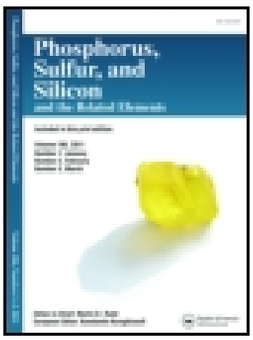

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ISSN: 1042-6507 (Print) 1563-5325 (Online) Journal homepage: http://www.tandfonline.com/loi/gpss20

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To cite this article: Derya Osmaniye, Serkan Levent, Cankız Mina Ardıç, Özlem Atlı, Yusuf Özkay & Zafer Asım Kaplancıklı (2017): Synthesis and anticancer activity of some novel benzothiazole-thiazolidine derivatives, Phosphorus, Sulfur, and Silicon and the Related Elements, DOI: 10.1080/10426507.2017.1395878

To link to this article: https://doi.org/10.1080/10426507.2017.1395878

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https://doi.org/10.1080/10426507.2017.1395878

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Synthesis and anticancer activity of some novel benzothiazole-thiazolidine derivatives

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ABSTRACT

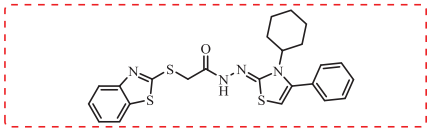
Sixteen new 2-(benzothiazol-2-ylthio)-N′-(3-substituted-4-(3,4-substitutedphenyl)thiazol-2(3H)-ylidene) acetohydrazide derivatives (**4a**-**4p**) were synthesized. The structures of the synthesized compounds were elucidated using FT-IR, ^{1}H-NMR, ^{13}C-NMR, and HRMS spectral data. Anticancer activity of the compounds **4a**-**4p** against C6 (rat brain glioma) and A549 (human lung adenocarcinoma) cell lines was evaluated by using MTT, inhibition of DNA synthesis, and flow cytometric analysis assays. According to MTT assay, **4a** and **4d** were found to be the most active compounds against C6 cell line with an IC_{50} value of 0.03 mM. Moreover, IC_{50} values of **4a** (0.2 mM) and **4d** (0.1 mM) against NIH3T3 (mouse embryo fibroblast cell line) were higher than their IC_{50} values (0.03 mM) against C6 cell line. Accordingly, selectivity of compound **4a** against C6 cell line was two-fold higher than that of compound **4d.** Flow cytometry analysis showed that these compounds display anticancer activity by inducing apoptosis. As a result, compound **4a** has a remarkable anticancer activity and a good selectivity towards C6 cell lines.

ARTICLE HISTORY
Received 10 July 2017
Accepted 19 October 2017

KEYWORDS
Benzothiazole; Anticancer; C6 cell line; A549 cell line; Apoptosis

GRAPHICAL ABSTRACT

Selective anticancer compound towards C6 (rat brain glioma) cell line.



Compound 4a; C6 cell line IC_{50}: 0.03 mM and NIH3T3 cell line IC_{50}: 0.2 mM
Cisplatin; C6 cell line IC_{50}: 0.03 mM

Introduction

Global deaths caused by cancer are expected to carry on increasing with a probable 12 million in the year 2030.[1] The efficacy of conventional drugs in cancer treatment is adversely affected by reasons such as their unfavourable pharmacological and physico-chemical properties, involving in toxic effects to healthy cells and tissues, poor water affinity, and the development of multi-drug resistance.[2,3,4] Chemotherapy plays significant role in treatment of cancer.

In recent years, many new anticancer drugs have been used in the treatment of various types of cancer.[5] However, at present, anticancer chemotherapy still suffers from two major restrictions. The first is the absence of selectivity of chemotherapeutic agents, which carries side effects in the cancer treatment. The second is the development of multiple-drug resistance by cancer cells. In addition to this, cancer patients on chemotherapy are vulnerable to bacterial attack resulting from their falling immunity.[6] Thus, current situation highlights the need for the discovery and improvement of new principal compounds of simple structure, displaying optimal *in vivo* antitumor potency and different mechanisms of action.

Over the last five decades, a large number of benzothiazole derivatives have been used to design of new bioactive compounds because of their various pharmacological activities[7–12] especially anticancer.[13,14] Cytotoxicity assays on a panel of human cancer cell lines as well as preclinical and clinical studies of benzothiazoles showed that these molecules could be developed as promising chemotherapeutics for the treatment of cancer. Some of these new-generation benzothiazole conjugates indicated enhanced potency in comparison with the most of the typical anticancer agents. Based on the notable efficacy and potency data in animal models, many molecules of this type are being evaluated in the clinic and are at different phases. The rational drug design and development of these

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Supplemental data for this article can be accessed on the publisher's website at https://doi.org/10.1080/10426507.2017.1395878.

via rich preclinical screenings or computer-aided drug design need further detailed investigations for providing new leads with higher efficacy and reduced toxicity. These methodologies, and the development of benzothiazole based molecules therefore provides a possibility of clinical trials.[15]

Benzothiazole analogs perform their anticancer activity on different molecular targets. Some significant examples of such biotargets are replication and mitosis inhibitors,[16] topoisomerase II inhibitors,[17] tyrosine kinase inhibitors,[18] inhibitors of thioredoxin signalling system,[19] cytochrome P450 inhibitors,[20–22] heat shock protein 90 inhibitors,[23] epidermal growth factor receptor inhibitors,[24] and cathepsin D inhibitors.[25]

As a result of structure-based drug design approaches, the 3D structure data for therapeutic targets have enhanced the chances for rapid replication of biologically active benzothiazoles. A structure activity relationship profile have been successfully developed for the newly synthesized compounds through the systematic investigation of the functional groups in the C_2, C_5, and C_6 positions of benzothiazole.[26] Especially C_2 substituted derivatives of benzothiazole have been studied for their anticancer activity. However, while most investigators set their sights on designing new benzothiazoles by substituting 2-aminobenzothiazoles or 2-arylbenzothiazoles, only a few utilized 2-mercaptobenzothiazoles. Recent findings indicated that 2-mercaptobenzothiazole has also emerged as an important pharmacophore for the development of anticancer agents.[23,25,27–30]

In addition to benzothiazole, thiazoline is another sulphur containing structure that is often subjected to new anticancer development studies. In recent years, especially, imino-thiazoline containing compounds have received considerable attention owing to their diverse chemotherapeutic potentials, including antitumor activity.[31–35]

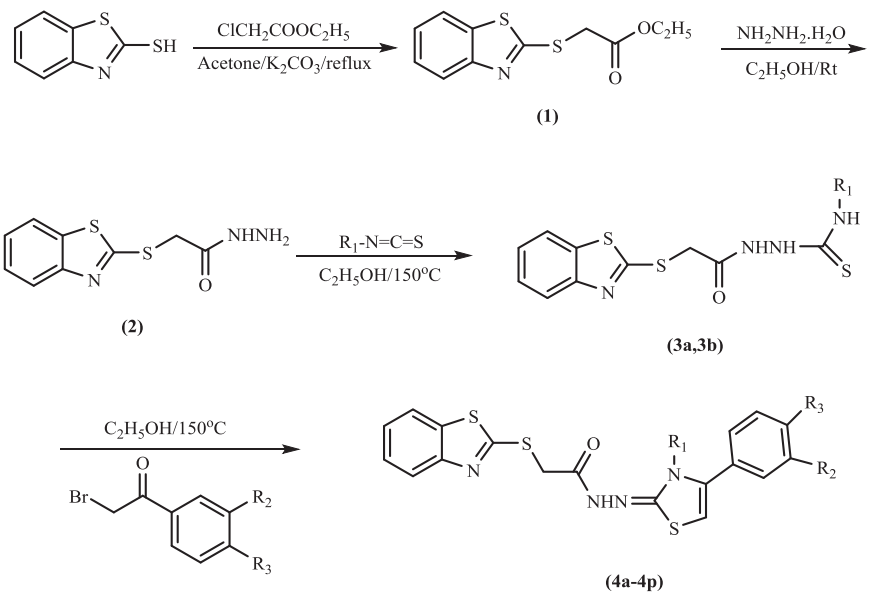
Taking all the above findings into consideration in the search for new lead compounds endowed with potent antitumor activity, we report herein the synthesis of new a series of benzothiazole-thiazoline derivatives and evaluation of their antitumor activity.

Results and discussion

Chemistry

The compounds **4a**-**4p** were synthesized as summarized in Scheme 1. First, ethyl 2-(benzothiazol-2-ylthio)acetate was prepared by the reaction of 2-mercaptobenzothiazole and ethyl 2-chloroacetate in the presence of potassium carbonate. In the second step, the reaction of ethyl 2-(benzothiazol-2-ylthio)acetate **(1)** and excess of hydrazine hydrate afforded 2-(benzothiazol-2-ylthio)acetohydrazide (**2**)**.** Thiosemicarbazide derivatives **(3a** and **3b)** were obtained via reaction of 2-(benzothiazol-2-ylthio)acetohydrazide **(2)** with cyclohexyl isothiocyanate or phenyl isothiocyanate. In the last step, target compounds **(4a-4p)** were prepared via ring closure reaction using thiosemicarbazide derivatives **(3a** and **3b)** and an appropriate 2-bromoacetophenone.

The structures of final compounds were elucidated by IR, ^{1}H-NMR, ^{13}C-NMR, and HRMS analysis. In the IR spectra, the stretching absorptions, which were seen around 3036 cm^{-1} showed the N-H bond of hydrazide group. Signals belonging to the carbonyl function appeared at 1651–1728 cm^{-1}. The stretching absorption at about 1203–1244 cm^{-1} were assigned to the C-N single bond. The cyano group in compounds **4c** and **4k** had signals at 2233 and 2231 cm^{-1}, respectively. In the ^{1}H-NMR spectra, aliphatic protons belonging to the cyclohexyl ring were recorded between 0.97 ppm and 2.64 ppm. singlets between 4.21 ppm and 4.35 ppm were assigned to the methylene group and the protons of aromatic rings were recorded at 6.14-8.07 ppm. The peaks corresponding to hydrogens of the benzothiazole overlapped in most cases, with the phenyl peaks. The proton of hydrazide was recorded over 10 ppm. In addition, in the ^{1}H-NMR spectra of compounds **4a**, **4b**, **4c**, and **4h** peak splitting indicated that these compounds were obtained as a mixture of E and Z isomers. In the ^{13}C-NMR spectra, aliphatic carbons were recorded between 21.2 ppm and 59.3 ppm. Aromatic carbons gave peaks from 97.9 ppm to 165.6 ppm. C2 of benzothiazole was recorded about 166 ppm. Carbonyl carbon had a peak over 167 ppm. Carbon-fluorine splitting was also



Scheme 1. Synthesis of compounds **4a**-**4p**.

observed for compounds **4e** and **4m**. In addition, 2D NMR studies including, HMBC, HSQC, and NOESY were performed for compound **4a**. Thus, all data observed in ^{1}H-NMR and ^{13}C-NMR were also confirmed by 2D NMR spectra. In the MS spectra, all masses matched well with the expected M+H values. Selected characterization spectra of compounds **3a**, **3b**, and **4a-4p** were presented in **Figure S 1-S 75 (Supplemental Materials)**.

Biological evaluation

Cytotoxicity test

In the research of discovering new anticancer agents, the most common screening methods are cytotoxicity tests towards cancer cell lines. These are high prevalent screening assays, exposing compounds with the cytotoxic activity.[36] MTT assay, attached to the capability of metabolically active cells that change the pale yellow MTT colour to a spectrophotometrically measurable blue formazan salt, is one of the most favoured cytotoxicity tests.[37] We assessed the antitumor potential of compounds **4a-4p** on A549 and C6 cell lines with various concentrations (1mM, 0.316 mM, 0.1 mM, 0.0316 mM, 0.01 mM, 0.00316 mM, 0.001 mM, 0.000316 mM). Besides, the cytotoxic activities of these compounds were evaluated against healthy NIH3T3 cells by virtue of showing the selectivity towards carcinogenic cells. The IC_{50} values of the compounds against cell lines are presented in **Table S 1 (Supplemental Materials).**

Compounds **4a, 4d** were found to have the highest cytotoxic activity against C6 cell line. These compounds indicated equal IC_{50} value (0.03 mM) to cisplatin against C6 cell line.

One of the important criteria so as to being anticancer agent candidate is to display minimum or no side-effects on healthy cells. Hence, cytotoxicity of compounds **4a-4p** against NIH3T3 cell line was evaluated. As seen in the Table S 1 (Supplemental Materials), IC_{50} values of compounds **4a, 4d** against C6 cells were lower than their IC_{50} values against NIH3T3 cell line. Moreover, selectivity of compound **4a** against C6 cells was two-fold higher than that of compound **4d**. Based on the obtained results, it can be interpreted that compound **4a** is a promising anticancer agent. Compared to the A549 and C6 cell line, synthesized compounds were found to be more effective on the C6 cell line. Therefore, C6 cell line was used in further tests, including DNA synthesis inhibition and flow cytometric analysis.

DNA synthesis inhibition assay

This immunostaining assay is related to detect the attendance of BrdU into nuclear DNA instead of thymidine during S-phase of cell cycle using specific anti-BrdU antibodies.[38,39] Thus, this study was performed to evaluate inhibitory effects of compounds **4a** and **4d** on proliferation of C6 cell line. Test compounds and reference agent cisplatin were used at the concentrations of $IC_{50}/4$, $IC_{50}/2$, IC_{50}, 2 x IC_{50}, and 4 x IC_{50}. **Figure S 76** represents the DNA synthesis inhibitory effect of compounds **4a** and **4d** on C6 cell line.

Compound **4a** indicated 4.24, 14.72, 18.37, 51.83 and 63.16% DNA synthesis inhibition at $IC_{50}/4$ to 4 x IC_{50} concentrations, respectively. Besides, compound **4d** displayed 6.34, 16.87, 45.00, 58.82 and 71.04% DNA synthesis inhibition, whereas cisplatin was found to have 18.20, 35.40, 48.49, 52.13 and 65.72% inhibition at the same concentrations, respectively.

Tested compounds exhibited concentration-dependent inhibitory activity on DNA synthesis of C6 cell line. When the DNA synthesis inhibitory activity of compounds is compared, it is clear that **4d** has higher inhibition potency than cisplatin at IC_{50}, 2 x IC_{50}, and 4 x IC_{50}, whereas **4a** has same inhibition potency with cisplatin at 2 x IC_{50} and 4 x IC_{50}. According to these results, it can be suggested that compound **4d** possesses higher antiproliferative activity against C6 cell line than cisplatin.

Flow cytometric analysis

Apoptosis is an important approach to express the cell death pathway since most of the anticancer drugs induce apoptosis in cancer cells. Annexin V-FITC is a reagent that can detect and measure quantity of apoptotic cells by flow cytometry. Colouring cells with PI and Annexin V-FITC show the differences between live, apoptotic, dead, and late apoptotic or necrotic cells.[40] Thus, this method provides an evidence on the pathway of the cell death.

Flow cytometry studies were carried out on C6 cells for compounds **4a**, **4d** and cisplatin with the aim of determining the stimulated cellular death pathway. Selected compounds were used at concentrations of $IC_{50}/2$, IC_{50}, and 2 x IC_{50}. Flow cytometric analysis diagrams of compound **4a** and **4d** are presented in **Figure S 77** and **Figure S 78 (Supplemental Materials)**, respectively. At the IC_{50} concentration, compound **4a** displayed the highest population of apoptotic cells (28.48%), whereas cisplatin displayed 20.90% apoptotic cells. Compound **4d** also generated an analogous population of apoptotic cells (19.53) to that of 20.57 at IC_{50}. As a result, findings of flow cytometric analysis revealed that compounds **4a** and **4d** provoked the apoptotic induction in C6 cell line after 24 h treatment.

Conclusion

A major challenge to medicinal chemists is determination of new structures that may be useful in the design of novel, selective and less-toxic anticancer agents. With this aim, we synthesized new 2-(benzothiazol-2-ylthio)-N′-(3-substituted-4-(3,4-substitutedphenyl)thiazol-2(3H)-ylidene)acetohydrazides (**4a-4p**) and elucidated their anticancer activity against A549 and C6 cells. Cytotoxic activities of the synthesized benzothiazoles against healthy NIH3T3 cell line were also examined.

It was determined that compounds **4a** and **4d** showed equal cytotoxicity with cisplatin against C6 cell line. Moreover, compound **4a** showed more selective inhibition than **4d** towards C6 cell line. Structures of the test compounds vary from each other in terms of substituents on imino-thiazoline and phenyl substructures. When the anticancer activity results are examined, it is clearly seen that cyclohexyl substituted derivatives (**4a-4h**) are more effective than phenyl substituted compounds (**4i-4p**) against the C6 cell line. This finding suggests that cyclohexyl group has a potency to induce anticancer activity. On the other hand, compound **4a**, including a nonsubstituted

phenyl group on the thiazolidine structure, possesses the most selective cytotoxic effects. This result shows that incorporation of an electron withdrawing or donating substituent on phenyl moiety decreases the selectivity. In conclusion, the anticancer activity screening of novel benzothiazole-thiazoline derivatives designated that compound **4a** is the lead compound of the series. According to flow cytometry analysis this compound has a greater induction potential of apoptosis than cisplatin.

Experimental

Chemistry

All chemicals were obtained either from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, MO, USA) or Merck (Merck KGaA, Darmstadt, Germany), and used without further chemical purifications. Melting points of the compounds were measured by using an automatic melting point determination instrument (MP90, Mettler-Toledo, OH, USA) and were presented as uncorrected. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 by a Bruker digital FT-NMR spectrometer (Bruker Bioscience, MA, USA) at 300 MHz and 75 MHz, respectively. The IR spectra of the compounds were recorded using an IRAffinity-1S Fourier transform IR (FTIR) spectrometer (Shimadzu, Tokyo, Japan). HRMS studies were performed on an LCMS-IT-TOF system (Shimadzu, Tokyo, Japan). Chemical purities of the compounds were checked by classical TLC applications performed on silica gel 60 F_{254} (Merck KGaA, Darmstadt, Germany). The Supplemental Materials contains sample ^{1}H NMR, ^{13}C NMR and HRMS spectra of the products **4a – 4p** (**Figures S 1 – S 75**).

Materials and methods

Ethyl 2-(benzothiazol-2-ylthio)acetate 1

A mixture of benzothiazole-2-thiol (5.51 g, 33 mmol) and ethyl 2-chloroacetate (4.24 ml, 39 mmol) were refluxed in acetone (100 mL) in the presence of potassium carbonate (4.55 g, 33 mmol) for 8 h. After completion of the reaction, acetone was removed under reduced pressure, the residue was washed with water, dried, and recrystallized from EtOH. Yield 69%, M.P. = 58–59°C (measured), M.P. = 58°C (reported.[41])

2-(benzothiazol-2-ylthio)acetohydrazide 2

Hydrazine hydrate (4.08 mL, 84 mmol) was added in portions into a solution of ethyl 2-(benzothiazol-2-ylthio)acetate **(1)** (7.18 g, 28 mmol) in EtOH and then the mixture was stirred for 12 h at room temperature. The precipitated product was filtered and the solid was washed with ethanol to remove the excess of the hydrazine hydrate, dried, and recrystallized from EtOH.

2-(2-(benzothiazol-2-ylthio)acetyl)-N-substitutedhydrazine-1-carbothioamide 3a-3b

2-(benzothiazol-2-ylthio)acetohydrazide (**2**) (3.05 g, 13 mmol) and substituted isothiocyanate (13 mmol) were refluxed for 2 h in EtOH (30 mL). After completion of the reaction the mixture was cooled in an ice-bath, precipitated product was filtered, dried, and recrystallized from EtOH. Yield 64%, m.p. = 192–193°C (measured), m.p. = 193°C (reported.[41])

2-(2-(Benzo[d]thiazol-2-ylthio)acetyl)-N-cyclohexylhydrazine-1-carbothioamide 3a

Yield: 88%, m.p. = 185–186°C, FTIR (ATR, cm^{-1}): 3360 (N–H), 1691 (C=O), 1230 (C–N), 1006. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.03-1.23 (6H, m, cyclohexyl), 1.52-1.77 (5H, m, cyclohexyl), 4.05 (1H, s, NH), 4.24 (2H, s, CH_2), 7.38 (1H, td, J_1 = 1.17 Hz, J_2 = 8.13 Hz, benzothiazole CH), 7.49 (1H, td, J_1 = 1.23 Hz, J_2 = 7.32 Hz, benzothiazole CH), 7.87 (1H, d, J = 7.77 Hz, benzothiazole CH), 8.03 (1H, d, J = 7.23 Hz, benzothiazole CH), 9.29 (1H, s. NH), 10.17 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 19.02, 25.25, 25.54, 32.13, 32.33, 35.62, 53.23, 121.67, 122.33, 125.08, 126.86, 135.27, 152.94, 166.41, 166.74, 180.81. ESI-MS (M+H): $C_{16}H_{20}N_4OS_3$: 381.20.

2-(2-(Benzothiazol-2-ylthio)acetyl)-N-phenylhydrazine-1-carbothioamide 3b

Yield: 89%, m.p. = 163–164°C, FTIR (ATR, cm^{-1}): 3323 (N–H), 1653 (C=O), 1232 (C–N), 1004, 873. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.29 (2H, s, CH_2), 7.17 (1H, t, J = 7.26 Hz, phenyl CH), 7.30-7.47 (6H, m, benzothiazole CH, phenyl CH), 7.82 (1H, d, J = 7.95 Hz, benzothiazole CH), 8.01 (1H, d, J = 7.98 Hz, benzothiazole CH), 9.63 (1H, s, NH), 9.77 (1H, s, NH), 10.44 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 121.6, 122.3, 125.4, 125.7, 126.8, 128.6, 135.2, 139.4, 152.9, 166.4, 181.3. ESI-MS (M+H): $C_{16}H_{14}N_4OS_3$: 375.15.

General procedure for the 2-(benzothiazol-2-ylthio)-N'-(3-substituted-4-(3,4-substitutedphenyl)thiazol-2(3H)-ylidene)acetohydrazide 4a-4p

A mixture of thiosemicarbazides **(3a** or **3b)** (0.7 mmol) and an appropriate 2-bromoacetophenone derivatives (0.7 mmol) in EtOH (20 mL) were refluxed for 4 h. When reaction completed water was added to the mixture for precipitation. The obtained product was filtered, dried, and recrystallized from EtOH.

2-(Benzothiazol -2-ylthio)-N-(3-cyclohexyl-4-phenylthiazol-2(3H)-ylidene)acetohydrazide 4a

Yield: 79%, m.p. = 183–184°C, FTIR (ATR, cm^{-1}): 3140 (N–H), 1651 (C=O), 1558-1427 (C=N, C=C), 1203 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 0.97-1.05 (4H, m, cyclohexyl), 1.46-1.67 (6H, m, cyclohexyl), 2.56-2.64 (1H, m, cyclohexyl), 4.21 (2H, s, CH_2), 6.14 (1H, s, thiazolylidene CH), 7.35-7.45 (3H, m, phenyl CH), 7.48-7.50 (4H, m, phenyl CH, benzothiazole CH), 7.87 (1H, d, J = 8.05 Hz, benzothiazole CH), 8.03 (1H, d, J = 8.05 Hz, benzothiazole CH), 10.04-10.43 (1H, s, NH).

^{13}C-NMR (75 MHz, DMSO-d_6): δ = 25.0 (cyclohexyl C4), 26.0 (cyclohexyl C3,3'), 27.9 (cyclohexyl C2,2'), 36.0 (-CH_2), 58.9 (cyclohexyl C1), 97.9 (thiazolylidene C3), 121.6 (benzothiazole C6), 122.3 (benzothiazole C3), 125.0 (benzothiazole C5), 126.9 (benzothiazole C4), 129.2 (phenyl C2,2'), 129.3 (phenyl C3,3'), 129.7 (phenyl C4), 132.2 (phenyl C1), 135.3 (benzothiazole C7), 141.5 (thiazolylidene C2), 153.1 (benzothiazole C2), 162.5 (C=O), 166.6 (benzothiazole C1), 167.9 (thiazolylidene C1). HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{24}N_4OS_3$: 481.1185; found: 481.1174.

2-(Benzothiazol-2-ylthio)-N-(3-cyclohexyl-4-(p-tolyl) thiazol-2(3H)-ylidene)acetohydrazide 4b

Yield: 82%, m.p. = 257–258°C, FTIR (ATR, cm^{-1}): 3049 (N–H), 1714 (C=O), 1583-1429 (C=N, C=C), 1238 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.13-1.21 (1H, m, cyclohexyl), 1.32-1.54 (4H, m, cyclohexyl), 1.63-1.67 (1H, m, cyclohexyl), 1.80-2.01 (4H, m, cyclohexyl), 2.10 (3H, s, -CH_3), 4.29 (2H, s, CH_2), 6.95 (2H, d, J = 7.98 Hz, phenyl CH), 7.12 (1H, s, thiazolylidene CH), 7.26 (2H, d, J = 8.10 Hz, phenyl CH), 7.37-7.43 (1H, m, benzothiazole CH), 7.46-7.52 (1H, m, benzothiazole CH), 7.79 (1H, d, J = 8.01 Hz, benzothiazole CH), 8.01 (1H, d, J = 8.01 Hz, benzothiazole CH), 10.13-10.15 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 21.2, 24.8, 25.1, 31.7, 34.8, 59.0, 102.1, 121.7, 122.3, 124.2, 125.1, 126.8, 128.8, 129.4, 135.3, 140.4, 141.4, 152.7, 165.4, 166.8, 167.8. HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{26}N_4OS_3$: 495.1342; found: 495.1333.

2-(Benzothiazol-2-ylthio)-N-(4-(4-cyanophenyl)-3-cyclohexylthiazol-2(3H)-ylidene)acetohydrazide 4c

Yield: 83%, m.p. = 221–222°C, FTIR (ATR, cm^{-1}): 3059 (N–H), 2233 (C≡N), 1728 (C=O), 1582-1427 (C=N, C=C), 1238 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.13-1.21 (1H, m, cyclohexyl), 1.32-1.54 (4H, m, cyclohexyl), 1.63-1.67 (1H, m, cyclohexyl), 1.80-2.01 (4H, m, cyclohexyl), 4.31 (2H, s, CH_2), 6.95 (2H, d, J = 7.98 Hz, phenyl CH), 7.12 (1H, s, thiazolylidene CH), 7.26 (2H, d, J = 8.1 Hz, phenyl CH), 7.37-7.43 (1H, m, benzothiazole CH), 7.46-7.52 (1H, m, benzothiazole CH), 7.79 (1H, d, J = 8.01 Hz, benzothiazole CH), 8.01 (1H, d, J = 8.01 Hz, benzothiazole CH), 10.13-10.29 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 21.2, 25.1, 31.7, 34.8, 59.0, 101.7, 119.0, 121.4, 122.4, 124.3, 125.4, 127.0, 128.8, 129.5, 135.9, 140.4, 141.4, 152.4, 165.4, 166.1, 167.1. HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{23}N_5OS_3$: 506.1137; found: 506.1132.

2-(Benzothiazol-2-ylthio)-N-(3-cyclohexyl-4-(4-nitrophenyl)thiazol-2(3H)-ylidene)acetohydrazide 4d

Yield: 77%, m.p. = 173–174°C, FTIR (ATR, cm^{-1}): 3365 (N–H), 1701 (C=O), 1517-1427 (C=N, C=C), 1217 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.13-1.21 (1H, m, cyclohexyl), 1.36-1.67 (5H, m, cyclohexyl), 1.79-2.00 (4H, m, cyclohexyl), 2.44 (1H, s, cyclohexyl), 4.33 (2H, s, CH_2), 7.30-7.44 (3H, m, benzothiazole CH, thiazolylidene CH), 7.64-7.68 (3H, m, phenyl CH, benzothiazole CH), 7.89-7.92 (3H, m, phenyl CH, benzothiazole CH), 10.35 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 24.8, 25.1, 31.7, 34.8, 59.3, 105.6, 121.5, 122.2, 123.7, 125.2, 126.8, 129.8, 130.2, 135.1, 139.2, 148.0, 152.4, 165.2, 166.2, 167.0. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{23}N_5O_3S_3$: 526.1036; found: 526.1018.

2-(Benzothiazol-2-ylthio)-N-(3-cyclohexyl-4-(4-fluorophenyl)thiazol-2(3H)-ylidene)acetohydrazide 4e

Yield: 85%, m.p. = 250–251°C, FTIR (ATR, cm^{-1}): 3053 (N–H), 1703 (C=O), 1508-1487 (C=N, C=C), 1242 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = ^{1}H-NMR (300 MHz, DMSO-d6): δ = 1.11-1.20 (1H, m, cyclohexyl), 1.29-1.41 (3H, m, cyclohexyl), 1.61-1.65 (3H, m, cyclohexyl), 1.76-1.80 (2H, m, cyclohexyl), 1.91 (2H, s, cyclohexyl), 4.33 (2H, s, CH_2), 7.00-7.06 (2H, m, phenyl CH), 7.36-7.41 (2H, m, phenyl CH), 7.47-7.52 (3H, m, benzothiazole CH, thiazolylidene CH), 7.76 (1H, d, J = 7.95 Hz, benzothiazole CH), 7.97 (1H, d, J = 8.04 Hz, benzothiazole CH), 10.73 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 24.9, 25.1, 31.6, 35.1, 59.3, 102.8, 116.1 (d, J = 22.1 Hz), 121.7, 122.2, 125.1, 126.1 (d, J = 2.9 Hz), 126.8, 129.1 (d, J = 9.4 Hz), 135.2, 152.8, 156.7, 162.1 (d, J = 248.9 Hz), 165.6, 166.8, 167.7. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{23}FN_4OS_3$: 499.1091; found: 499.1075.

2-(Benzothiazol-2-ylthio)-N-(4-(4-chlorophenyl)-3-cyclohexylthiazol-2(3H)-ylidene)acetohydrazide 4f

Yield: 76%, m.p. = 257–258°C, FTIR (ATR, cm^{-1}): 3057 (N–H), 1716 (C=O), 1591-1462 (C=N, C=C), 1238 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.13-1.21 (1H, m, cyclohexyl), 1.31-1.54 (5H, m, cyclohexyl), 1.63-1.67 (1H, m, cyclohexyl), 1.80 (2H, s, cyclohexyl), 1.90-1.93 (1H, m, cyclohexyl), 2.00-2.04 (1H, m, cyclohexyl), 4.28 (2H, s, CH_2), 7.23-7.25 (3H, m, phenyl CH, thiazolylidene CH), 7.39-7.42 (3H, m, phenyl CH, benzothiazole CH), 7.46-7.52 (1H, m, benzothiazole CH), 7.78 (1H, d, J = 8.04 Hz, benzothiazole CH), 8.00 (1H, d, J = 7.83 Hz, benzothiazole CH), 10.20 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 24.8, 25.1, 31.7, 34.8, 59.0, 103.4, 121.7, 122.3, 125.2, 125.9, 126.9, 129.0, 130.8, 135.3, 135.6, 140.1, 152.6, 165.3, 166.9, 167.8. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{23}ClN_4OS_3$: 515.0795; found: 515.0776.

2-(Benzothiazol-2-ylthio)-N-(4-(4-bromophenyl)-3-cyclohexylthiazol-2(3H)-ylidene)acetohydrazide 4g

Yield: 89%, m.p. = 266–267°C, FTIR (ATR, cm^{-1}): 3059 (N–H), 1714 (C=O), 1589-1475 (C=N, C=C), 1238 (C–N).^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.13-1.21 (1H, m, cyclohexyl), 1.32-1.53 (5H, m, cyclohexyl), 1.63-1.67 (1H, m, cyclohexyl), 1.80 (2H, s, cyclohexyl), 1.90-2.04 (2H, m, cyclohexyl), 4.28 (2H, s, CH_2), 7.23 (1H, s, thiazolylidene CH), 7.32-7.35 (2H, m, phenyl CH), 7.38-7.40 (2H, m, phenyl CH) 7.40-7.43 (1H, m, benzothiazole CH), 7.47-7.52 (1H, m, benzothiazole CH), 7.79 (1H,

d, $J = 7.59$ Hz, benzothiazole CH), 8.01 (1H, d, $J = 8.04$ Hz, benzothiazole CH), 10.19 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta = 24.8, 25.1, 31.7, 34.8, 59.0, 103.4, 121.7, 122.4, 124.4, 125.2, 126.3, 126.9, 131.0, 132.0, 135.3, 140.2, 152.6, 165.3, 166.9, 167.8$. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{23}BrN_4OS_3$: 559.0290; found: 559.0264.

2-(Benzothiazol-2-ylthio)-N-(3-cyclohexyl-4-(3,4-dichlorophenyl)thiazol-2(3H)-ylidene)acetohydrazide 4h

Yield: 75%, m.p. = 277–278°C, FTIR (ATR, cm^{-1}): 3057 (N–H), 1703 (C=O), 1577-1462 (C=N, C=C), 1240 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta =$ 1.13-1.22 (1H, m), 1.32-1.56 (4H, m, cyclohexyl), 1.63-1.67 (2H, m, cyclohexyl), 1.80 (2H, s, cyclohexyl), 1.89-2.03 (2H, m, cyclohexyl), 4.27 (2H, s, CH_2), 7.31 (1H, s, thiazolylidene CH), 7.35-7.41 (3H, m, dichlorophenyl CH, benzothiazole CH), 7.44-7.49 (1H, m, benzothiazole CH), 7.71-7.73 (2H, m, dichlorophenyl CH, benzothiazole CH), 7.98 (1H, d, $J =$ 7.86 Hz, benzothiazole CH), 10.26-10.29 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta = 24.8, 25.0, 31.7, 34.8, 59.1, 104.5, 121.6, 122.3, 125.2, 126.9, 127.4, 129.1, 130.9, 131.0, 132.0, 133.7, 135.2, 138.8, 152.6, 165.2, 167.0, 167.9$. HRMS (m/z): [M+H]+ calcd for $C_{24}H_{22}Cl_2N_4OS_3$: 549.0406; found: 549.0381

2-(Benzothiazol-2-ylthio)-N-(3,4-diphenylthiazol-2(3H)-ylidene)acetohydrazide 4i

Yield: 77%, m.p. = 233–234°C, FTIR (ATR, cm^{-1}): 3059 (N–H), 1705 (C=O), 1564-1444 (C=N, C=C), 1219 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta = 4.35$ (2H, s, CH_2), 7.03 (1H, s, thiazolylidene CH), 7.29 (2H, d, $J = 7.50$ Hz, phenyl CH), 7.30-7.36 (4H, m, phenyl CH), 7.39-7.47 (4H, m, phenyl CH, benzothiazole CH), 7.55 (2H, d, $J = 7.50$ Hz, phenyl CH), 7.78 (1H, d, $J = 7.50$ Hz, benzothiazole CH), 7.99 (1H, d, $J = 7.50$ Hz, benzothiazole CH), 12.18 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta =$ 34.6, 121.4, 121.7, 122.2, 124.7, 125.0, 126.7, 128.0, 128.8, 129.8, 130.1, 130.2, 130.8, 135.3, 138.5, 149.0, 152.9, 155.2, 165.8, 167.0. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{18}N_4OS_3$: 475.0715; found: 475.0712.

2-(Benzothiazol-2-ylthio)-N-(3-phenyl-4-(p-tolyl)thiazol-2(3H)-ylidene)acetohydrazide 4j

Yield: 73%, m.p. = 233–234°C, FTIR (ATR, cm^{-1}): 3049 (N–H), 1714 (C=O), 1572-1427 (C=N, C=C), 1234 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta =$ 2.14 (3H, s, CH_3), 4.32 (2H, s, CH_2), 6.93 (1H, s, thiazolylidene CH), 7.01 (2H, d, $J = 8.05$ Hz, methylphenyl CH), 7.31 (2H, d, $J = 8.05$ Hz, methylphenyl CH), 7.37-7.42 (4H, m, phenyl CH, benzothiazole CH), 7.48 (1H, dt, $J =$ 7.35 Hz – 1.30 Hz, benzothiazole CH), 7.55 (1H, d, $J =$ 7.65 Hz, Phenyl CH), 7.78 (1H, d, $J =$ 7.35 Hz, benzothiazole CH), 8.01 (1H, d, $J =$ 7.35 Hz, benzothiazole CH), 12.06 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta =$ 21.2, 34.9, 100.6, 121.7, 122.3, 123.8, 123.9, 124.8, 124.9, 125.1, 126.8, 129.5, 130.8, 135.3, 140.1, 141.0, 152.8, 165.5, 167.1. HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{20}N_4OS_3$: 489.0872; found: 489.0866.

2-(Benzothiazol-2-ylthio)-N-(4-(4-cyanophenyl)-3-phenylthiazol-2(3H)-ylidene)acetohydrazide 4k

Yield: 77%, m.p. = 254–255°C, FTIR (ATR, cm^{-1}): 3049 (N–H), 2231 (C≡N), 1714 (C=O), 1572-1425 (C=N, C=C), 1244 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta = 4.32$ (2H, s, CH_2), 6.93 (1H, s, thiazolylidene CH), 6.97 (2H, d, $J =$ 8.10 Hz, cyanophenyl CH), 7.35-7.40 (4H, m, phenyl CH, benzothiazole CH), 7.45 (2H, d, $J =$ 8.10 Hz, cyanophenyl CH), 7.50 (1H, dt, $J =$ 7.40 Hz – 1.30 Hz, benzothiazole CH), 7.58 (1H, d, $J =$ 7.65 Hz, phenyl CH), 7.78 (1H, d, $J =$ 7.40 Hz, benzothiazole CH), 8.00 (1H, d, $J =$ 7.40 Hz, benzothiazole CH), 12.03 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta =$ 34.9, 100.0, 118.8, 122.0, 122.4, 123.7, 123.9, 124.8, 124.9, 125.2, 126.9, 129.6, 131.1 135.7, 140.1, 141.5, 152.0, 165.6, 167.5. HRMS (m/z): $[M+H]^+$ calcd for $C_{25}H_{17}N_5OS_3$: 500.0668; found: 500.0659.

2-(Benzothiazol-2-ylthio)-N-(4-(4-nitrophenyl)-3-phenylthiazol-2(3H)-ylidene)acetohydrazide 4l

Yield: 75%, m.p. = 256–257°C, FTIR (ATR, cm^{-1}): 3036 (N–H), 1714 (C=O), 1570-1427 (C=N, C=C), 1244 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta =$ 4.32 (2H, s, CH_2), 6.96 (1H, s, thiazolylidene CH), 7.36-7.40 (4H, m, phenyl CH, benzothiazole CH), 7.50 (1H, dt, $J =$ 7.40 Hz – 1.25 Hz, benzothiazole CH), 7.60 (1H, d, $J =$ 7.60 Hz, phenyl CH), 7.78 (1H, d, $J =$ 7.40 Hz, benzothiazole CH), 8.00 (1H, d, $J = 7.40$ Hz, benzothiazole CH), 8.07 (2H, d, $J =$ 8.30 Hz, nitrophenyl CH), 8.38 (2H, d, $J =$ 8.30 Hz, nitrophenyl CH), 12.01 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta =$ 34.8, 103.1, 121.2, 121.5, 122.1, 123.2, 123.8, 125.1, 126.7, 129.6, 129.9, 130.2, 130.7, 131.2, 135.1, 138.6, 147.8, 152.5, 165.3, 167.4. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{17}N_5O_3S_3$: 520.0566; found: 520.0552.

2-(Benzothiazol-2-ylthio)-N-(4-(4-fluorophenyl)-3-phenylthiazol-2(3H)-ylidene)acetohydrazide 4m

Yield: 79%, m.p. = 226–227°C, FTIR (ATR, cm^{-1}): 3049 (N–H), 1714 (C=O), 1572-1429 (C=N, C=C), 1227 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): $\delta = 4.34$ (2H, s, CH_2), 6.88 (1H, s, thiazolylidene CH), 7.08 (2H, t, $J = 8.85$ Hz, fluorophenyl CH), 7.30-7.35 (2H, m, phenyl CH), 7.38 (1H, dt, $J = 7.60$ Hz – 1.90 Hz, benzothiazole CH), 7.45-7.53 (4H, m, phenyl CH, benzothiazole CH), 7.76 (1H, d, $J = 8.25$ Hz, benzothiazole CH), 7.96 (1H, d, $J = 8.25$ Hz, benzothiazole CH), 12.23 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): $\delta =$ 35.0, 103.1, 116.0 (d, $J =$ 21.7 Hz), 121.7, 122.2, 123.3, 123.4, 125.1, 126.8, 130.0, 130.2, 130.3, 130.6, 131.2 (d, $J = 8.8$ Hz), 135.2, 139.7, 152.8, 163.2 (d, $J = 246.3$ Hz), 165.6, 167.0. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{17}N_4OFS_3$: 493.0621; found: 493.0607.

2-(Benzothiazol-2-ylthio)-N-(4-(4-chlorophenyl)-3-phenylthiazol-2(3H)-ylidene)acetohydrazide 4n

Yield: 81%, m.p. = 248–249°C, FTIR (ATR, cm^{-1}): 3048 (N–H), 1717 (C=O), 1572-1427 (C=N, C=C), 1203 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.32 (2H, s, CH_2), 6.92 (1H, s, thiazolylidene CH), 7.23 (2H, t, J = 8.70 Hz, chlorophenyl CH), 7.30-7.35 (2H, m, phenyl CH), 7.39 (1H, dt, J = 7.55 Hz – 1.90 Hz, benzothiazole CH), 7.45-7.53 (4H, m, phenyl CH, benzothiazole CH), 7.78 (1H, d, J = 8.25 Hz, benzothiazole CH), 7.99 (1H, d, J = 8.25 Hz, benzothiazole CH), 12.16 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 34.8, 101.2, 121.3, 121.7, 122.3, 123.5, 124.6, 125.1, 126.8, 129.0, 130.2, 130.5, 130.7, 131.6, 135.2, 137.4, 149.2, 152.7, 165.5, 167.1. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{17}N_4OClS_3$: 509.0326; found: 509.0314.

2-(Benzothiazol-2-ylthio)-N-(4-(4-bromophenyl)-3-phenylthiazol-2(3H)-ylidene)acetohydrazide 4o

Yield: 80%, m.p. = 249–250°C, FTIR (ATR, cm^{-1}): 3048 (N–H), 1717 (C=O), 1572-1429 (C=N, C=C), 1246 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.33 (2H, s, CH_2), 6.99 (1H, s, thiazolylidene CH), 7.32-7.35 (3H, m, phenyl CH, benzothiazole CH), 7.38 (2H, d, J = 8.55 Hz, bromophenyl CH), 7.45 (2H, d, J = 8.55 Hz, bromophenyl CH), 7.48-7.53 (4H, m, Phenyl CH, benzothiazole CH), 7.77 (1H, d, J = 7.50 Hz, benzothiazole CH), 8.00 (1H, d, J = 7.50 Hz, benzothiazole CH), 12.00 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 34.8, 101.4, 121.3, 121.7, 122.3, 123.5, 124.0, 125.1, 126.9, 127.1, 127.6, 130.2, 130.7, 130.8, 132.0, 135.3, 139.6, 152.7, 165.5, 167.1. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{17}N_4OBrS_3$: 552.9821; found: 552.9800.

2-(Benzothiazol-2-ylthio)-N-(4-(3,4-dichlorophenyl)-3-phenylthiazol-2(3H)-ylidene)acetohydrazide 4p

Yield: 79%, m.p. = 257–258°C, FTIR (ATR, cm^{-1}): 3046 (N–H), 1719 (C=O), 1568-1427 (C=N, C=C), 1244 (C–N). ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 4.30 (2H, s, CH_2), 6.94 (1H, dd, J = 7.50 Hz- 1.60 Hz, dichlorophenyl CH), 7.03 (1H, s, thiazolylidene CH), 7.32-7.35 (3H, m, phenyl CH, benzothiazole CH), 7.48-7.53 (5H, m, phenyl CH, dichlorophenyl CH, benzothiazole CH), 7.62 (1H, d, J = 1.60 Hz, dichlorophenyl CH), 7.72 (1H, d, J = 7.50 Hz, benzothiazole CH), 7.96 (1H, d, J = 7.50 Hz, benzothiazole CH), 11.96 (1H, s, NH). ^{13}C-NMR (75 MHz, DMSO-d_6): δ = 34.8, 101.8, 121.2, 121.6, 122.2, 123.3, 125.1, 126.8, 128.6, 130.0, 130.2, 130.5, 130.7, 131.0, 131.6, 131.9, 133.1, 135.2, 138.2, 152.7, 165.4, 167.3. HRMS (m/z): $[M+H]^+$ calcd for $C_{24}H_{16}N_4OCl_2S_3$: 542.9936; found: 542.9927.

Cytotoxicity test

Metabolic activity of viable cells were measured by MTT assay based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium salt to formazan product, which can be quantified spectrophotometrically to determine percent of viable cells.[42]

NIH3T3, A549, and C6 cells were used in the MTT assay. NIH3T3 cells were incubated in DMEM medium (Sigma Aldrich, St. Louis, MO, USA), added with fetal calf serum, penicillin (100 IU/mL), streptomycin (100 mg/mL), and 7.5% $NaHCO_3$ at 37°C in a humidified atmosphere of 95% air and 5% CO_2. A549 and C6 cells were incubated in RPMI (Hyclone, Thermo Scientific, USA) medium, supplemented with fetal calf serum, penicillin (100 IU/mL), streptomycin (100 mg/mL), and 7.5% $NaHCO_3$ at 37°C in a humidified atmosphere of 95% air and 5% CO_2. NIH3T3, A549, and C6 cell lines were seeded into the 96-well plates at a density of 1×10^4 cells. After 24 h of incubating period, the culture mediums were removed and test compounds were added at concentrations of 0.000316 – 1 mM. After 24 h incubation period, colorimetric measurements were performed by a microplate reader (Biotek, USA) at 540 nm. Inhibition % at all concentrations was determined using formula below and the IC_{50} values were calculated from a dose-response curve obtained by plotting the percentage inhibition versus the log concentration with the use of Microsoft Excel 2013. The results were displayed as mean ± standard deviation (SD).[43-46] Cisplatin was used as a positive control.

$$\%\ \text{inhibition} = 100 - (\text{mean sample} \times 100/\text{mean solvent})$$

DNA synthesis inhibition assay

The BrdU (5-bromo-20-deoxy-uridine) cell proliferation method was performed to analyse the effects of compounds **4a** and **4d** on proliferation of C6 cells. C6 cells were seeded into the 96-well plates at a density of 1×10^4 cells. Compounds were added into the each well at five different concentrations (IC_{50}/4, IC_{50}/2, IC_{50}, 2 x IC_{50}, and 4 x IC_{50}) and the plates were incubated for 24 h. At the end of the incubation period, BrdU solution was added and cells were reincubated for 2h at 37°C. Anti-BrdU-POD (100 mL) was added and the mixture was incubated for 90 min. Microplates were washed with phosphate buffered saline for three times. After adding substrate solution, the mixture was incubated for 15 minutes. Colorimetric measurements were performed at 492 nm. Proliferation of control cells was assessed as percentage of normal and growth inhibition % of cells, treated with **4a**, **4d** and cisplatin were calculated.[47] The results were displayed as mean ± SD.

Flow cytometric analysis

Death pathway of the carcinogenic cell lines was detected by Annexin V-FITC Apoptosis Detection Kit (BD, Pharmingen) as reported in the manufacturer's instruction. Cisplatin and compounds **4a** and **4d**, which possess the highest cytotoxic activity, were used at their IC_{50}/2, IC_{50}, and 2 x IC_{50} concentrations. After 24 h incubation period, cells were harvested by centrifugation at 1200 rpm for 5 min. at room temperature, rinsed with cold water twice, and then suspended at 1×10^6 cells/mL concentration in Annexin V-FITC binding buffer. Propidium iodide (5 mL) and annexin V-FITC (5 mL) were added for staining the cells, and the fluorescence measurements were performed using a flow cytometer. FCSExpress software was used to display the percent of normal and apoptotic cells at different stages.[47] In

the diagrams, Q1, Q2, Q3, and Q4 demonstrate the necrotic cells (positive for PI and negative for annexin/ FITC), late apoptotic or necrotic cells (positive for annexin and PI), live cells (negative for annexin and PI), and apoptotic cells (negative for PI and positive for annexin), respectively. The experiments were carried out in triplicates.

Acknowledgment

This study was financially supported by Anadolu University Scientific Projects Fund, Project No: 1601S019.

Conflicts of Interest

The authors declare no conflict of interest.

References

[1] Sharma, P.C.; Sinhmar, A.; Sharma, A.; Rajak, H.; Pthak, D.P. *J. Enzyme Inhib. Med. Chem.* **2013**, 28, 240-266.
[2] Luo, Y.; Prestwich, G.D. *Curr. Cancer Drug Targets* **2002**, 2, 209-226.
[3] Luo, J.; Solimini, N.L.; Elledge, S.J. *Cell.* **2009**, 136, 823-837.
[4] Jemal, A.; Siegel, R.; Xu, J.; Ward, E. *CA Cancer J. Clin.* **2010**, 60, 277-300.
[5] Andrade, S.F.; Oliveira, B.G.; Pereira, L.C.; Ramos, J.P.; Joaquim, A.R.; Steppe, M.; Souza-Fagundes, E.M.; Alves, R.J. *Eur. J. Med. Chem.* **2017**, 138, 13-25.
[6] Gill, R.K.; Kumar, V.; Robijns, S.C.A.; Steenackers, H.P.L.; Van der Eycken, E.V.; Bariwal, J. *Eur. J. Med. Chem.* **2017**, 138, 152-169.
[7] Paramashivappa, R.; Phanikumar, P.; Subbarao, P.; Srinivasarao, A. *Bioorg. Med. Chem. Lett.* **2003**, 13, 657.
[8] Keri, R.S.; Patil, M.R.; Patil, S.A.; Budagumpi, S. *Eur. J. Med. Chem.* **2005**, 89, 207.
[9] Kaya, B.; Sağlık, B.N.; Levent, S.; Özkay, Y.; Kaplancıklı, Z.A. *J. Enzyme Inhib. Med. Chem.* **2016**, 31, 1654-1661.
[10] Mohsen, U.A.; Kaplancıklı, Z.A.; Özkay, Y.; Yurttas, L. *Drug Res.* **2015**, 65, 176-183.
[11] Demir-Özkay, Ü.; Can, O.D.; Özkay, Y.; Özturk, Y. *Pharmacol. Rep.* **2012**, 64, 834-847.
[12] Yurttaş, L.; Özkay, Y.; Duran, M.; Turan-Zitouni, G.; Özdemir, A.; Cantürk, Z.; Küçükoğlu, K.; Kaplancıklı, Z.A. *Phosphorus Sulfur Silicon Relat. Elem.* **2016**, 191, 1166-1173.
[13] Abdelgawad, M.A.; Belal, A.; Ahmed, O.M. *J. Chem. and Pharm. Res.* **2013**, 5, 318.
[14] Turan-Zitouni, G.; Özkay, Y.; Özdemir, A.; Kaplancıklı, Z.A.; Altıntop, M.D. *Lett. Drug Des. Disc.* **2011**, 8, 830-837.
[15] Ahmed, K.; Venkata, S.Y.V.; Mohammed, N.A.K.; Sultana, F.; Methuku, K.R. *Expert Opin. Investig. Drugs* **2012**, 21, 619-635.
[16] Tuylu, B.A.; Zeytinoğlu H.S., Isikdağ I. *Biologia* **2007**, 62, 626-632.
[17] Choi, S.J.; Park, H.J.; Lee, S.K.; Kim, S.W.; Han, G.; Choo, H.Y. *Bioorg. Med. Chem.* **2006**, 14, 1229-1235.
[18] Bhuya, H.A.; Kini, S.G. *J. Mol. Graph. Model.* **2010**, 29, 32-37.
[19] Lion, C.J.; Matthews, C.S.; Wells, G.; Bradshaw, T.D.; Stevens, M.F.; Westwell, A.D. *Bioorg. Med. Chem. Lett.* **2006**, 16, 5005-5008.
[20] Hutchinson, I.; Chua, M.S.; Browne, H.L.; Trapani, V.; Bradshaw, T.D.; Westwell, A.D.; Stevens, M.F. *J. Med. Chem.* **2001**, 44, 1446-1455.
[21] Hutchinson, I.; Jennings, S.A.; Vishnuvajjala, B.R.; Westwell, A.D.; Stevens, M.F. *J. Med. Chem.* **2002**, 45, 744-747.
[22] Hutchinson, I.; Bradshaw, T.D.; Matthews, C.S.; Stevens, M.F.; Westwell, A.D. *Bioorg. Med. Chem. Lett.* **2003**, 13, 471-474.
[23] Zhang, L.; Fan, J.; Vu, K.; Hong, K.; Le Brazidec, J.Y.; Shi, J.; Biamonte, M.; Busch, D.J.; Lough, R.E.; Grecko, R.; Ran, Y.; Sensintaffar, J.L.; Kamal, A.; Lundgren, K.; Burrows, F.J.; Mnsfield, R.; Timony, G.A.; Ulm, E.H.; Kasibhatla, S.R.; Boehm, M.F. *J. Med. Chem.* **2006**, 49, 5352-5362.
[24] Singh, M.; Kumar Singh, S.; Thakur, B.; Ray, P.; Singh, S.K. *Anticancer Agents Med. Chem.* **2016**, 16, 722-739.
[25] Dumas, J.; Brittelli, D.; Chen, J.; Dixon, B.; Hatoum-Mokdad, H.; König, G.; Sibley, R.; Witowsky, J.; Wong, S. *Bioorg. Med. Chem. Lett.* **1999**, 9, 2531-2536.
[26] Singh, M.; Singh, S.K. *Anticancer Agents Med. Chem.* **2014**, 14, 127-146.
[27] Wang, Z.; Shi, X.H.; Wang, J.; Zhou, T.; Xu, Y.Z.; Huang, T.T.; Li, Y.F.; Zhao, Y.L.; Yang, L.; Yang, S.Y.; Yu, L.T.; Wei, Y.Q. *Bioorg. Med. Chem. Lett.* **2011**, 21, 1097-1101.
[28] Fréchette, S.; Leit, S.; Woo, S.H.; Lapointe, G.; Jeannotte, G.; Moradei, O.; Paquin, I.; Bouchain, G.; Raeppel, S.; Gaudette, F.; Zhou, N.; Vaisburg, A.; Fournel, M.; Yan, P.T.; Trachy-Bourget, M.C.; Kalita, A.; Robert, M.F.; Lu, A.; Rahil, J.; MacLeod, A.R.; Besterman, J.M.; Li, Z.; Delorme, D. *Bioorg. Med. Chem. Lett.* **2008**, 18, 1502-1506.
[29] Shi, X.H.; Wang, Z.; Xia, Y.; Ye, T.H.; Deng, M.; Xu, Y.Z.; Wei, Y.Q.; Yu, L.T. *Molecules* **2012**, 17, 3933-3944.
[30] Wang, Z.; Shi, X.H.; Wang, J.; Zhou, T.; Xu, Y.Z.; Huang, T.T.; Li, Y.F.; Zhao, Y.L.; Yang, L.; Yang, S.Y.; Yu, L.T.; Wei, Y.Q. *Bioorg. Med. Chem. Lett.* **2011**, 21, 1097-1101.
[31] Miller, D.D.; Dalton, J.T.; Gududuru, V.; Hurh, E.; *PCT. Int. Appl.* **2005**, WO 2005086638
[32] Ng, R.A.; Sui, Z.; *PCT. Int. Appl.* **2005**, WO 2004113309.
[33] Pine, M.J.; Mirand, E.A.; Ambrus, J.L.; Bock, F.G. *J. Med.* **1983**, 14, 433-449.
[34] Sondhi, S.M.; Rani, R.; Gupta, P.P.; Agrawal, S.K.; Saxena, A.K. *Mol. Divers.* **2009**, 13, 357-366.
[35] Altıntop, M.D.; Kaplancıklı, Z.A.; Akalın Çiftçi, G.; Demirel, R. *Eur. J. Med. Chem.* **2014**, 74, 264-277.
[36] Popiołkiewicz, J.; Polkowski, K.; Skierski, J.S.; Mazurek, A.P. *Cancer Lett.* **2005**, 229, 67-75.
[37] Varache-Lembege, M.; Moreau, S.; Larrouture, S.; Montaudon, D.; Robert, J.; Nuhrich, A. *Eur. J. Med. Chem.* **2008**, 43, 1336-1343.
[38] Yurttaş, L.; Özkay, Y.; Akalın-Çiftçi, G.; Ulusoylar-Yıldırım, Ş. *J. Enzyme Inhib. Med. Chem.* **2014**, 29, 175-184.
[39] Maghni, K.; Nicolescu, O.M.; Martin, J.G. *J. Immunol. Methods* **1999**, 223, 185-194.
[40] Fulda, S.; Debatin, K.M. *Oncogene* **2006**, 25, 4798-4811.
[41] Desai, K.G. *J. Sulfur Chem.* **2006**, 27, 315-328.
[42] Berridge, M.V.; Herst, P.M.; Tan, A.S.; *Biotechnol. Annu. Rev.* **2005**, 11, 127-152.
[43] Demir-Özkay, Ü.; Can, Ö.D.; Sağlık, B.N.; Acar Çevik, U.; Levent, S.; Özkay, Y.; Ilgın, S.; Atlı, Ö. *Bioorg. Med. Chem. Lett.* **2016**, 26, 5387-5394.
[44] Sağlık, B.N.; Ilgın, S.; Özkay, Y. *Eur. J. Med. Chem.* **2016**, 124, 1026-1040.
[45] Altıntop, M.D.; Özdemir, A.; Turan-Zitouni, G.; Ilgın, S.; Atlı, Ö.; Demirel, R.; Kaplancıklı, Z.A. *Eur. J. Med. Chem.* **2015**, 92, 342-352.
[46] Altintop, M.D.; Atli, O.; Ilgın, S.; Demirel, R.; Özdemir, A.; Kaplancikli, Z.A. *Eur. J. Med. Chem.* **2016**, 108, 406-414.
[47] Yurttaş, L.; Demirayak, S.; Ilgın, S.; Atlı, Ö. *Bioorg. Med. Chem.* **2014**, 22, 6313-6323.