5}$, $J_1 = 1.6$ Hz, $J_2 = 5$ Hz), 7.26–7.28 (m, 1H, benzothiazole H_5), 7.63 (s, 1H, benzothiazole H_7), 7.67 (d, 1H, benzothiazole H_4, $J = 8.4$ Hz), 8.30–8.32 (dd, 2H, pyridine $H_{2,6}$, $J_1 = 1.6$ Hz, $J_2 = 5.2$ Hz), 10.37 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{19}H_{21}N_5OS$ (367.468): C, 61.76; H, 6.27; N, 18.95; S, 8.68. Found: C, 61.73; H, 6.29; N, 18.96; S, 8.66.

*N-(6-methylbenzothiazol-2-yl)-2-[4-(3,4-dichlorophenyl)piperazinyl]acetamide (**1e**)*

White powder. 16% (0.196 g), m.p. 243.8 °C. UV (MeOH, λ_{max}, nm); 293 (log ε: 4.25). FT-IR (KBr, cm^{-1}); 3278 (N–H), 3017 (C–H, aromatic), 2893 (C–H, aliphatic), 1707 (C=O, amide), 1605 (C=C, aromatic), 1240 (C–N). ^{1}H-NMR ($CDCl_3$, ppm); 2.48 (s, 3H, Ar–CH_3), 2.80 (t, 4H, piperazine $H_{2,6}$, $J = 4.8$ Hz), 3.27 (t, 4H, piperazine $H_{3,5}$, $J = 5.2$ Hz), 3.35 (s, 2H, –COCH_2N–), 6.74–6.77 (dd, 1H, phenyl H_2, $J_1 = 3.2$ Hz, $J_2 = 8.6$ Hz), 6.98 (d, 1H, phenyl H_6, $J = 3.2$ Hz), 7.25–7.26 (m, 1H, benzothiazole H_5), 7.30 (d, 1H, phenyl H_5, $J = 9.2$ Hz), 7.62 (s, 1H, benzothiazole H_7), 7.68 (d, 1H, benzothiazole H_4, $J = 8.4$ Hz), 10.33 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{20}H_{20}Cl_2N_4OS$ (435.37): C, 54.92; H, 5.07; N, 12.81; S, 7.33. Found: C, 54.91; H, 5.04; N, 12.83; S, 7.31.

*N-(6-methylbenzothiazol-2-yl)-2-[4-(4-chlorobenzyl)piperazinyl]acetamide (**1f**)*

Cream-colored powder. 12% (0.115 g), m.p. 189.6 °C. UV (MeOH, λ_{max}, nm); 288 (log ε: 4.27). FT-IR (KBr, cm^{-1}); 3100 (N–H), 3038 (C–H, aromatic), 2929 (C–H, aliphatic), 1692 (C=O, amide), 1607 (C=C, aromatic), 1271 (C–N). ^{1}H-NMR ($CDCl_3$, ppm); 2.47 (s, 3H, Ar–CH_3), 2.54 (bs, 4H, piperazine $H_{3,5}$), 2.66 (bs, 4H, piperazine $H_{2,6}$), 3.27 (s, 2H, –N–CH_2–), 3.51 (s, 2H, –COCH_2N–), 7.22–7.28 (m, 4H, phenyl), 7.29–7.31 (m, 1H, benzothiazole H_5), 7.61 (s, 1H, benzothiazole H_7), 7.68 (d, 1H, benzothiazole H_4, $J = 8.4$ Hz), 10.4 (bs, 1H, –NHCOCH_2–). Anal. calcd. for $C_{21}H_{23}ClN_4OS$ (414.952): C, 60.49; H, 6.04; N, 13.44; S, 7.69. Found: C, 60.41; H, 6.08; N, 13.41; S, 7.70.

*N-(6-ethoxybenzothiazol-2-yl)-2-[4-(p-toluyl)piperazinyl]acetamide (**2a**)*

Honey colored irregular crystals. 36.5% (0.375 g), m.p. 146.7 °C. UV (MeOH, λ_{max}, nm); 294 (log ε: 4.18). FT-IR (KBr, cm^{-1}); 3315 (N–H), 3006 (C–H; aromatic), 2980 (C–H; aliphatic), 1698 (C=O; amide), 1608 (C=C; aromatic) and 1219 (C–N). ^{1}H-NMR (DMSO, ppm); 1.46 (t, 3H, –O$CH_2$$CH_3$, $J = 7.2$ Hz), 2.29 (s, 3H, –Ph–CH_3), 2.81 (t, 4H, piperazine $H_{2,6}$, $J = 5.2$ Hz), 3.23 (t, 4H, piperazine $H_{3,5}$, $J = 5.2$ Hz), 2.29 (s, 2H, –COCH_2N–), 4.06–4.12 (m, 2H, –O$CH_2$$CH_3$), 6.86 (d, 2H, phenyl $H_{2,6}$, $J = 8.8$ Hz), 7.03–7.06 (dd, 2H, phenyl $H_{3,5}$, $J_1 = 2.4$ Hz, $J_2 = 8.8$ Hz), 7.09–7.11 (dd, 1H, benzothiazole H_5, $J = 8.8$ Hz), 7.26–7.29 (m, 1H, benzothiazole H_7), 7.67 (d, 1H, benzothiazol H_4, $J = 8.8$ Hz), 10.36 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{22}H_{26}N_4O_2S$ (410.532): C, 64.05; H, 6.84; N, 13.58; S, 7.77. Found: C, 64.04; H, 6.82; N, 13.55; S, 7.80.

*N-(6-ethoxybenzothiazol-2-yl)-2-[4-(o-chlorophenyl)piperazinyl]acetamide (**2b**)*

Light brown colored powder. 47.5% (0.512 g), m.p. above 300 °C. UV (MeOH, λ_{max}, nm); 293 (log ε: 4.16). FT-IR (KBr, cm^{-1}); 3334 (N–H), 3065 (C–H; aromatic), 2971 (C–H; aliphatic), 1702 (C=O; amide), 1605 (C=C; aromatic), 1226 (C–N). ^{1}H-NMR (DMSO, ppm); 1.47 (t, 3H, –O$CH_2$$CH_3$, $J = 7.2$ Hz), 2.85 (t, 4H, piperazine $H_{2,6}$, $J = 4.4$ Hz), 3.17 (bs, 4H, piperazine $H_{3,5}$), 3.36 (s, 2H, –COCH_2N–), 4.07–4.12 (m, 2H, –O$CH_2$$CH_3$), 6.99–7.09 (m, 3H, phenyl $H_{4,5,6}$), 7.25 (d, 1H, phenyl H_3, $J = 1.2$ Hz), 7.26–7.29 (m, 1H, benzothiazole H_7), 7.38–7.39 (dd, 1H, benzothiazole H_5, $J_1 = 1.2$ Hz, $J_2 = 8$ Hz), 7.68 (d, 1H, benzothiazol H_4, $J = 8.8$ Hz), 10.41 (bs, 1H, –NHCOCH_2–). Anal Calcd for $C_{21}H_{23}ClN_4O_2S$ (430.951): C, 58.25; H, 5.82; N, 12.94; O, 7.39; S, 7.41. Found: C, 58.22; H, 5.82; N, 12.94; S, 7.40.

*N-(6-ethoxybenzothiazol-2-yl)-2-[4-(p-cyanophenyl)piperazinyl]acetamide (**2c**)*

Beige colored powder. 42.7% (0.45 g), m.p. above 300 °C. UV (MeOH, λ_{max}, nm); 292 (log ε: 4.21). FT-IR (KBr, cm^{-1}); 3194 (N–H), 3060 (C–H; aromatic), 2972 (C–H; aliphatic), 2215 (C≡N), 1699 (C=O; amide), 1605 (C=C; aromatic), 1225 (C–N). ^{1}H-NMR (DMSO, ppm); 1.46 (t, 3H, –$OCH_2\underline{CH_3}$, $J = 7.2$ Hz), 2.80 (t, 4H, piperazine $H_{2,6}$, $J = 5.2$ Hz), 3.42 (t, 4H, piperazine $H_{3,5}$, $J = 5.2$ Hz), 3.35 (s, 2H, –$CO\underline{CH_2}$N–), 4.06–4.12 (m, 2H, –$O\underline{CH_2}CH_3$), 6.88 (d, 2H, phenyl $H_{2,6}$, $J = 8.8$ Hz), 7.53 (d, 2H, phenyl $H_{3,5}$, $J_1 = 8.8$ Hz), 7.03–7.06 (dd, 1H, benzothiazole H_5, $J_1 = 2.4$ Hz, $J_2 = 8.6$ Hz), 7.26–7.29 (m, 1H, benzothiazole H_7), 7.67 (d, 1H, benzothiazole H_4, $J = 8.8$ Hz), 10.28 (bs, 1H, –$\underline{NH}COCH_2$–). Anal Calcd for $C_{22}H_{23}N_5O_2S$ (421.515): C, 62.39; H, 5.95; N, 16.54; S, 7.57. Found: C, 62.35; H, 5.96; N, 16.56; S, 7.58.

*N-(6-ethoxybenzothiazol-2-yl)-2-[4-(o-cyanophenyl)piperazinyl]acetamide (**2d**)*

Beige colored powder. 25.6% (0.27 g), m.p. above 300 °C. UV (MeOH, λ_{max}, nm); 290 (log ε: 4.23). FT-IR (KBr, cm^{-1}); 3290 (N–H), 3068 (C–H; aromatic), 2825 (C–H; aliphatic), 2221 (C≡N), 1703 (C=O; amide), 1605 (C=C; aromatic), 1225 (C–N). ^{1}H-NMR (DMSO, ppm); 1.45 (t, 3H, –$OCH_2\underline{CH_3}$, $J = 7.2$ Hz), 2.88 (t, 4H, piperazine $H_{2,6}$, $J = 4.4$ Hz), 3.31 (t, 4H, piperazine $H_{3,5}$, $J = 4.4$ Hz), 3.38 (s, 2H, –$CO\underline{CH_2}$N–), 4.07–4.12 (m, 2H, –$O\underline{CH_2}CH_3$), 7.03–7.09 (m, 3H, phenyl $H_{4,5,6}$), 7.51–7.56 (m, 1H, phenyl H_3), 7.26–7.29 (m, 1H, benzothiazole H_7), 7.58–7.61 (dd, 1H, benzothiazole H_5, $J_1 = 1.6$ Hz, $J_2 = 8$ Hz), 7.68 (d, 1H, benzothiazol H_4, $J = 9.2$ Hz), 10.36 (bs, 1H, –$\underline{NH}COCH_2$–). Anal Calcd for $C_{22}H_{23}N_5O_2S$ (421.515): C, 62.39; H, 5.95; N, 16.54; S, 7.57. Found: C, 62.37; H, 5.96; N, 16.55; S, 7.58.

Biological activity

The cytotoxic activity of the synthesized compounds **1a–f** and **2a–d** was investigated on liver (HUH-7), breast (MCF-7) and colon (HCT-116) cancer cell lines, by means of sulphorhodamine B (SRB) assays in triplicate. As shown in Table 2, all tested compounds were screened with mean 50% growth inhibition concentration (GI_{50}) in micromolar concentration range. The cytotoxicity results show that most of the substituted benzothiazole-piperazine derivatives are active against tested cancer cell lines. Further investigation of compound **1d** by Hoechst staining and FACS revealed that this compound causes apoptosis by cell cycle arrest at subG_1 phase.

Discussion

The synthesis of benzothiazole-piperazine derivatives is outlined in Figure 1. The final compounds are obtained by two stages which are *N*-acetylation of the primary amine and *N*-alkylation of the secondary amine. In order to obtain target compounds, firstly it is necessary to synthesize 2-chloro-*N*-(6-methylbenzothiazole-2-yl)acetamide (**1**) and 2-chloro-*N*-(6-ethoxybenzothiazole-2-yl)acetamide (**2**) starting from 2-amino-6-methylbenzothiazole and 2-amino-6-ethoxybenzothiazole, respectively. Finally, compounds **1** and **2** are reacted with various piperazine derivatives under basic conditions in which piperazines are *N*-alkylated to yield the final products. Structural and physical properties of the synthesized benzothiazole-piperazine derivatives are summarized in Table 1.

Synthesized compounds were identified with UV, IR and ^{1}H-NMR spectra. In UV spectrum of compounds there is one significant band at 290 nm which represents $n \rightarrow \pi^*$ transition of the series. In IR spectrum of benzothiazole-piperazine derivatives, characteristic N–H stretching band was observed nearly at 3330 cm^{-1}. Other stretching bands were observed approximately at 3050 cm^{-1} (C–H; aromatic), 2980 cm^{-1} (C–H; aliphatic), 1700 cm^{-1} (C=O; amide), 1605 cm^{-1} (C=C; aromatic) and 1240 cm^{-1} (C–N). In ^{1}H-NMR spectra of benzothiazole-piperazine derivatives, the protons of piperazine were seen approximately at 2.70 and 3.33 ppm as triplets ($J = 8.2$ Hz). Amide N–H gave broad singlet nearly at 10.33 ppm. Methylene protons of –$CO\underline{CH_2}$N– gave singlet nearly at 3.35 ppm. The protons of benzothiazole were seen approximately at 7.67 ppm as doublet

Table 1. Structural and physical properties of compounds **1a–f** and **2a–d**.

Compounds	R_1	R_2	M.P. (°C)	Yield %	Log *P**
1a	Methyl	2-Methoxyphenyl	121.9†	48	4.15
1b	Methyl	2-Methoxyethyl	81.1	12	2.04
1c	Methyl	Cyclohexyl	181.3	18	3.74
1d	Methyl	4-Pyridyl	100.8	22	2.94
1e	Methyl	3,4-Dichlorophenyl	243.8	16	5.39
1f	Methyl	4-Chlorobenzyl	189.6	12	4.49
2a	Ethoxy	4-Methylphenyl	146.7	36.5	4.76
2b	Ethoxy	2-Chlorophenyl	>300	47.5	4.83
2c	Ethoxy	4-Cyanophenyl	>300	42.7	4.31
2d	Ethoxy	2-Cyanophenyl	>300	25.6	4.31

*Log *P* were calculated using ChemDraw Ultra V.9.0 (ChembridgeSoft Corp., Cambridge, MA).

†Melting point of compound **1a** in the original reference[15] is reported as 122–124 °C.

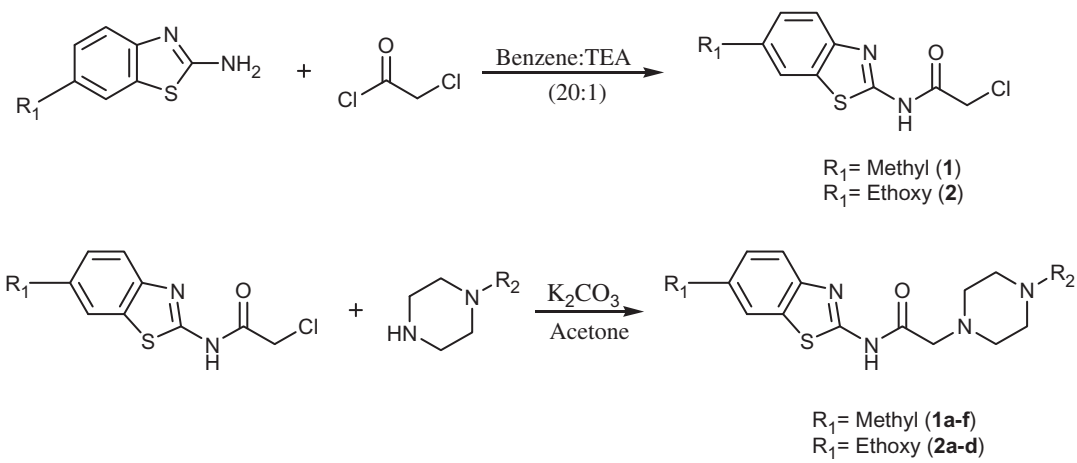

Figure 1. Synthesis of compounds **1a–f** and **2a–d**.

($J = 8$ Hz) (H_4) at 7.25–7.27 ppm as multiplets (H_5) and 7.62 ppm as singlets (H_7). Methyl protons of Ar-CH_3 (**1a–f**) gave singlet nearly at 2.48 ppm. Methyl protons of Ar-OCH_2CH_3 (**2a–d**) gave triplets nearly at 1.45 ($J = 7.2$ Hz) ppm and methylene protons of Ar-OCH_2CH_3 (**2a–d**) gave multiplets nearly at 4.06–4.12 ppm.

Table 2. Cytotoxic activity data for compounds **1a–f** and **2a–d**.

			Cancer cell line (GI_{50}, μM)			
Compounds	HCT-116	R^2	MCF-7	R^2	HUH-7	R^2
1a	4.8	1.0	15.1	1.0	5.7	1.0
1b	7.7	0.8	no inh	–	10.7	0.8
1c	15.1	0.9	26.9	0.8	7.9	0.9
1d	7.9	0.9	9.2	0.9	3.1	0.9
1e	8.0	0.9	16.6	0.9	9.7	1.0
1f	5.0	0.8	no inh	–	7.0	0.9
2a	4.5	1.0	61.4	0.9	9.4	1.0
2b	5.8	0.9	20.4	0.9	6.5	0.9
2c	6.3	0.8	29.8	0.9	7.4	0.8
2d	25.3	0.9	19.8	0.9	6.7	0.9
5-FU	30.66		3.51		18.67	

In HCT-116 colorectal cancer cell line, the GI_{50} values for benzothiazole-piperazine derivatives were in range of 4–25 μM. Among series, 4-methylphenyl substituted compound **2a** (GI_{50}: 4.5 μM) had the highest cytotoxicity. Cyclohexyl ring (**1c**, GI_{50}: 15.1 μM) lowered the activity of 6-methylbenzothiazole derivatives. In addition, compounds **2c** (GI_{50}: 6.3 μM) and **2d** (GI_{50}: 25.3 μM) showed that electron withdrawing cyano group should be on *para* position rather than *ortho* position of phenyl ring for higher cytotoxicity.

In MCF-7 breast cancer cell line, the GI_{50} values for benzothiazole-piperazine derivatives were in range of 9–60 μM. Among series, 4-pyridyl substituted compound **1d** (GI_{50}: 9.2 μM) and 2-methoxyphenyl substituted compound **1a** (GI_{50}: 15.1 μM) had highest cytotoxicities. Also compounds **1b** (R_2: 2-methoxyethyl) and **1f** (R_2: 4-chlorobenzyl) showed no inhibition against MCF-7 cell line.

The activity data showed that all synthesized compounds have good cytotoxic activity against HUH-7 liver cancer cell line in range of 3–10 μM. The most active compound against this cell

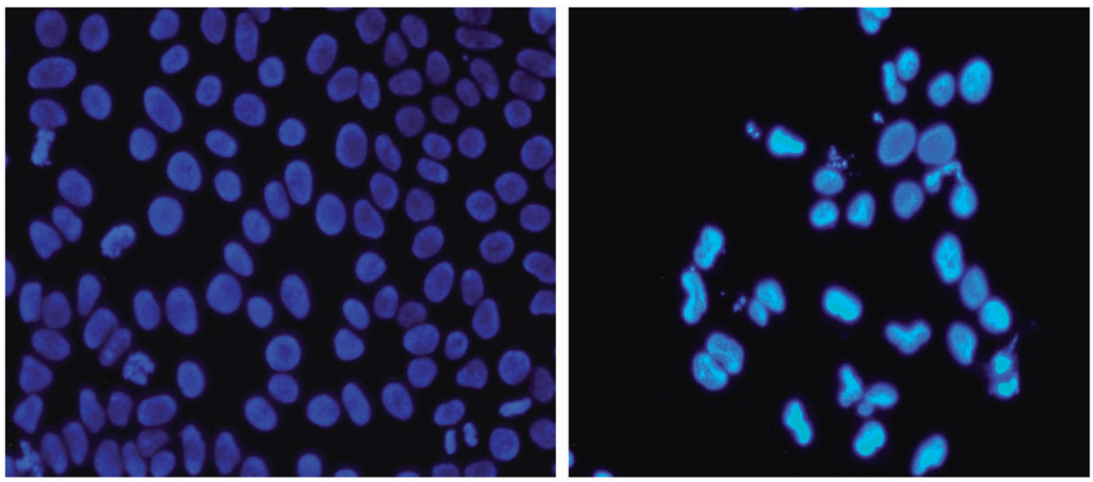

Figure 2. Fluorescence images of liver cancer (HUH7) cells[a,b]. [a]Cells were placed on cover slips and treated with GI_{50} concentration of compound **1d** or DMSO control for 72 h. [b]Nuclear Hoechst 33258 stain was used to stain the cells.

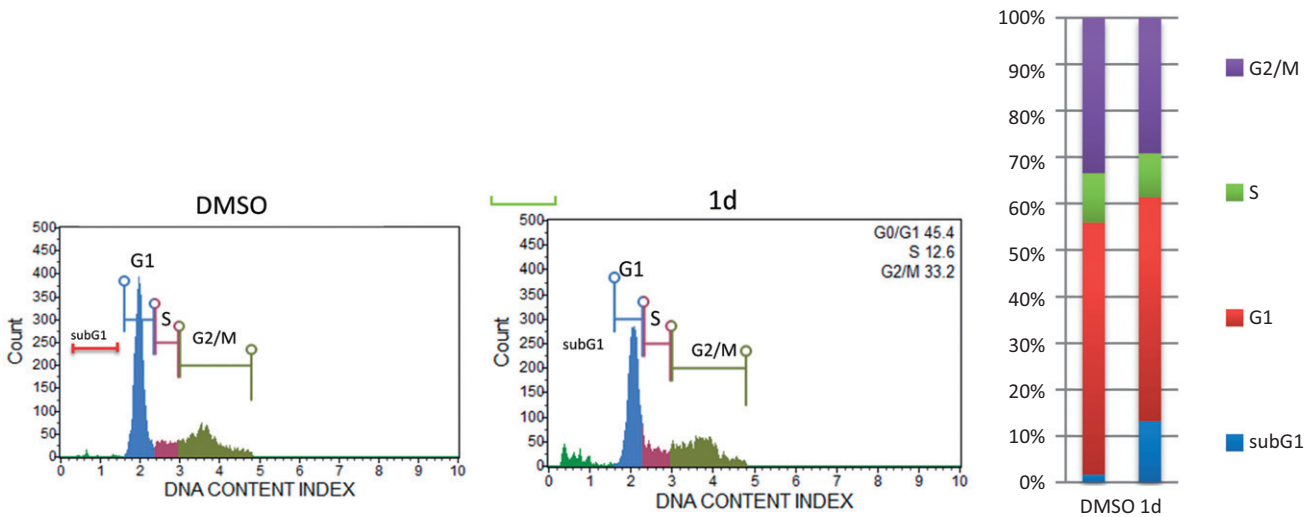

Figure 3. Cell cycle distribution analysis of compound **1d**.

line was **1d** (R_2: 4-pyridyl, GI_{50}: 3.1 μM). Compounds **1a** (R_2: 2-methoxyphenyl, GI_{50}: 5.7 μM) and **2b** (R_2: 2-chlorophenyl, GI_{50}: 6.5 μM) were other highly active derivatives against HUH-7 cell line.

We did not find any significant correlation between GI_{50} values and log *P* values of the compounds **1a–f** and **2a–d** (Table 1). Therefore, the difference in lipophilicity may not be a significant factor for the difference in cytotoxicity of the series in our study.

Compound **1d** (R_1 = methyl, R_2 = 4-pyridyl) was found highly active against all tested cancer cell lines. Therefore, we carried out Hoechst staining (Figure 2) and Fluorescence-Activated Cell Sorting Analysis (FACS, Figure 3) for compound **1d** to gain insight into its mechanism of action.

To examine the type of cell death, induction of apoptosis was investigated by Hoechst staining. Human liver cancer cell line HUH-7 was treated with compound **1d**. Hoechst staining showed condensed nuclei that indicated apoptotic cells in treated samples. Whereas in HUH-7 cell group treated with DMSO, no apoptotic cells were present. This result indicated that apoptosis is most likely to be the cell death type induced by compound **1d**.

The effect of the compound **1d** on the cell cycle was further characterized by FACS analysis, using a propidium iodide stain. This analysis revealed elevated $subG_1$ phased cells indicating the $subG_1$ cell cycle arrest compared to control cells treated with DMSO. This result that indicates the presence of cells arrested at $subG_1$ phase supports the induction of apoptotic cell death in those cells treated with compound **1d**.

Conclusions

In this study, ten benzothiazole-piperazine derivatives were synthesized, purified and characterized by various analysis methods. *In vitro* cytotoxic activities were screened against colorectal (HCT-116), breast (MCF-7), and hepatocellular (HUH-7) cancer cell lines by sulphorhodamine B assay. Most of the compounds showed high cytotoxic activity against all tested cell lines. Hoechst staining and Fluorescence-activated cell sorting analysis, were also performed with compound **1d** to understand the mechanism of cytotoxicity which revealed that this compound causes apoptosis by cell cycle arrest at $subG_1$ phase.

Declaration of interest

The authors have declared no conflict of interest. This work is supported by The Scientific & Technological Research Council of Turkey (TUBITAK) (Project Number: 114S115).

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