Anticancer and antiangiogenesis activities of novel synthesized 2-substituted benzimidazoles molecules

Adem GÜNER1, Elifsu POLATLI2, Tamer AKKAN1, Hakan BEKTAŞ3, Canan ALBAY3

1Department of Biology, Faculty of Science and Art, Giresun University, Güre, Giresun, Turkey
2Department of Bioengineering, Faculty of Engineering, Ege University, Bornova, İzmir, Turkey
3Department of Chemistry, Faculty of Science and Art, Giresun University, Güre, Giresun, Turkey

Abstract: In this study, novel 2-substituted benzimidazoles molecules having triazole, thia diazole, and oxadia zole rings were synthesized and were evaluated by anticancer, antioxidant/oxidant status, genotoxicity, and antiangiogenesis assays. Anticancer activity of the compounds was determined by MTT (0.5, 5, and 50 µg/mL) and lactate dehydrogenase (LDH) release assays against human prostate and breast cancer cells. Oxidative status of cells was elicited by total oxidative stress and total antioxidant capacity methods. Chick chorioallantoic membrane assay was used to evaluate the antiangiogenic activity. Genotoxicity was evaluated by the sister chromatid exchange (SCE) and micronucleus (MN) tests in lymphocyte cultured human blood. Our results showed that some of the compounds synthesized had significant antiproliferative activity against both cancer cell lines (between 4.54 ± 0.35 and 20.17 ± 3.15 µg/mL), with higher inhibition of the breast cancer, and caused inhibition of LDH release with a linear correlation to MTT results. Moreover, the 5 µg/mL dose of these molecules led to an increase in antioxidant levels. Compounds had antiangiogenic effectiveness in a dose-dependent manner. Additionally, all of the compounds did not affect SCE and MN levels compared to controls. In conclusion, these newly synthesized molecules can be a resource of new anticancer agents with their nongenotoxic, antiproliferative, and antiangiogenic properties.

Key words: Antiangiogenesis, anticancer, benzimidazole, cytotoxicity, genotoxicity

1. Introduction
Cancer is a serious health problem characterized by capability of metastasis to surrounding tissue, fast growth of uncontrolled cells, and significant morbidity and mortality [1]. According to the American Cancer Society, prostate and breast cancers account for approximately 12% of all cancer deaths and 433,360 new cancer cases were estimated in 2018 [2]. Furthermore, these cancers are estimated to be responsible for approximately 4 million deaths worldwide in 2018 [3]. Despite development in diagnostic and treatment methods, these cancers for both males and females generally have a dismal prognosis. Given the difficult and probable side effects of the current treatments and high morbidity and prevalence of these disorders, the discovery of novel therapeutic strategies is an urgent need. Currently, active therapeutic compounds derived from natural and chemical products are among the most important options against cancer.

A popular approach in drug research is the design of new therapeutic subunits with improved efficiency from a bioactive molecular compound. Benzimidazole is a well-known heterocyclic organic compound consisting...
of benzene and imidazole. Benzimidazole is an isostere of nucleotides, easily reacting with biomacromolecules and able to affect their normal function [4]. Therefore, benzimidazole and its derivatives have received great attention in medicinal chemistry areas and their antioxidant, antimicrobial, anticancer, antiproliferative, and antitumor activities have been revealed in a wide spectrum [5–9]. A number of previous reports have revealed that compounds bearing a benzimidazole ring showed antiproliferative activities against different types of cancer cell lines [10]. Studies conducted with different types of cancer have shown that benzimidazole-derived agents have remarkable chemotherapeutic effectiveness through both inhibitions of poly (ADP-ribose) polymerases (PARP family) and induction of apoptosis in cancer cells [11]. Additionally, the insertion of different groups into the structure of the benzimidazole ring has led to the formation of many patented anticancer drugs that are effective on different intrinsic and extrinsic cellular pathways [12,13]. In light of this promising approach, this study was conducted to evaluate anticancer activity on prostate and breast cancer cells as well as antiangiogenic activity and genotoxic effects of novel synthesized 2-substituted benzimidazole molecules containing heterocyclic moieties such as triazole, thiadiazole, and oxadiazole rings.

2. Results and discussion
Benzimidazole derivatives attached to other heterocyclic moieties such as triazole, thiadiazole, and oxadiazole rings have attracted great interest in medicinal chemistry due to their wide range of antitumor activities [14]. In this study, the anticancer potentials of novel synthesized 2-substituted benzimidazole compounds including triazole, thiadiazole, and oxadiazole skeletons such as compounds 1, 2, 3A, 3B, 4, 5, 6, 7, 8, 9A, and 9B were evaluated on human prostate (PC-3) and breast (SK-BR-3) cancer cells causing the highest male and female mortality, respectively.

Our results showed that although compounds 1, 4, 5, and 7 had no effect against cancer cells, the other tested compounds exhibited marked antiproliferative activity against both tumor cell lines, with an efficient half maximal inhibitory concentration (IC₅₀), increasing dose-dependently (Table 1; Figure 1). Molecules 3A and 3B bearing oxadiazole rings had different anticancer activity, which tended to be more effective against the SK-BR-3 cell line (IC₅₀ 7.68 ± 2.84 and 5.67 ± 1.67 μg/mL, respectively) than against the PC-3 cell line (IC₅₀ 17.11 ± 4.79 and 21.15 ± 3.06 μg/mL, respectively). In addition, compound 2 having oxadiazole moiety as well as a morpholine nucleus at the N-3 position of the oxadiazole ring displayed significant cytotoxicity against only breast cancer cells with a low value of IC₅₀ of 14.93 ± 1.84 μg/mL. This value provided that, given that this molecule showed very low cytotoxicity (>50 IC₅₀) against noncancerous HEK-293 cells, compound 2 bearing morpholine may have a selective effect against breast cancer. Lipophilicity has often been correlated to biological activity. Compound 6 bearing a thiadiazole ring with alkyl and the propyl chain was far more effective against both SK-BR-3 (4.54 ± 0.35 μg/mL) and PC-3 (13.53 ± 5.82 μg/mL) cell lines. Although compound 5 was synthesized in the same step with compound 6, the absence of its activity can be explained by the presence of the propyl group in compound 6. These have added lipophilicity with the propyl chain on the thiadiazole nucleus when compared to 5, which suggests a cause for the higher activity. Compound 8 bearing a triazole ring showed a cytotoxic effect with IC₅₀ values of 20.17 ± 3.15 μg/mL against SK-BR-3, while there was no cytotoxic effect observed against PC-3 cells. Given the efficacy of compound 8 in breast cancer, the absence of an effect of compound 7 carrying similar molecules as compound 8 was associated with the presence of triazole. Molecules 9A and 9B bearing triazole rings compounds were found to possess different anticancer activities depending on the cell line used; generally, all the tested samples tended to be more effective against the SK-BR-3 cell line (12.99 ± 1.74 and 9.86 ± 1.74 μg/mL, respectively) than against the PC-3 cell line (17.93
Abdel-Fattah et al. revealed that 1,2,4-triazole derivatives attached to benzimidazole significantly increased the anticancer activity of molecules against breast cancer cells [15]. Furthermore, we found that long-chain alkyl-substituted target compounds like compounds 3A, 3B, 9A, and 9B showed greater antiproliferative activity than the corresponding 3-thiol compounds. These differences have been associated with both the different sensitivities of the cells tested and the interactions with different receptors in the cell membrane of the synthesis structures. In addition to this overall information, effective anticancer compounds showed less toxic effects in noncancerous HEK-293 cells than the standard anticancer drug doxorubicin, and this is a promising point for future cancer drug development.

Table 1. Correlations (R²) and IC₅₀ values for breast cancer (SK-BR-3), prostate (PC-3), and noncancerous human embryonic kidney (HEK-293) cells after exposure to synthesized compounds and doxorubicin that used as a positive control.

<table>
<thead>
<tr>
<th>Sample</th>
<th>HEK-293</th>
<th>SK-BR-3</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>IC50 (µg/mL)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Compound 2</td>
<td>IC50 (µg/mL)</td>
<td>&gt; 50</td>
<td>14.93 ± 1.84</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9249</td>
<td>0.7994</td>
</tr>
<tr>
<td>Compound 3A</td>
<td>IC50 (µg/mL)</td>
<td>3.42 ± 0.62</td>
<td>7.68 ± 2.84</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9731</td>
<td>0.953</td>
</tr>
<tr>
<td>Compound 3B</td>
<td>IC50 (µg/mL)</td>
<td>8.35 ± 0.39</td>
<td>5.67 ± 1.67</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9018</td>
<td>0.9162</td>
</tr>
<tr>
<td>Compound 4</td>
<td>IC50 (µg/mL)</td>
<td>&gt; 50</td>
<td>-</td>
</tr>
<tr>
<td>Compound 5</td>
<td>IC50 (µg/mL)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Compound 6</td>
<td>IC50 (µg/mL)</td>
<td>14.72 ± 2.62</td>
<td>4.54 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9949</td>
<td>0.8551</td>
</tr>
<tr>
<td>Compound 7</td>
<td>IC50 (µg/mL)</td>
<td>&gt; 50</td>
<td>-</td>
</tr>
<tr>
<td>Compound 8</td>
<td>IC50 (µg/mL)</td>
<td>30.08 ± 1.58</td>
<td>20.17 ± 3.15</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9982</td>
<td>0.9176</td>
</tr>
<tr>
<td>Compound 9A</td>
<td>IC50 (µg/mL)</td>
<td>12.52 ± 2.52</td>
<td>12.99 ± 1.74</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9912</td>
<td>0.9412</td>
</tr>
<tr>
<td>Compound 9B</td>
<td>IC50 (µg/mL)</td>
<td>23.94 ± 2.06</td>
<td>9.86 ± 1.74</td>
</tr>
<tr>
<td></td>
<td>Correlation (R²)</td>
<td>0.9313</td>
<td>0.9149</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>IC50 (µg/mL)</td>
<td>1.09 ± 0.26</td>
<td>0.92 ± 0.04</td>
</tr>
</tbody>
</table>

The values are means ± standard errors of experiments carried out in triplicate.

Lactate dehydrogenase (LDH) is a sign of cell membrane impairment and has recently been introduced as the most popular marker for determining the cytotoxic/nontoxic levels of novel therapeutic samples [16,17]. In other words, an increase in LDH levels is an important indicator of cytotoxicity. In this regard, the potential cytotoxic effect of the synthesized compounds was also evaluated by LDH release assay in concentrations of 0.5, 5, and 50 µg/mL to support the anticancer MTT results. As seen in Figure 2, compounds 3A, 3B, 6, 8, 9A, and 9B caused a significant increase in LDH levels at 50 µg/mL concentrations on noncancerous HEK-293 cells compared to the untreated control, while compound 2 did not lead to any alterations in LDH levels. When LDH level change was examined after the treatment of synthesized molecules on cancer cells, all the synthesized compounds significantly (P < 0.05) inhibited LDH leakage at rates between 29.4% and 62.2% for SK-BR-3.
cells as compared to those of the Triton-X-treated group (98.6%). However, LDH level was inhibited at rates between 40.2% and 63.4% in PC-3 cells after 48 h, except for treatment with compounds 2 and 8. At the same time, these results revealed a significant linear correlation between MTT and LDH assays (Table 1).

The cellular antioxidant system is controlled by a balance between production and destruction of reactive oxygen species [18], but various endogenous and exogenous-origin agents cause an imbalance in oxidant and antioxidant levels. This state is called oxidative stress and may result in damage to many cellular components [19]. Many current methods and therapeutics are based on normalization and determination of oxidative stress and antioxidant levels in cells [20]. In this study, oxidant and antioxidant statuses after treatment with different concentrations of novel synthesized 2-substituted benzimidazole compounds on PC-3, SK-BR-3, and noncancerous HEK-293 cells were determined by using automated colorimetric measurement techniques. As seen in Figure 3, 5 and 50 $\mu$g/mL concentrations of compounds 2 and 8 caused statistically significant increases in total antioxidant capacity (TAC) levels in SK-BR-3 cells in a dose-dependent manner compared with the untreated control, while only the 5 $\mu$g/mL concentration of compounds 3A, 3B, 6, 9A, and 9B caused a statistically significant increase (P < 0.05) in TAC levels. When the TAC levels were examined in PC-3 cells (Figure 3), there was no statistically significant change in TAC levels after treatment with compounds 2 and 8 compared to the untreated control group. Compounds 3A, 3B, 9A, and 9B caused a statistically significant increase (P < 0.05) at 5 $\mu$g/mL concentration while compound 6 led to a statistically significant elevation (P < 0.05) in
TAC level at 5 and 50 μg/mL concentrations compared to the untreated control group. Briefly, the addition of compounds to the cell medium led to an increase in the antioxidant level. In addition, the morpholine structure against breast cancer cells and the propyl chain, alkyl chain, and lipophilicity structures against prostate cancer cells enhanced the activity of compounds. However, in noncancerous HEK-293 cells, while TAC levels after treatment with compounds 2 and 8 were statistically significantly increased (P < 0.05) at 5 and 50 μg/mL concentrations, while compounds 3A, 3B, 6, 9A, and 9B caused a statistically significant increase (P < 0.05) only at 5 μg/mL concentration. Taken together, this revealed that the synthesized compounds having oxadiazole, triazole, and thiadiazole rings caused an increase in the antioxidant levels in cancer and noncancerous cells, generally at 5 μg/mL concentration. In support of our results, in previous studies, benzimidazole molecules containing oxadiazole, triazole, thiadiazole, and morpholine rings have been reported to have antioxidant activity [21].

When total oxidant status (TOS) activities of cancer cells were investigated after exposure to the compounds (Figure 4), the concentration of 50 μg/mL of synthesized molecules led to statistically important (P < 0.05) increases in TOS levels in both cancer cell lines in comparison with control values, except for...
Figure 3. TAC levels in cells after synthesized compounds were treated at different concentrations. A) SK-BR-3 cell line, B) PC-3 cell line, C) HEK-293 cell line. Each value is expressed as mean ± SD (n = 3). Values followed by different small symbols differ significantly at P < 0.05. a Mean difference is significant (P < 0.05) when compared with the control group. b Mean difference is significant (P < 0.05) when compared with ascorbic acid-treated group.

compounds 2 and 8 in PC-3 cells. This phenomenon supports that the compounds cause inhibition of cancer cells through oxidative stress. The concentration of 50 μg/mL of compounds 3A, 3B, 6, 8, 9A, and 9B caused a statistically significant increase (P < 0.05) in the levels of oxidative stress while concentrations of 0.5 and 5 μg/mL of all compounds yielded no different TOS value compared to the untreated control in HEK-293 cells. The results suggested that a dose of 50 μg/mL of synthesized compounds had a toxic effect on noncancerous and cancer cells. These toxic effects at high concentrations can occur by the triggering of oxidative stress via different cellular mechanisms, but high TAC activities and no induction of oxidative stress at the 5 μg/mL concentration of compounds indicated that the compounds can be developed to target only the cancer cells.

Angiogenesis is the formation of new blood vessels from existing vessels in wound healing, tissue growth, and regeneration processes [22]. This formation is often an indispensable source of healthy tissue, but it also has life-threatening risks in cancer cases having fast metabolic processes. In recent years, strategies that prevent the feeding or metastasis of tumor cells have become more popular than directly intervening with the tumor in the cancerous tissue [23,24]. The chick chorioallantoic membrane (CAM) assay is a method that quantitatively and visually confirms the anti- or proangiogenic effectiveness of the tested sample [25]. In the present study, antiangiogenic effects of novel synthesized 2-substituted benzimidazoles bearing triazole, thiazole, and oxadiazole rings were first demonstrated by a CAM assay. Some criteria of the CAM reaction against the tested compounds and the obtained antiangiogenic scores are displayed in Figure 5. Suramin (50 μg/pellet), used as the positive control, indicated moderate activity with a value of 0.95 while there were no antiangiogenic effects with DMSO or in the untreated control group. Each concentration (0.5, 5, and 50
M) of synthesized compounds was separately evaluated for antiangiogenic activity/no activity. The average antiangiogenic score after treatment with the synthesized compounds showed differences in a dose-dependent manner. All tested compounds had no antiangiogenic effect at 0.5 μM concentration. However, compound 2 bearing an oxadiazole ring and 6 bearing a thiadiazole ring had moderate dose-dependent activity with values of 0.78 and 0.85, respectively, while other tested molecules (3A, 3B, 8, 9A, and 9B) had a weak antiangiogenic activity with scores of 0.68, 0.71, 0.74, 0.73, and 0.71, respectively, at 50 μM concentrations. It is noteworthy that the structure consisting of oxadiazole and morpholine as well as the structure consisting of the propyl chain, alkyl chain, and lipophilicity contributed to the increase of antiangiogenic activity. As previously stated, tumor progression and cancer cell survival have been attenuated by blockage of the capillary system or growth factors in cancerous tissue [26]. Many studies revealed that benzimidazole derivatives can be used as an important pharmacophore against cancer through antitumor, anticancer, and antiangiogenic activities [27,28]. More specifically, in vivo and in vitro studies revealed that benzimidazole-substituted compounds contribute to anticancer activities by disrupting endothelial cells or reducing vascular endothelial growth factor secretion in cancer cells [29,30]. In view of this evidence, this animal study evaluating antiangiogenic effects declared that the antiangiogenesis strategies of benzimidazole compounds bearing triazole, thiadiazole, and oxadiazole rings were thought to be closely related to the destruction of the cancer tissue by possibly inhibiting metastasis and cell growth following capillary impairment.

Genotoxicity tests play an important role in the safety assessment of a variety of substances, especially in preclinical evaluations. Many well-established in vitro assays are used successfully to predict genotoxicity. The SCE and MN tests are among the important in vitro tests that reveal chromosome aberrations, breakage, and loss structurally and numerically in aneugenic and clastogenic conditions. The benzimidazole ring is an
Figure 5. Average antiangiogenic score and some scoring criteria for determining antiangiogenic effects on CAM surface after exposure of synthesized compounds and suramin. A, B, and C) Untreated samples. D) Strong effect after treatment. E) Moderate effect after treatment. F) Weak effect after treatment. Each value is expressed as mean ± SD (n = 3).

An important resource of many therapeutic agents such as anthelmintic, antiulcer, antipsychotic, antiprotozoal, and antifungal agents [31]. However, risk assessment of substances having this ring system is an important step for future drug formations. In the present study, possible genotoxic effects of newly synthesized compounds
on human lymphocytes were tested for the first time by SCE and MN assays. The results of the present study did not show any significant increases in the frequencies of SCEs and MN in lymphocytes after exposure to all synthesized compounds as compared to the control values in all three dose treatments (Table 2). The toxicological screening of novel benzimidazole-morpholine derivatives performed by Ames test showed that the synthesized compounds had no genotoxic effects, in accordance with our findings [32]. In contrast to our results, the previous studies conducted by in vitro cytochalasin-B micronucleus test on human lymphocytes revealed that thiabendazole, carbendazim, and mebendazole containing benzimidazole rings caused an increase in MN frequency in a dose-dependent manner [33]. Barale et al. revealed the clastogenic and aneugenic effects of three benzimidazoles, i.e., benomyl, methyl thiophanate, and methyl 2-benzimidazole carbamate [34]. These discrepancies in terms of the toxicological experiments can be explained by differences in both the ring structure of the synthesized compound and the test method used.

In conclusion, our novel synthesized compounds from 2-substituted benzimidazole compounds bearing triazole, thiadiazole, and oxadiazole rings showed potent anticancer activity by highly inhibiting the proliferation of breast and prostate cancer cells and had dose-dependent antiangiogenic activity. However, doses of 5 μg/mL of compounds led to an increase in the antioxidant level of cell lines. In particular, the selective toxic effect of compound 2, the high antiproliferative effects on both cancer cell lines of compound 6, and the significant antiangiogenic effectiveness of these molecules may provide pioneering information on the synthesis of new anticancer agents. In other words, oxadiazole structures bearing morpholine against breast cancer and lipophilicity structures consisting of thiaadiazole, propyl, and alkyl chain against prostate cancer can be accepted as a critical strategy in the development of novel anticancer agents. Taken together, considering they did not cause any genotoxic effect, the present results suggest that our newly synthesized molecules, especially compounds 2 and 6, can be developed as effective agents against cancer via their antioxidative and antiangiogenic mechanisms.

3. Experimental

3.1. Chemistry

$^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance II 400 MHz NMR spectrometer (400.13 MHz for $^1$H and 100.13 MHz for $^{13}$C) in DMSO-$d_6$, using TMS as an internal standard. Mass spectra were recorded on a Thermo Scientific TSQ Quantum Access MAX LC-MS instrument (ESI). The elemental analysis was carried out on a Costech Elemental Combustion System CHNS-O elemental analyzer. Melting points were determined in capillary tubes on an Electrothermal 9100 and Automated Melting Point System melting point apparatus (Digital Image Processing Technology) and were not corrected. All chemicals were purchased from Merck or Sigma-Aldrich and used without further purification.

3.2. General procedure for the synthesis of compounds

As seen in Figure 6, the synthesis of 2-substituted benzimidazole molecules was performed as follows.

3.2.1. Synthesis of compound A

Compound A was synthesized from appropriate benzimidazole derivative by the reported method in the literature [35,36].
Table 2. The evaluation of SCEs and MN frequencies and in human lymphocytes after exposure to different concentrations of benzimidazole-derived compounds for 72 h.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Concentration(µg/mL)</th>
<th>SCEs/cell ± SD</th>
<th>MN/1000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>-</td>
<td>5.42 ± 0.87</td>
<td>4.02 ± 0.72</td>
</tr>
<tr>
<td>Positive control</td>
<td>-</td>
<td>10.02 ± 1.12 a</td>
<td>9.53 ± 0.93 a</td>
</tr>
<tr>
<td>Compound 1</td>
<td>0.5</td>
<td>5.74 ± 0.91</td>
<td>3.58 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.82 ± 0.88</td>
<td>3.67 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.83 ± 0.75</td>
<td>3.98 ± 0.82</td>
</tr>
<tr>
<td>Compound 2</td>
<td>0.5</td>
<td>5.46 ± 0.82</td>
<td>5.12 ± 0.78</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.54 ± 0.89</td>
<td>4.92 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.49 ± 0.79</td>
<td>4.94 ± 0.91</td>
</tr>
<tr>
<td>Compound 3A</td>
<td>0.5</td>
<td>5.98 ± 0.73</td>
<td>5.21 ± 0.93</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.03 ± 1.2</td>
<td>5.11 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.01 ± 0.96</td>
<td>5.14 ± 0.74</td>
</tr>
<tr>
<td>Compound 3B</td>
<td>0.5</td>
<td>5.81 ± 0.88</td>
<td>3.98 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.82 ± 0.81</td>
<td>4.12 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.92 ± 0.96</td>
<td>4.15 ± 0.92</td>
</tr>
<tr>
<td>Compound 4</td>
<td>0.5</td>
<td>5.61 ± 0.97</td>
<td>4.58 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.65 ± 0.73</td>
<td>4.64 ± 0.96</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.68 ± 0.78</td>
<td>4.72 ± 0.87</td>
</tr>
<tr>
<td>Compound 5</td>
<td>0.5</td>
<td>5.54 ± 0.86</td>
<td>5.21 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.56 ± 0.85</td>
<td>5.18 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.61 ± 0.88</td>
<td>5.22 ± 0.63</td>
</tr>
<tr>
<td>Compound 6</td>
<td>0.5</td>
<td>5.59 ± 0.77</td>
<td>4.58 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.58 ± 0.95</td>
<td>4.67 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.62 ± 0.93</td>
<td>4.75 ± 0.79</td>
</tr>
<tr>
<td>Compound 7</td>
<td>0.5</td>
<td>5.52 ± 0.91</td>
<td>3.75 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.54 ± 0.87</td>
<td>3.85 ± 0.61</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.54 ± 0.74</td>
<td>3.97 ± 0.64</td>
</tr>
<tr>
<td>Compound 8</td>
<td>0.5</td>
<td>5.77 ± 0.94</td>
<td>4.14 ± 0.78</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.81 ± 1.02</td>
<td>4.21 ± 0.78</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.83 ± 0.95</td>
<td>4.36 ± 0.82</td>
</tr>
<tr>
<td>Compound 9A</td>
<td>0.5</td>
<td>6.01 ± 0.94</td>
<td>5.11 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.03 ± 0.83</td>
<td>5.14 ± 0.86</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>6.03 ± 0.87</td>
<td>5.16 ± 0.84</td>
</tr>
<tr>
<td>Compound 9B</td>
<td>0.5</td>
<td>5.38 ± 0.86</td>
<td>4.98 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.43 ± 0.79</td>
<td>5.01 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5.47 ± 0.78</td>
<td>5.14 ± 0.83</td>
</tr>
</tbody>
</table>

Values are means ± SD (n = 6). "Significantly different from the control at P < 0.05. Each nontreated whole blood culture was studied as a negative control group. Treatment with mitomycin C alone (10⁻⁷ M) was accepted as a positive control.
3.2.1. 2-(2-Benzyl-1H-benzimidazol-1-yl)acetohydrazide (A) Yield 82%, mp: 186–187 °C, \(^1\)H NMR spectrum (400 MHz, DMSO-\(d_6\)), \(\delta\), ppm \((J, \text{Hz})\): 9.58 (1H, s, NH, D\(_2\)O exc.), 7.59 (1H, d, \(J=7\) Hz, ArH), 7.45 (1H, d, \(J=6.8\) Hz, ArH), 7.32–7.30 (4H, m, ArH), 7.25–7.19 (3H, m, ArH), 4.82 (2H, s, NCH\(_2\)), 4.38 (2H, bs, NH\(_2\), D\(_2\)O exc.), 4.27 (2H, s, CH\(_2\)). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)), \(\delta\), ppm: 33.50 (CH\(_2\)), 45.95 (NCH\(_2\)), ArC [110.40, 110.48, 119.02, 121.95, 122.26, 126.99, 128.89, 129.40 (2C), 136.08, 137.29, 142.72, 154.59 (C=N), 166.42 (C=O). LC-MS, \(m/z\): 281.35 [M + H]+. Anal. calcd. (%) for C\(_{16}\)H\(_{16}\)N\(_4\)O: C, 68.55; H, 5.75; N, 19.99. Found: C, 68.59; H, 5.68; N, 19.92.

3.2.2. Synthesis of compound 1
To a solution of compound A (0.010 mol) in ethanol (20 mL), CS\(_2\) (0.010 mol) and a solution of KOH (0.010 mol) in 50 mL of water were added, and then the reaction mixture was refluxed for 3 h. The reaction mixture was cooled at room temperature and was acidified with cold dilute HCl (1 : 1). Then the solid formed was filtered off, washed with sufficient water, and dried. The product was recrystallized from a solution of ethanol and water (1 : 1).

3.2.2.1. 5-[(2-Benzyl-1H-benzimidazol-1-yl)methyl]-1,3,4-oxadiazole-2-thiol (1) Yield 78%, mp: 208–209 °C, \(^1\)H NMR spectrum (400 MHz, DMSO-\(d_6\)), \(\delta\), ppm \((J, \text{Hz})\): 13.12 (1H, bs, SH), 7.64–7.57 (2H, m, ArH), 7.46–7.44 (1H, d, Ar-H), 7.32–7.17 (6H, m, ArH), 5.70, 4.84 (2H, s, NCH\(_2\)), 4.36, 4.28 (2H, s, CH\(_2\)). \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)), \(\delta\), ppm: 33.12 (CH\(_2\)), 44.96 (NCH\(_2\)), ArC [110.58, 119.22, 122.53, 122.88, 127.09, 128.84, 128.91, 128.22, 129.41, 136.72, 142.80, 154.12], 159.43 (benzimidazole, C=N), 166.31 (oxadiazole, C-2), 178.36 (oxadiazole, C-5). LC-MS, \(m/z\): 281.38 [M + H]+. Anal. calcd. (%) for C\(_{17}\)H\(_{14}\)N\(_4\)OS: C, 63.34; H, 4.38; N, 17.38; S, 9.94. Found: C, 63.38; H, 4.42; N, 17.43; S, 9.96.
3.2.3. Synthesis of compound 2

To the solution of corresponding compound 1 (10 mmol) in dichloromethane, formaldehyde (37%, 1.55 mmol) and morpholine (10 mmol) were added and the mixture was stirred at room temperature for 3 h. After removing the solvent under reduced pressure, a solid was obtained. This crude product was treated with water, filtered off, and recrystallized from ethyl acetate/petroleum ether (1 : 2) to yield the desired compound.

3.2.3.1. 5-[(2-Benzyl-1H-benzimidazol-1-yl)methyl]-3-(morpholin-4-ylmethyl)-1,3,4-oxadiazole-2(3H)-thione (2) Yield 68%, mp: 153–154 °C, 1H NMR spectrum (400 MHz, DMSO-d6), δ, ppm (J, Hz): 7.71–7.60 (2H, m, ArH), 7.44–7.18 (7H, m, ArH), 5.75 (2H, s, NCH2), 4.75 (2H, s, CH2), 4.22 (2H, m, CH2), 2.53 (8H, m, 4CH2). 13C NMR (100 MHz, DMSO-d6), δ, ppm: 33.18 (CH2), 38.71 (CH2), 50.21 (NCH2), 66.42 (2CH2), 70.16 (2CH2), ArC [110.70, 119.29, 122.54, 122.87, 127.09, 129.23 (2C), 129.29, 135.76, 136.77, 142.62, 154.11], 159.38 (benzimidazole, C=N), 162.48 (oxadiazole, C-2), 178.40 (oxadiazole, C-5). LC-MS, m/z: 422.56 [M+H]+. Anal. calcd. (%) for C22H23N5O2S: C, 62.69; H, 5.50; N, 16.61; S, 7.61. Found: C, 62.72; H, 5.48; N, 16.65; S, 7.65.

3.2.4. Synthesis of compounds 3A and 3B

K2CO3 (0.020 mol) was added to an acetone (15 mL) solution of compound 1 (0.010 mol), and the reaction mixture was stirred at room temperature for 30 min. After that, to synthesize compounds 3A and 3B, 1-bromopropane (0.012 mol) and 1-bromohexane (0.012 mol) were added to the mixture, respectively. The reaction mixture was further stirred for 10 h at room temperature. The reaction was monitored by TLC (ethyl acetate). The K2CO3 was filtered off and separated from the reaction mixture. The solvent (acetone) was evaporated under reduced pressure. After that, a white solid was appeared. The final solid product was recrystallized with acetone.

3.2.4.1. 2-Benzyl-1-[(5-propylthio)-1,3,4-oxadiazol-2-yl]methyl]-1H-benzimidazole (3A) Yield 64%, mp: 139–140 °C, 1H NMR spectrum (400 MHz, DMSO-d6), δ, ppm (J, Hz): 7.62 (1H, d, ArH), 7.55 (1H, d, ArH), 7.28–7.18 (7H, m, ArH), 5.83 (2H, s, NCH2), 4.36 (2H, s, CH2), 3.08 (2H, t, CH2, J = 7.2), 1.63 (2H, m, CH2, J = 7.2), 0.89 (3H, t, CH3, J = 7.2). 13C NMR (100 MHz, DMSO-d6), δ, ppm: 13.15 (CH3), 22.71 (CH2), 33.17 (CH2), 34.27 (CH2), 38.43 (NCH2), ArC [110.62, 119.27, 122.46, 122.79, 127.04, 128.81 (2C), 129.18 (2C), 135.62, 136.77, 142.70], 154.00 (benzimidazole, C=N), 163.34 (oxadiazole, C-2), 164.79 (oxadiazole, C-5). LC-MS, m/z: 365.48 [M+H]+. Anal. calcd. (%) for C22H23N5O2S: C, 65.91; H, 5.53; N, 15.37; S, 8.80. Found: C, 65.94; H, 5.58; N, 15.32; S, 8.85.

3.2.4.2. 2-Benzyl-1-[(5-hexylthio)-1,3,4-oxadiazol-2-yl]methyl]-1H-benzimidazole (3B) Yield 68%, mp: 104–105 °C, 1H NMR spectrum (400 MHz, DMSO-d6), δ, ppm (J, Hz): 7.63 (1H, d, ArH), 7.55 (1H, d, ArH), 7.25–7.19 (7H, m, ArH), 5.83 (2H, s, NCH2), 4.36 (2H, s, CH2), 3.08 (2H, t, CH2, J = 7.2), 1.59 (2H, m, CH2, J = 7.2), 1.29–1.17 (6H, m, 3CH2), 0.83 (3H, t, CH3, J = 7.2). 13C NMR (100 MHz, DMSO-d6), δ, ppm: 14.28 (CH3), 22.36, 27.89, 29.29, 31.00, 32.39 (5CH2), 33.17 (CH2), 38.42 (NCH2), ArC [110.60, 119.27, 122.45, 122.78, 127.03, 128.80 (2C), 129.17 (2C), 135.61, 136.77, 142.70], 153.97 (benzimidazole,
3.2.5. Synthesis of compound 4

To a solution of compound A (0.010 mol) in ethanol (25 mL) 4-chlorophenylisothiocyanate (0.010 mol) was added. Then the reaction mixture was refluxed for 3 h. The reaction was monitored by TLC (ethyl acetate : ethanol, 2 : 1). The resultant reaction mixture was cooled at room temperature, dried, and then recrystallized from a solution of ethanol and water (1 : 2).

3.2.5.1. 2-[(2-Benzyl-1H-benzimidazol-1-yl)acetyl]-N-(4-chlorophenyl)hydrazinecarbothioamide (4)

Yield 82%, mp: 211–212 °C, 1H NMR spectrum (400 MHz, DMSO-d$_6$), δ, ppm (J, Hz): 10.56 (1H, bs, NH, D$_2$O exc.), 9.86 (2H, bs, 2NH, D$_2$O exc.), 7.59 (1H, s, ArH), 7.49–7.43 (7H, m, ArH), 7.31–7.21 (5H, m, ArH), 4.99 (2H, s, NCH$_2$), 4.28 (2H, s, CH$_2$). 13C NMR (100 MHz, DMSO-d$_6$), δ, ppm: 33.38 (CH$_2$), 44.95 (NCH$_2$), ArC [110.59, 119.04, 122.03, 127.03, 128.65, 129.93, 129.37, 136.11, 137.23, 138.47, 142.70], 154.56 (C=N), 168.03 (C=O), 177.46 (C=S). LC-MS, m/z: 450.95 [M + H]$^+$

Anal. calcd. (%) for C$_{23}$H$_{20}$ClN$_5$S: C, 61.40; H, 4.48; N, 16.21; S, 7.42. Found: C, 61.45; H, 4.52; N, 16.26; S, 7.48.

3.2.6. Synthesis of compound 5

A solution of corresponding carbothioamide derivative (4) (0.010 mol) in concentrated 10 mL of sulfuric acid was stirred for 10 min. Then the mixture was allowed to cool to room temperature. After stirring for an additional 30 min. the resulting solution was added as controlled into ice-cold water and raised to pH 8 with ammonia. Afterwards, the precipitated solid was filtered off. Final product was washed with water and recrystallized with EtOH.

3.2.6.1. 5-[(2-Benzyl-1H-benzimidazol-1-yl)methyl]-N-(4-chlorophenyl)-1,3,4-thiadiazol-2-amine (5)

Yield 82%, mp: 225–226 °C, 1H NMR spectrum (400 MHz, DMSO-d$_6$), δ, ppm (J, Hz): 10.12 (1H, s, NH), 7.63–7.55 (4H, m, ArH), 7.37–7.35 (5H, m, ArH), 7.33–7.18 (4H, m, ArH), 5.80 (2H, s, NCH$_2$), 4.39 (2H, s, CH$_2$). 13C NMR (100 MHz, DMSO-d$_6$), δ, ppm: 33.37 (CH$_2$), 42.32 (NCH$_2$), ArC [110.75, 119.26 (2C), 119.44, 122.41 (2C), 122.73, 125.95 (2C), 127.15 (2C), 129.00, 129.36, 135.35 (2C), 137.01 (2C), 142.84], 153.92 (benzimidazole, C=N), 155.70 (thiadiazole, C-2), 165.34 (thiadiazole, C-5). LC-MS, m/z: 433.96 [M + H]$^+$

Anal. calcd. (%) for C$_{23}$H$_{21}$ClN$_5$S: C, 63.96; H, 4.20; N, 16.21; S, 7.42. Found: C, 63.92; H, 4.26; N, 16.26; S, 7.48.

3.2.7. Synthesis of compound 6

K$_2$CO$_3$ (0.020 mol) was added to an acetone solution (15 mL) of compound 5 (0.010 mol), and the reaction mixture was stirred at room temperature for 30 min. After that, 1-bromopropane (0.012 mol) was added to the mixture and stirred for 10 h at room temperature. The reaction was monitored by TLC (ethyl acetate). The K$_2$CO$_3$ was filtered off and separated from the reaction mixture. The solvent (acetone) was evaporated under reduced pressure. After that, a white solid appeared. The final solid product was recrystallized with acetone.
3.2.7.1. 5-[(2-Benzyl-1H-benzimidazol-1-yl)methyl]-N-(4-chlorophenyl)-N-propyl-1,3,4-thiadiazol-2-amine (6) Yield 68%, mp: 194–195 °C, 1H NMR spectrum (400 MHz, DMSO-d6), δ, ppm (J, Hz): 7.63–7.53 (3H, ArH), 7.25–7.19 (10H, ArH), 5.82 (2H, s, NCH2), 4.36 (2H, s, CH2), 3.08 (2H, t, CH2), 1.64 (2H, m, CH2), 0.90 (3H, t, CH3). 13C NMR (100 MHz, DMSO-d6), δ, ppm: 13.15 (CH3), 22.72 (CH2), 33.16 (CH2), 34.28 (CH2), 38.43 (NCH2), ArC [110.62, 119.27 (2C), 122.46 (2C), 122.80 (2C), 127.04 (2C), 128.81 (2C), 129.17 (2C), 135.62 (2C), 136.77 (2C), 142.70], 153.99 (benzimidazole, C=N), 163.34 (thiadiazole, C-2), 164.79 (thiadiazole, C-5). LC-MS, m/z: 475.03 [M + H]+. Anal. calcd. (%) for C26H24ClN5S: C, 65.88; H, 5.10; N, 14.77; S, 6.76. Found: C, 65.92; H, 5.15; N, 14.72; S, 6.70.

3.2.8. Synthesis of compound 7

An ethanol solution (15 mL) of compound 4 (0.010 mol) was refluxed in the presence of 15 mL of 2 N NaOH for 4 h. After that, the resultant reaction mixture was cooled at room temperature and pH was adjusted to 4–5 with 37% HCl. The separated product was filtered, washed with sufficient water, and recrystallized from a solution of ethanol and water (1 : 2).

3.2.8.1. 5-[(2-Benzyl-1H-benzimidazol-1-yl)methyl]-4-(4-chlorophenyl)-4H-1,2,4-triazole-3-thiol (7) Yield 78%, mp: 210–211 °C, 1H NMR spectrum (400 MHz, DMSO-d6), δ, ppm (J, Hz): 13.90 (1H, s, NH), 7.61–7.56 (4H, m, ArH), 7.41–7.32 (3H, m, ArH), 7.27–7.15 (6H, m, ArH), 5.36, 5.04 (2H, s, NCH2), 4.10 (2H, s, CH2). 13C NMR (100 MHz, DMSO-d6), δ, ppm: 33.10 (CH2), 45.12 (NCH2), ArC [110.76, 118.97, 122.23, 122.50, 127.05, 128.84 (2C), 128.98, 129.18, 129.40 (2C), 130.03, 130.39, 132.18 (2C), 135.03, 136.62, 142.28], 147.89 (triazole, C-2), 153.88 (benzimidazole, C=N), 169.03 (triazole, C-5). LC-MS, m/z: 422.52 [M + H]+. Anal. calcd. (%) for C23H18ClN5S: C, 63.96; H, 4.20; N, 16.21; S, 7.42. Found: C, 63.92; H, 4.26; N, 16.26; S, 7.46.

3.2.9. Synthesis of compound 8

To a solution of corresponding compound 7 (10 mmol) in dichloromethane, formaldehyde (37%, 1.55 mmol) and morpholine (10 mmol) were added and the mixture was stirred at room temperature for 3 h. After removing the solvent under reduced pressure, a solid was obtained. This crude product was treated with water, filtered off, and recrystallized from ethyl acetate and petroleum ether (1 : 2) to yield the desired compound.

3.2.9.1. 5-[(2-Benzyl-1H-benzimidazol-1-yl)methyl]-4-(4-chlorophenyl)-2-(morpholin-4-ylmethyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione (8) Yield 68%, mp: 95–96 °C, 1H NMR spectrum (400 MHz, DMSO-d6), δ, ppm (J, Hz): 7.56–7.44 (5H, m, ArH), 7.27–6.59 (8H, m, ArH), 5.43 (2H, s, NCH2), 4.93 (2H, s, CH2), 4.08 (2H, s, CH2), 2.61 (4H, m, 2CH2), 2.51 (m, 4H, 2CH2). 13C NMR (100 MHz, DMSO-d6), δ, ppm: 33.16 (2 CH2), 50.56 (2 CH2), 66.51 (CH2), 69.28 (NCH2), 71.16 (CH2), ArC [110.70 (2C), 118.97, 122.23, 122.50, 127.05, 128.84 (2C), 128.98, 129.18, 129.40 (2C), 130.03, 130.39, 132.18 (2C), 135.03, 136.62, 142.28], 147.89 (triazole, C-2), 153.88 (benzimidazole, C=N), 169.03 (triazole, C-5). LC-MS, m/z: 532.06 [M + H]+. Anal. calcd. (%) for C28H27ClN6OS: C, 63.33; H, 5.12; N, 15.82; S, 6.04. Found: C, 63.37; H, 5.16; N, 15.78; S, 6.08.
3.2.10. Synthesis of compounds 9A and 9B

To a solution of compound 7 (0.010 mol) in acetone, K$_2$CO$_3$ (0.020 mol) was added and the mixture was stirred at room temperature for 30 min. After that, to synthesize compounds 9A and 9B, 1-bromopropane (0.012 mol) and 1-bromohexane (0.012 mol) were added to the mixture, respectively. The reaction mixture was stirred for 10 h at room temperature. The reaction was monitored by TLC (ethyl acetate). The K$_2$CO$_3$ was filtered off from the reaction mixture. After that, the solvent was evaporated under reduced pressure and a white solid appeared. The final product was recrystallized with acetone.

3.2.10.1. 2-Benzyl-1-\{[4-(4-chlorophenyl)-5-(propylthio)-4H-1,2,4-triazol-3-yl]methyl\}-1H-benzimidazole (9A)

Yield 75%, mp: 118–119 °C, $^1$H NMR spectrum (400 MHz, DMSO-$d_6$), δ, ppm (J, Hz): 7.61–7.50 (4H, m, ArH), 7.38–7.08 (9H, m, ArH), 5.75, 5.49 (2H, s, NCH$_2$), 4.37, 4.00 (2H, s, CH$_2$), 3.04 (2H, t, $J = 7.2$ Hz, SCH$_2$), 1.65–1.61 (2H, m, CH$_2$), 0.89 (3H, t, $J = 7.2$ Hz, CH$_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$), δ, ppm: 13.30 (CH$_3$), 22.74 (CH$_2$), 33.16 (CH$_2$), 34.57 (CH$_2$), 38.94 (NCH$_2$), ArC [110.57 (2C), 118.97 (2C), 121.98, 122.22, 126.99, 128.82, 129.23 (2C), 129.34, 130.33 (2C), 131.44, 135.48, 136.75, 136.80, 142.40], 151.66 (triazole, C-2), 152.07 (triazole, C-5), 153.72 (benzimidazole, C=N). LC-MS, m/z: 475.10 [M + H]$^+$. Anal. calcd. (%) for C$_{26}$H$_{24}$ClN$_5$S: C, 65.88; H, 5.10; N, 14.77; S, 6.76. Found: C, 65.82; H, 5.14; N, 14.72; S, 6.70.

3.2.10.2. 2-Benzyl-1-\{[4-(4-chlorophenyl)-5-(hexylthio)-4H-1,2,4-triazol-3-yl]methyl\}-1H-benzimidazole (9B)

Yield 74%, mp: 113–114 °C, $^1$H NMR spectrum (400 MHz, DMSO-$d_6$), δ, ppm (J, Hz): 7.61–7.50 (4H, m, ArH), 7.38–7.08 (9H, m, ArH), 5.75, 5.49 (2H, s, NCH$_2$), 4.37, 4.00 (2H, s, CH$_2$), 3.04 (2H, t, $J = 7.2$ Hz, SCH$_2$), 1.65–1.57 (2H, m, CH$_2$), 1.29–1.22 (m, 6H, CH$_2$), 0.89 (3H, t, $J = 7.2$ Hz, CH$_3$). $^{13}$C NMR (100 MHz, DMSO-$d_6$), δ, ppm: 14.31 (CH$_3$), 22.40, 27.97, 29.30, 31.09, 323.73 (5CH$_2$), 33.17 (CH$_2$), 38.95 (NCH$_2$), ArC [110.56 (2C), 118.98 (2C), 121.98, 122.22, 126.98, 128.82, 131.45, 135.48, 136.75 (2C), 142.42], 151.66 (triazole, C-2), 152.10 (triazole, C-5), 153.71 (benzimidazole, C=N). LC-MS, m/z: 517.12 [M + H]$^+$. Anal. calcd. (%) for C$_{29}$H$_{30}$ClN$_5$S: C, 67.49; H, 5.86; N, 13.57; S, 6.21. Found: C, 67.52; H, 5.92; N, 13.52; S, 6.26.

3.3. Bioassay

3.3.1. Cell culture and cytotoxic activity

SK-BR-3 (human breast cancer cells), PC-3 (human prostate adenocarcinoma), and HEK-293 (noncancerous human embryonic kidney) cell lines were used for the cytotoxicity assay (American Type Culture Collection, Rockville, MD, USA). All cell lines were maintained in Dulbecco’s modified Eagle’s medium: nutrient mixture F-12 with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Serox GmbH, Germany). All cells were incubated at 37 °C in a 95% humidified atmosphere of 5% CO$_2$.

The cytotoxicity of samples was determined using the MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide) assay, which determines the activity of mitochondrial reductase of viable cells colorimetrically [37,38].
All cell lines were cultivated in 96-well plates at $1 \times 10^5$ cells/mL initial cell concentrations. Cells were treated with samples (1, 2, 3A, 3B, 4, 5, 6, 7, 8, 9A, and 9B) at different concentrations (0.5, 5, and 50 μg/mL) after 24 h and doxorubicin was used for the positive control (0.2, 2, and 20 μg/mL). Optical densities of the dissolved material were determined at 570 nm (reference filter 620 nm) with a UV-Vis spectrophotometer after 48 h. The percentage of living cells was determined by spectrophotometric measurement.

IC$_{50}$ is the concentration of the samples causing 50% inhibition of cell viability. The mean IC$_{50}$ concentration of the samples reduces cell growth by 50% under laboratory conditions.

### 3.3.2. LDH assay

As an indicator of cytotoxicity, LDH activity released from damaged cells in culture medium was determined by LDH assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). A total of 100 μL of LDH reaction solution was added to the relevant well of a 96-well plate and then the absorbance of the cells was measured at 490 nm. Triton X-100 was applied as a positive control to stimulate maximized LDH secretion.

### 3.3.3. TAC and TOS activity

TAC and TOS levels were measured in cellular media using a commercial kit (Rel Assay Diagnostics, Gaziantep, Turkey) according to the manufacturer’s instructions. Another group of cells for these experiments was treated with different concentrations (0.5, 5, and 50 μg/mL) of synthesized compounds and incubated at 37 °C in a humidified 5% CO$_2$ for 2 h.

In the TAC assay, potential antioxidants in culture medium cause reduction of ABTS (2,2'-azino-bis 3-ethyl benzothiazoline-6-sulfuric acid) radical. Briefly, 500 μL of Reagent 1 solution in the kit’s contents was added to a quartz cuvette containing 30 μL of plasma sample and the initial absorbance was measured at 660 nm after 30 min. Then 75 μL of Reagent 2 solution was added to the same cuvette and the absorbance was measured at 660 nm after 5 min incubation. The assay was calibrated with Trolox and the results were expressed in terms of mM Trolox equivalent per liter (mmol Trolox equiv./L) [39,40].

The TOS assay was based on the conversion of ferrous ion-chelator complex to ferric ion via oxidants present in the culture medium. To determine the TOS level, 500 μL of Reagent 1 was mixed with 75 μL of each plasma sample and the absorbance of each sample was measured at 530 nm after 30 min. Then 15 μL of Reagent 2 was added to the mixture and the absorbance was read again at 530 nm. The assay was calibrated with H$_2$O$_2$ and the results were expressed in terms of μM H$_2$O$_2$ equivalent per liter (μmol H$_2$O$_2$ equiv./L) [40,41].

### 3.3.4. Antiangiogenic activity

Antiangiogenic effects of samples were determined on fertile Leghorn chicken eggs weighing 50–60 g by using a CAM method modified by Ulus et al. [25]. Fertilized eggs were placed into an incubator with a conveyor rotation system at 37 ± 1 °C and 80 ± 2% humidity for 6 days. On day 6, a round shell piece of 3–4 cm in diameter from one side of the eggs was carefully removed with forceps. Normal development of the CAM was verified and deformed embryos were removed. The opened cavity was masked with laboratory tape and incubated under the same conditions. When the diameter of CAM reached 2 cm (approximately 72 h), synthesized compounds that dissolved in DMSO (0.05%) were treated applied to the CAM with different concentrations (0.5, 5, and 50 μM) and the eggs were incubated for another 24 h. The eggs were divided into ten groups as follows: untreated
group (Group 1); treatment with only DMSO (Merck, Darmstadt, Germany) as a solvent (Group 2); suramin (Sigma-Aldrich, St. Louis, MO, USA), a known antiangiogenic agent, as a positive control (Group 3); and three different concentration treatments of the synthesized compounds (2, 3A, 3B, 6, 8, 9A, and 9B) (Groups 4–10). Scores were calculated by using the following formulations. No change in capillary area was scored as 0; a very weak effect (area with reduced density of capillary area around the pellet not larger than its own area) was scored as 0.5; a moderate effect (small capillary-free area not larger than double the size of its own area) was scored as 1; and a strong antiangiogenic effect (a capillary-free area around the pellet that was equal to or twice the size of its own area) was scored as 2. The average antiangiogenesis score was calculated as follows: 

\[
\text{Average score} = \frac{[\text{Number of eggs (Score 2) } \times 2 + \text{Number of eggs (Score 1) } \times 1]}{\text{Total number of eggs (Score 0, 1, 2)}}.
\]

According to this formulation, a score of <0.5 = no antiangiogenic effect, score of 0.5 to 0.75 = weak antiangiogenic effect, score of 0.75 to 1 = moderate antiangiogenic effect, and score of >1 = strong antiangiogenic effect. Fifteen eggs were used and all the tests were duplicated. NaCl (0.9%) as a negative control and suramin (50 µg/pellet), an antiangiogenic agent approved by the Federal Drug Administration, as a positive control were also tested.

3.3.5. SCE assay

This study was performed with blood samples obtained from five males aged between 20 and 40 years with no record of contact with any genotoxic agents and no smoking. Experiments involving volunteer individuals were conducted in accordance with the decisions of the Declaration of Helsinki and the Giresun University Local Ethics Committee. Approximately 6 mL of blood was collected by venous puncture from the participants on an empty stomach to minimize the potential effects of nutritional factors. Samples collected on the same day of the beginning of the trials were analyzed primarily for hematological and biochemical parameters and no disease was detected in the samples. The SCE assay was performed with minor modifications of the methods of Moorhead et al [42]. A blood sample of 500 µL and different concentrations (0.5, 5, and 50 µg/mL) of synthesized compounds were added to culture medium containing 10 µg/mL 5-bromo-20-deoxyuridine (BrdU; Sigma), 7 mL of Chromosome Medium B (Biochrom, Berlin, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.5 mL of phytohemagglutinin (Biochrom) and the cell culture was incubated at 37 °C for 72 h. At 70 h of incubation, 0.075 g/mL of colcemid (Sigma), a mitotic inhibitor, was added to the culture medium. At the end of the incubation period, the culture medium was centrifuged at 900 × g for 10 min and lymphocyte cells obtained were hypotonized by 0.075 M cold potassium chloride for 30 min and then cells were fixed with ice-cold methanol and acetic acid (3 : 1, v/v). Microscope slides were prepared in triplicate using the standard procedure, air-dried, and stained according to fluorescence plus Giemsa as described by Perry and Wolff [43]. For scoring of SCE cells, 25 well-spread second division metaphases containing 46 (±2) chromosomes were counted using a light microscope at 1000× magnification by a single observer. A total of 1000 metaphases were counted for each concentration and the values obtained were calculated as SCEs per cell.

3.3.6. MN assay

The MN assay was carried out in accordance with procedures previously described by Fenech and Morley [44]. Following the collection of blood samples as mentioned above, 500 µL of blood sample and different concentrations (0.5, 5, and 50 µg/mL) of synthesized compounds were added to 7 mL of Chromosome Medium B (Biochrom) containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.5 mL of phytohemagglutinin
(Biochrom) and the cell culture was incubated at 37 °C for 72 h. Cytochalasin B (Sigma) was added to the culture medium at 44 h of incubation. At the end of the incubation period, the culture medium was centrifuged at 900 × g for 10 min and the obtained lymphocyte cells were hypotonized by 0.075 M cold potassium chloride for 30 min, and then cells were fixed with ice-cold methanol and acetic acid (3 : 1, v/v). The fixed cells were put directly on slides using Cytospin and stained with Giemsa solution. The counting of MN cells was performed under a light microscope in accordance with the criteria declared by Fenech [45]. At least 1000 binucleated cells were counted per concentration (duplicate cultures for each concentration) for the formation of one, two, or more MN.

3.3.7. Statistical analysis
Statistical analysis was performed using SPSS 18.0 (SPSS, Chicago, IL, USA). Duncan’s test was performed to examine whether there were any differences between the application and control groups. IC_{50} was calculated by using GraphPad Prism 5 software (San Diego, CA, USA). P < 0.05 was accepted as significant. All assays were run in triplicate.

References
7. Huang ST, Hsei IJ, Chen C. Synthesis and anticancer evaluation of bis (benzimidazoles), bis (benzoxazoles), and benzothiazoles. Bioorganic & Medicinal Chemistry Letters 2006; 14 (17): 6106-6119. doi: 10.1016/j.bmc.2006.05.007
17. Specian AFL, Serpeloni JM, Tuttis K, Ribeiro DL, Cilião HL et al. LDH, proliferation curves and cell cycle analysis are the most suitable assays to identify and characterize new phytotherapeutic compounds. Cytotechnology 2016; 68 (6): 2729-2744. doi: 10.1007/s10616-016-9998-6


