Full Paper

Synthesis and Biological Evaluation of Some 1,2-Disubstituted Benzimidazole Derivatives as New Potential Anticancer Agents

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The synthesis of some new 1-(2-aryl-2-oxoethyl)-2-[(morpholine-4-yl)thioxomethyl]benzimidazole derivatives and investigation of their anticancer activities were the aims of this work. 2-(Chloromethyl)benzimidazole compound was reacted with sulfur and morpholine *via* Willgerodt–Kindler reaction to give 2-[(morpholine-4-yl)thioxomethyl]benzimidazole. Then, the obtained compound was reacted with appropriate α-bromoacetophenone derivatives in the presence of potassium carbonate to give the final products. Structure elucidation of the final compounds was achieved by FT-IR, ^{1}H NMR spectroscopy and MS spectrometry. The anticancer activities of the final compounds were evaluated by MTT assay, BrdU method, and flow cytometric analysis on C6, MCF-7, and A549 tumor cells. Most of the synthesized compounds exhibited considerable selectivity against the MCF-7 and C6 cell lines.

Keywords: Anticancer activity / Benzimidazole / BrdU / Flow cytometric analysis / Thioamide

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Introduction

Cancer treatment often encompasses more than one approach, and the strategy adopted depends largely on the nature of the cancer and how far it has progressed. At the present time, the main treatment strategies are still surgery, radiotherapy, and chemotherapy [1]. In cancer chemotherapy, besides drugs in use, there is much interest in the design of new anticancer agents especially small molecules that bind to DNA with sequence selectivity and noncovalent interactions [2].

Benzimidazoles are the privileged components of many bioactive compounds. Because of their synthetic utility and broad range of pharmacological activities such as antifungal, anti-helmintic, anti-HIV, antihistaminic, antiulcer, cardiotonic, and antihypertensive; they have become a key building block for a variety of compounds that play crucial roles in the function of a number of biologically important molecules [3, 4]. In addition, it has been extensively utilized as a drug scaffold in medicinal chemistry and is an important pharmacophore due to being a structural isostere of naturally occurring nucleotides [5–8]. In recent years, benzimidazole derivatives have attracted particular interest due to their anticancer activity or as potential *in vitro* anticancer agents [9, 10]. In particular, they have been explored as topoisomerase I, PARP-1, kinase Chk2, Pgp and tyrosine kinases, metallo and serine protease inhibitors [11, 12]. Benzimidazole as ''lead'' molecule binds with other heterocyclic compounds. It acts by intercalation or blocks cell growth by inhibiting the enzymes directly responsible for the formation of nucleic acids. This inhibition is believed to prevent DNA transcription, which ultimately leads to cell death, which explains the use of these drugs to treat cancer [13, 14]. Nocodazole [15],

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oncodazole, carbandazim [16], bendamustin [17], Hoechst-33258 [18], Hoechst-33342 [19], and IMET 3393 [12] are some of the benzimidazole carrying anticancer drugs which are in clinic or preclinic stage.

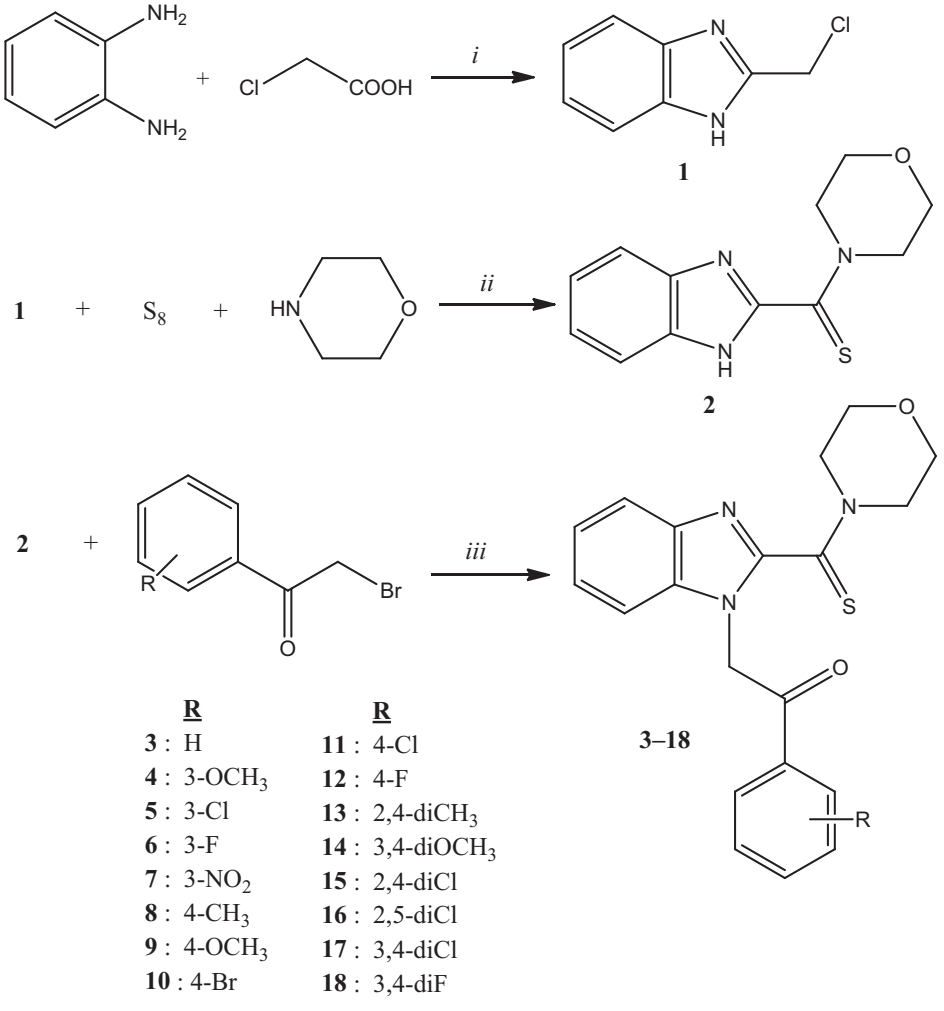
Additionally, in recent studies thioamide linkage has been preferred to amide linkage in newer peptide drug design studies because of the conformational changes. The slightly changed electron distribution of the thioamide structure with regard to the amide structure represents a conservative modification of the peptide backbone, which has been reported to improve the enzymatic stability and bioactivity [20, 21]. On the other hand, it is known that gefitinib [22] is one of the morpholine carrying anticancer drugs. There are also a lot of studies about anticancer activity of benzimidazole and morpholine including compounds [11, 23, 24].

Motivated by the above observations and as an extension of our previous works on pyrazino[1,2-a]benzimidazoles [25–27] in which intermediate product 1-(2-aryl-2-oxoethyl)-2-substituted benzimidazole derivatives exhibited higher anticancer activity than final pyrazino[1,2-a]benzimidazole compounds, we planned to synthesize 1-(2-aryl-2-oxoethyl)-2-[(morpholine-4-yl)thioxomethyl]benzimidazole derivatives *via* Willgerodt–Kindler reaction and to investigate their prospective anticancer activities.

Results and discussion

Chemistry

Target molecules (**3**–**18**) were synthesized in three steps as shown in Scheme 1. In the first step, 2-(chloromethyl)benzimidazole (**1**) was synthesized *via* Phillips method. 1,2-Diaminobenzene and chloroacetic acid were refluxed in 4 N HCl solution to obtain an irritant intermediate product. In the second step, 1*H*-2-[(morpholine-4-yl)thioxomethyl]-



R		R	
3 :	H	**11** :	4-Cl
4 :	3-OCH_3	**12** :	4-F
5 :	3-Cl	**13** :	2,4-diCH_3
6 :	3-F	**14** :	3,4-diOCH_3
7 :	3-NO_2	**15** :	2,4-diCl
8 :	4-CH_3	**16** :	2,5-diCl
9 :	4-OCH_3	**17** :	3,4-diCl
10 :	4-Br	**18** :	3,4-diF

Scheme 1. The synthetic protocol of the compounds (**3**–**18**). Reagents and conditions: (i) 4 N HCl, reflux; (ii) Et_3N, DMF; (iii) K_2CO_3, acetone.

benzimidazole (**2**) was obtained from 2-(chloromethyl)benzimidazole (**1**) *via* Willgerodt–Kindler reaction. Basically, the Willgerodt-Kindler reaction is the reaction of aryl, alkyl ketones and secondary amines with sulfur to give thioamides *via* oxidation and rearrangement [28]. In this study, unlike classical procedures, we used benzylic structure (**1**) as a starting material instead of aryl, alkyl ketone to obtain the thioamide structure [29]. 2-(Chloromethyl)benzimidazole (**1**), sulfur, triethylamine and morpholine were used with a relative ratio of 1:2.5:3:1.2 to obtain 1*H*-2-[(morpholine-4-yl)thioxomethyl]benzimidazole (**2**) compound and the reaction was realized with 63% yield. Finally, 1*H*-2-[(morpholine-4-yl)thioxomethyl]benzimidazole (**2**) was reacted with appropriate α-bromoacetophenone derivatives *via* bimolecular nucleophilic substitution (S_N2) reaction to give the intended 1-(2-aryl-2-oxoethyl)-2-[(morpholine-4-yl)thioxomethyl]benzimidazole derivatives (**3**–**18**).

Structures of the obtained compounds were elucidated by spectral data. In the IR spectra, significant stretching bands belonging to −C=N and −C=C were observed in between 1612–1350 cm^{-1} and bands belonging to −C–N and −C–O were observed at about 1286–981 cm^{-1}. In the NMR spectra of the final compounds, a singlet peak at about 6.20 ppm was observed belonging to $-CH_2$ of the oxoethyl moiety; a peak about 195.73–191.94 ppm belonging to the −C=O structure and 186.38–184.97 ppm belonging to the −C=S structure. Peaks belonging to the morpholine ring were assigned as four different triplets, commonly. Hydrogen and carbon atoms close to the oxygen atom were observed at 4.30–3.81 ppm and 66.93–66.04 ppm. Hydrogen and carbon atoms close to the nitrogen atom were observed at 3.75–3.69 ppm and 51.91–48.84 ppm in the NMR spectra. The other peaks belonging to aromatic and aliphatic protons of variable side chains were observed at the estimated areas. In the mass spectra of the copmpounds, M+1 peaks agreed well with the calculated molecular weight of the target compounds.

Biological results

Cytotoxicity of the compounds

Cytotoxical potentials of the compounds against tumor cells were assessed by the colorimetric MTT assay. The MTT test is based on the cleavage of the yellow tetrazolium salt to form a soluble blue formazan product by mitochondrial enzymes. The amount of formazan produced is directly proportional to the number of living cells [30]. The cytotoxic activities of the synthesized compounds (**3**–**18**) were compared against positive controls by using A549 (human non-small cell lung cancer), C6 (rat glioma), and MCF-7 (human breast carcinoma) cell lines. Tested concentrations for compounds were in between 3.9 and 500 μg/mL and for controls (doxorubicin and cisplatin) were in between 0.98 and 500 μg/mL. The corresponding IC_{50} values are listed in Table 1.

Table 1. IC_{50} values[a] (μg/mL) for compounds **3**–**18** in A549, C6, MCF-7 cancer cell lines.

Compounds	A549	C6	MCF-7
3	146.67 ± 2.4	131.67 ± 1.6	106.67 ± 2.7
4	>500	200 ± 2.8	303 ± 3.4
5	270 ± 3.2	161.67 ± 3.2	98.3 ± 2.5
6	353.33 ± 1.8	>500	440 ± 2.9
7	330 ± 2.2	>500	475 ± 4.3
8	58.33 ± 2.5	48 ± 2.2	17.3 ± 2.8
9	27.7 ± 2.7	24.33 ± 1.2	13 ± 3.7
10	230 ± 4.5	55 ± 2.3	22.7 ± 3.2
11	15.66 ± 1.8	9.33 ± 4.5	15.3 ± 1.7
12	>500	>500	>500
13	303.33 ± 3.3	>500	140 ± 2.8
14	108.33 ± 4.6	17.33 ± 3.2	13.8 ± 3.6
15	>500	205 ± 2.5	420 ± 3.2
16	323.33 ± 4.8	386.67 ± 1.7	>500
17	>500	415 ± 4.1	295 ± 2.4
18	90 ± 1.8	123.33	39.7 ± 2.3
Cisplatin	19 ± 1.8	14.67	7.8 ± 2.8
Doxorubicin	17.33 ± 2.4	9.67	5.17 ± 3.9

[a] Cytotoxicity of the compounds. Incubation for 24 h. IC_{50} is the drug concentration required to inhibit 50% of the cell growth. The values represent mean ± SD of triplicate determinations.

The IC_{50} values of the compounds were determined for A549 cell line in the range of 15.66–500 μg/mL. Compounds **8**, **9**, and **11**, which were including methyl, methoxy, and chloro substituents on the phenyl ring, had significant cytotoxic activity with IC_{50} values lower than 58.33 μg/mL. Compound **11** possessed the lowest IC_{50} value, which was 15.66 μg/mL, whereas cisplatin and doxorubicin IC_{50} values were 19 and 17.33 μg/mL against A549 cells, respectively. Compounds **8**, **9**, **10**, **11**, and **14** exhibited remarkable cytotoxic activities against C6 cell line. Compound **11** showed the highest cytotoxic activity with a IC_{50} value of 9.33 μg/mL whereas cisplatin and doxorubicin had IC_{50} values of 14.67 and 9.67 μg/mL, respectively. MCF-7 cells were the most susceptible cells to the compounds **8**, **9**, **10**, **11**, **14**, and **18**.

Cytotoxic properties of the 1-(2-aryl-2-oxoethyl)-2-[(morpholine-4-yl)thioxomethyl]benzimidazole derivatives (**3**–**18**) varied according to the substituents on the phenyl ring. In general, compounds **3**, **5**, **8**, **9**, **14**, and **18** were the most cytotoxic molecules. Compounds **3**, **5**, **8**, **9**, **10**, **14**, and **17** showed maximum activities against MCF-7 cells and minimum activities against A549 cells. Compounds **6**, **7**, and **16** exhibited the highest activities against A549 cells, on the other hand compounds **4**, **11**, and **15** displayed the highest actitivity to C6 cells. Compound **10** failed to provide 50% cytotoxicity on the three cell types even with the highest concentration (500 μg/mL).

Synthesized compounds (**3**–**18**) exhibited the highest cytotoxic activities against MCF-7 cells, the lowest cytotoxical

activities against A549 cells. Compounds **8**, **9**, **10**, **11**, **14**, and **18** destroyed 50% of the MCF-7 cells at doses up to 40 μg/μL. All studied concentrations of compounds **6**, **7**, **12**, and **13** showed unsufficient cytotoxical effect over C6 cells.

With regard to the substituent effect in cytotoxic activities of the studied compounds, 4-methyl, 4-methoxy, and 4-chloro substituents had remarkable influence on the cytotoxic activities and also 3-chloro, 4-bromo, 3,4-dimethoxy, 3,4-difluoro substituents had attracted attention with comparable effects. In addition, compounds **15** and **16** which had substitution on *ortho* position of the phenyl ring and compound **7** which was bearing electron withdrawing substituent were both observed with low cytotoxic activities. The compound **9** which was including a methoxy substituent on *para* position of the phenyl ring exhibited remarkable activity, but this was not observed for the *meta* methoxy including derivative (compound **4**). Besides compound **18** bearing 3,4-difluoro substituent had good cytotoxic activity, it could not be determined for the compound **6** with 3-fluoro substituent and compound **12** with 4-fluoro substituent.

DNA synthesis inhibition

Proliferation of the tumor cells (A549, C6, MCF-7) was measured by incorporation of BrdU, a thymidine analog that is incorporated into DNA during the S phase, and the method is based on a colorimetric measurement of the carcinogenic DNA synthesis inhibition ratio [31]. This study was performed for the highest cytotoxic eight compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, and **18**, which were determined by the MTT test. A549, C6, and MCF-7 cells were incubated with three different concentrations ($IC_{50}/2$, IC_{50}, and $IC_{50} \times 2$) of the compounds for 24 and 48 h time periods. The tested compounds showed time- and dose-dependent inhibitory activity on DNA synthesis of the tumor cells. Cisplatin and doxorubicin were used as positive controls.

Figure 1 shows the DNA % synthesis inhibitory activity of the compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, **18**, and standard drugs on A549 cells. Among all of the compounds, compound **11** was determined as the most active compound. Compound **11** was found to have 59.82 and 68.86% DNA synthesis inhibition at 15.66 and 23.49 μg/mL doses after 48 h of incubation whereas cisplatin was found to have 59.23 and 62.26% inhibition at 19 and 27.5 μg/mL doses, respectively. According to these results, it was observed that compound **11** had more antiproliferative activity on A549 cells than cisplatin. Furthermore, it was determined that compounds **9**, **14**, and **18** had lower antiproliferative activities and compounds **3**, **5**, and **8** were inactive on the A549 cell line.

The DNA % synthesis inhibitory activity of the compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, **18**, and standard drugs on C6 cells is seen in

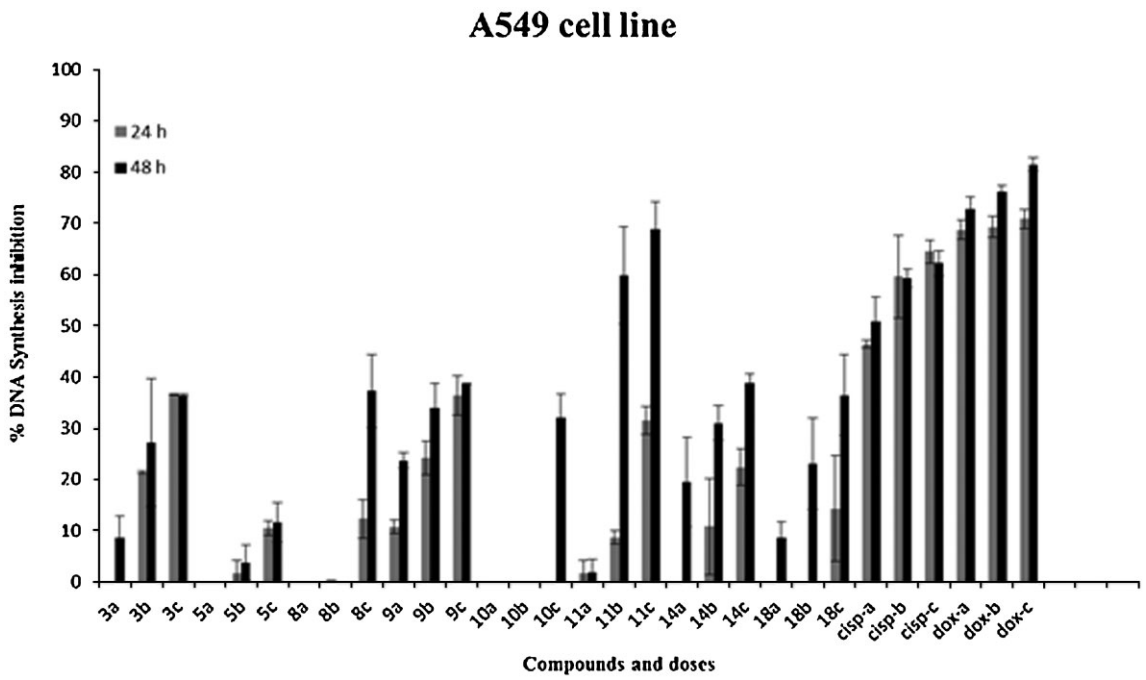


Figure 1. DNA % synthesis inhibitory activity of the compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, **18** and standard drugs on A549 cells. Mean percent absorbance of the untreated control (assessed in the presence of DMSO used as a solvent and assumed as 0%), and three different concentrations ($a = IC_{50}/2$, $b = IC_{50}$, $c = 2 \times IC_{50}$) of test compounds and cisplatin are given. Data points represent means for three independent experiments ± SD of nine independent wells. $p < 0.05$.

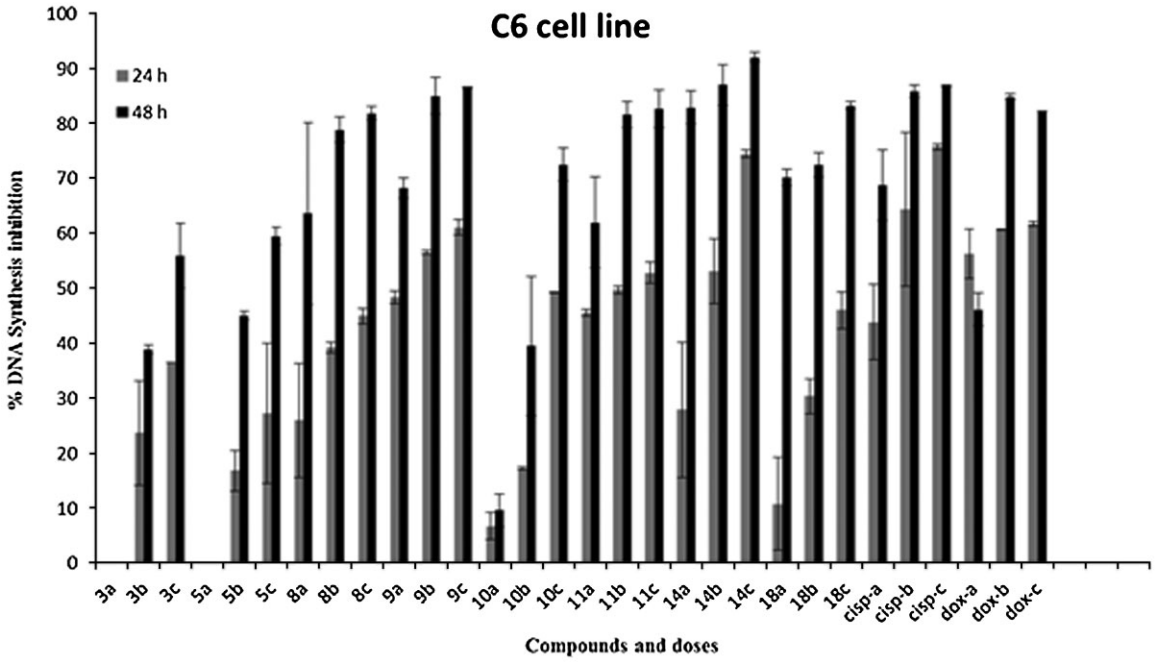


Figure 2. DNA % synthesis inhibitory activity of the compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, **18**, and standard drugs on C-6 cells. Mean percent absorbance of the untreated control (assessed in the presence of DMSO used as a solvent and assumed as 0%), and three different concentrations ($a = IC_{50}/2$, $b = IC_{50}$, $c = 2 \times IC_{50}$) of test compounds and cisplatin are given. Data points represent means for three independent experiments ± SD of nine independent wells. $p < 0.05$.

Fig. 2. DNA % inhibition was increased with the increasing incubation period (24 and 48 h) for all of the compounds. This increase can be clearly appreciated in particular for the compound **18**. Measurement of the inhibition for the compound **18** at lower dose ($IC_{50}/2$) after 48 h incubation was seven times higher than at 24 h of measurement and there was a twofold increase at the other two doses (IC_{50}, and $IC_{50} \times 2$). It was observed that compounds **8**, **9**, **11**, **14**, and **18** had the highest inhibitory activities on C6 cells DNA synthesis. In fact, compound **11** had higher activity than both cisplatin and doxorubicin with values of 45.53 and 61.96% at a concentration of 4.67 μg/mL. Also compound **14** had 27.85 and 82.88% inhibitory activity after 24 and 48 h incubation, at 8.67 μg/mL whereas cisplatin had 43.84 and 68.70% inhibitions at 7.34 μg/mL. In addition to these, compounds **3** and **5** exhibited moderate inhibitor activity on C6 cell line.

Figure 3 shows the DNA % synthesis inhibitory activity of the compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, **18**, and standard drugs on MCF-7 cells. Although it was not up to standard drugs, it was seen that many tested compounds showed significant antiproliferative activity at IC_{50} concentrations, after 48 h incubation period. At IC_{50} concentrations, DNA % inhibitions of the compounds **8**, **10**, and **14** were below 48% while the inhibitions of the other compounds were increased up to 80.51%. According to the 24 h incubation antiproliferative activity results, only compound **9** exceeded 50%. Time-dependent increase of % inhibition was seen at most for compounds **5** and **9**. Among all of the tested compounds, compound **9** was found to cause maximum inhibition of DNA and compound **5** was found to cause minimum inhibition. Compound **9** was observed to have an approximate inhibitory activity with a value of 80.51% at 13 μg/mL concentration whereas cisplatin had 76.54% inhibition at 11.7 μg/mL concentration after 48 h incubation.

In all evaluation of DNA synthesis inhibition results, A549 cells were the most resistant cells against all tested compounds. Compounds had antiproliferative activity mostly against the MCF-7 cell line. Among the tested compounds, compound **11** had significant antiproliferative activity against all three cell lines and also compounds **8** and **9** had high activity against C6 and MCF-7 cell lines. These three compound (**8**, **9**, and **11**) have attracted attention with *para* substituted phenyl moiety. It was also determined that 4-methyl, 4-methoxy, and 4-chloro substituents support the antiproliferative effect positively.

Induction of apoptosis

Apoptosis is a regulated process of cell death that occurs during embryonic development as well as maintenance of tissue homeostasis. Annexin V labeled with FITC can identify

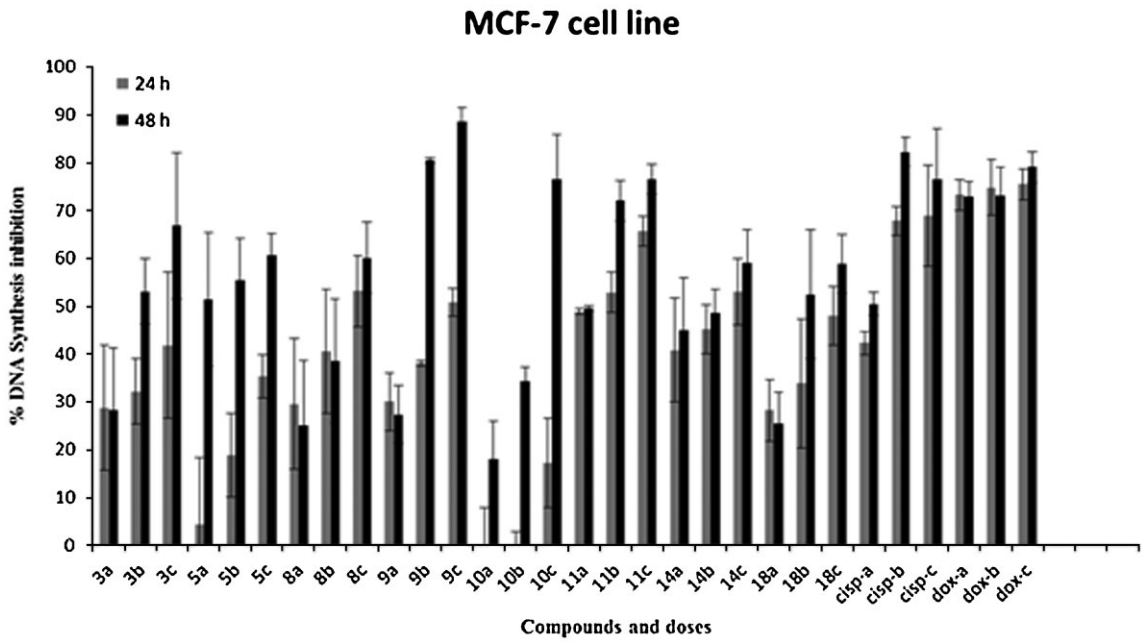


Figure 3. DNA % synthesis inhibitory activity of the compounds **3**, **5**, **8**, **9**, **10**, **11**, **14**, **18**, and standard drugs on MCF-7 cells. Mean percent absorbance of the untreated control (assessed in the presence of DMSO used as a solvent and assumed as 0%), and three different concentrations ($a = IC_{50}/2$, $b = IC_{50}$, $c = 2 \times IC_{50}$) of test compounds and cisplatin are given. Data points represent means for three independent experiments ± SD of nine independent wells. $p < 0.05$.

and quantify apoptotic cells on a single-cell basis by flow cytometry. Staining cells with propidium iodide and Annexin V-FITC enables the distinction of live, apoptotic, dead and late apoptotic or necrotic cells [32]. The percentages detected in this test for each cell type, at different concentrations and periods of time, provide information on the mechanism involved in the cell death. With the purpose of detecting the induced cellular death (necrosis or apoptosis), studies of flow cytometry were performed on compounds **8**, **9**, **11**, **14**, and cisplatin in C6 and MCF-7 cells. These compounds were incubated for 24 h at IC_{50} concentration and the results are shown in Figs. 4 and 5. The four areas in the diagrams stand for necrotic cells (Q1, positive for PI and negative for Annexin/FITC, left square on the top), live cells (Q3, negative for annexin and PI, left square at the bottom), late apoptotic or necrotic cells (Q2, positive for annexin and PI, right square on the top) and apoptotic cells (Q4, negative for PI and positive for annexin, right square at the bottom), respectively.

In Fig. 4, as for C6 cell line, compound **14** showed the highest population of apoptotic cells (20.5%) of the tested compounds which was 1.1-fold higher than for cisplatin. Compound **8** and **9** produced a comparable population of apoptotic cells with a percentage of 10.2 and 13.0%, respectively according to cisplatin's percentage of 17.8%. However compound **11** provoked necrotic induction in C6 cells after 24 h treatment.

In Fig. 5, it is seen the flow cytometric analysis diagram for MCF-7 cell line. The populations of apoptotic cells induced by compounds **8**, **9**, **11**, and **14** were 22.1, 7.2, 9.1, and 6.8%, respectively whereas cisplatin induced 34.5% apoptosis. The results above demonstrated that the synthesized compounds, in general, induced apoptosis of C6 and MCF-7 tumor cells.

Conclusion

The synthesis and anticancer activity of sixteen 1-(2-aryl-2-oxoethyl)2-[(morpholine-4-yl)thioxomethyl]benzimidazole derivatives (**3**–**18**) have been reported in this work. It was determined that many synthesized compounds had considerable anticancer activity against the C6 and MCF-7 cell lines. However compound **11** including 4-chlorophenyl substituent was the most active compound against the A549 cell line. Additionally, compound **11** possessed higher cytotoxic activity than doxorubicin and cisplatin with a IC_{50} value of 9.33 μg/mL against A549 cells and 15.66 μg/mL. According to the DNA synthesis inhibition studies, compounds **8** and **9** inhibited DNA synthesis on C6 and MCF-7 cells and compound **11** inhibited DNA synthesis on all three cells and there was a time dependent increase of inhibition ratios. Compounds **8**, **9**, and **14** were determined that they affected C6 tumor cells by the apoptotic pathway and compound **11** by the necrotic pathway. Also compound **8** and **11** affected MCF-7 cells by the apoptotic way.

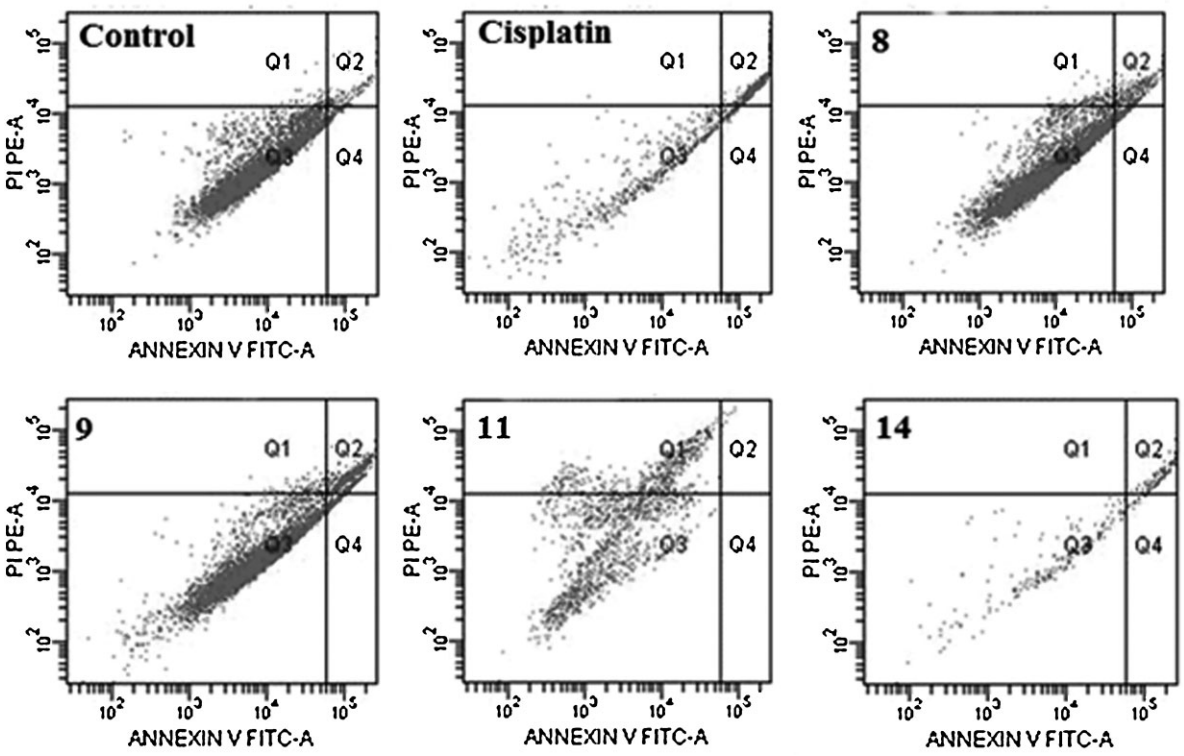


Figure 4. Flow cytometric analysis of the distribution of C6 cells treated with the selected compounds and cisplatin. Annexin V-PI analysis in C6 cells, following 24 h of drug treatment of cisplatin and compounds **8**, **9**, **11**, and **14** by Annexin-V-FITC method at IC_{50} concentrations, which are 14.67, 48.0, 24.33, 9.33, and 17.33 μg/mL in the order indicated in figure. Each condition was analyzed in a histogram, which displays two parameters, Annexin V-FITC and PI, as represented. The dual parametric dot plots show the necrotic cells in the upper left quadrant, Q1 (Annexin V-negative/PI-positive; the late apoptotic cells in the upper right quadrant, Q2 (Annexin V-positive/PI-positive); the viable cell population in the lower left quadrant, Q3 (Annexin V-negative/PI-negative) and the early apoptotic cells in the lower right quadrant, Q4 (Annexin V-positive/PI-negative). Results are representative of one of three independent experiments. Percentages of Q1, Q2, Q3, and Q4 are measured as 0.3, 12.8, 81.9, and 5.0% for cisplatin; 1.4, 5.6, 88.5, and 4.6% for compound **8**; 1.1, 8.3, 85.9, and 4.7% for compound **9**; 26.0, 3.3, 70.7, and 0% for compound **11**; 1.1, 14.5, 78.4, and 6.0% for compound **14**, respectively.

4-Methyl, 4-methoxy, 4-chloro, and 3,4-dimethoxy phenyl including derivatives have been identified as the most effective compounds for anticancer activity. Accordingly, methoxy and chloro substituents at the *para* position of the phenyl ring increased anticancer activity. In this study, it was seen once again that 1-(2-aryl-2-oxoethyl)-2-substituted benzimidazole derivatives were anticancer compounds. Substitution of the benzimidazole ring on second position with thiomorpholinide moiety did not contribute a significant increase in activity. In future studies, it is thought to substitute 1-(2-aryl-2-oxoethyl)benzimidazole derivatives with amide and/or chloro, methoxy including aryl moieties.

Experimental

Chemistry

All chemicals were purchased from Merck or Sigma–Aldrich Chemical companies. All melting points (m.p.) were determined by Electrothermal 9100 digital melting point apparatus and were uncorrected. NMR data was recorded by Bruker 500 MHz spectrometer. M+1 peaks were determined by AB Sciex-3200 Q-TRAP LC/MS/MS system.

*2-(Chloromethyl)benzimidazole (**1**)*

1,2-Diaminobenzene (200 mmol, 21.6 g) and chloroacetic acid (300 mmol, 28.4 g) were refluxed in 300 mL 4 N HCl solution for 3–4 h. Then the reaction mixture was cooled to room temperature and neutralized with sodium bicarbonate. The precipitate was filtered and washed with water to give pure product. Yield: 75%, m.p. 162°C (ref. 165°C) [33].

*1H-2-[(Morpholine-4-yl)thioxomethyl]benzimidazole (**2**)*

A mixture of 2-(chloromethyl)benzimidazole (120 mmol, 19.9 g), sulfur (300 mmol, 9.6 g), and triethylamine (360 mmol, 50.50 mL) in 150 mL dimethylformamide was kept for 30 min, morpholine (144 mmol, 12.56 mL) was added, and the reaction mixture was stirred for 3 h at room temprature. The mixture was then poured into water, the precipitate was filtered off and the

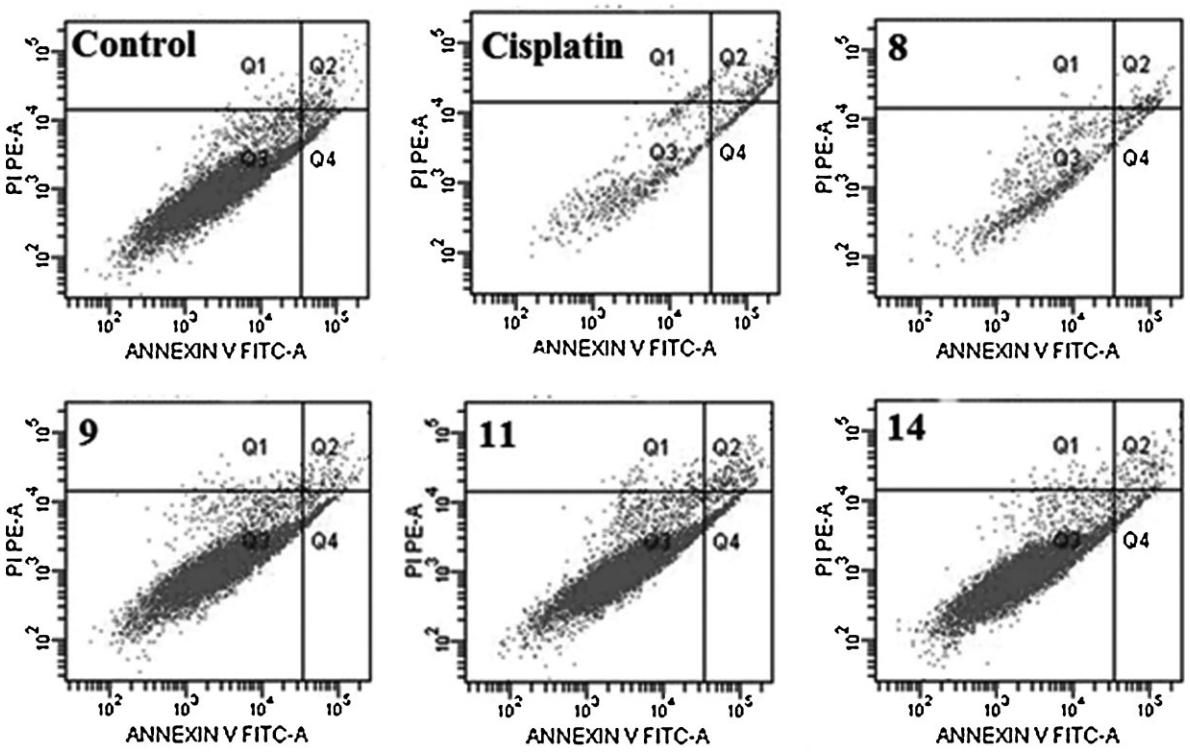


Figure 5. Flow cytometric analysis of the distribution of MCF-7 cells treated with the selected compounds and cisplatin. Annexin V-PI analysis in C6 cells, following 24 h of drug treatment of cisplatin and compounds **8**, **9**, **11**, and **14** by Annexin-V-FITC method at IC_{50} concentrations, which are 7.8, 17.3, 13, 15.3, and 13.8 μg/mL in the order indicated in figure. Each condition was analyzed in a histogram, which displays two parameters, Annexin V-FITC and PI, as represented. The dual parametric dot plots show the necrotic cells in the upper left quadrant, Q1 (Annexin V-negative/PI-positive; the late apoptotic cells in the upper right quadrant, Q2 (Annexin V-positive/PI-positive); the viable cell population in the lower left quadrant, Q3 (Annexin V-negative/PI-negative) and the early apoptotic cells in the lower right quadrant, Q4 (Annexin V-positive/PI-negative). Results are representative of one of three independent experiments. Percentages of Q1, Q2, Q3, and Q4 are measured as 3.8, 20.7, 61.8, and 13.8% for cisplatin; 1.5, 8.5, 76.5, and 13.6% for compound **8**; 1.1, 2.6, 91.6, and 4.6% for compound **9**; 1.3, 3.5, 89.7, and 5.6% for compound **11**; 0.9, 2.6, 92.3, and 4.2% for compound **14**, respectively.

obtained compound was crystallized from ethanol. Yield: 63%, m.p. 250°C (ref. 248–249°C) [34]. IR (KBr, cm^{-1}): ν_{max} 3250–2623 (N–H), 1593–1446 (C=C and C=N), 1280–1114 (C–O, C–N and C=S). ^{1}H NMR (500 MHz, dimethyl sulfoxide (DMSO)-d_6, ppm): δ 3.71 (t, J = 4.74 Hz, 2H, N–CH_2), 3.82 (t, J = 4.84 Hz, 2H, N–CH_2), 4.24 (t, J = 4.73 Hz, 2H, O–CH_2), 4.38 (t, J = 4.85 Hz, 2H, O–CH_2), 7.25 (t, J = 7.59 Hz, 1H, Ar–H), 7.31 (t, J = 7.55 Hz, 1H, Ar–H), 7.54 (d, J = 7.95 Hz, 1H, Ar–H), 7.70 (d, J = 8.09 Hz, 1H, Ar–H), 12.96 (1H, s, NH). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 50.88, 53.64, 66.72, 67.42, 113.24, 121.18, 123.68, 125.09, 135.19, 143.49, 150.18, 186.55.

*1-(2-Aryl-2-oxoethyl)-2-[(morpholine-4-yl)thioxomethyl]-benzimidazole derivatives (**3**–**18**)*

1*H*-2-[(Morpholine-4-yl)thioxomethyl]benzimidazole (2.02 mmol, 0.5 g), appropriate α-bromoacetophenone derivative (2.02 mmol) and potassium carbonate (2.02 mmol, 0.28 g) were stirred for 5 h. After the reaction was finished, the solvent was evaporated. The residue was washed with water and filtered to obtain pure product.

*1-(2-Phenyl-2-oxoethyl)-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**3**)*

Yield: 78%, m.p. 194°C. IR (KBr, cm^{-1}): ν_{max} 3057 (aromatic C–H), 2972 (aliphatic C–H), 1693 (C=O), 1452 (C=C, C=N), 1277–1112 (C–O, C–N and C=S), 761–744 (monosubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.72 (m, 4H, N(CH_2)$_2$), 3.83 (t, J = 4.69 Hz, 2H, O–CH_2), 4.26 (t, J = 4.76 Hz, 2H, O–CH_2), 6.20 (s, 2H, CO–CH_2), 7.29–7.35 (m, 2H, Ar–H), 7.62–7.68 (m, 3H, Ar–H), 7.73–7.78 (m, 2H, Ar–H), 8.13 (d, J = 7.16 Hz, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.14, 51.52, 53. 89, 66.49, 66.89, 112.07, 121.02, 123.93, 124.70, 129.51, 130.29, 135.54, 135.59, 136.73, 142.22, 150.66, 186.31, 194.82. MS (EI) m/z: 366 (100%), 279 (69%), 251 (75%).

*1-[2-(3-Methoxyphenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**4**)*

Yield 82%, m.p. 170°C. IR (KBr, cm^{-1}): ν_{max} 3043 (aromatic C–H), 2980–2918 (aliphatic C–H), 1687 (C=O), 1596–1433 (C=C, C=N), 1271–1031 (C–O, C–N and C=S), 752 (1,3-disubstituted benzene).

^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.69–3.71 (t, J = 4.02 Hz, 4H, N(CH_2)$_2$), 3.82 (t, J = 4.09 Hz, 2H, O–CH_2), 3.86 (s, 3H, O–CH_3), 4.26 (t, J = 4.69 Hz, 2H, O–CH_2), 6.17 (s, 2H, $COCH_2$), 7.28–7.34 (m, 3H, Ar–H), 7.55 (t, J = 7.94 Hz, 1H, Ar–H), 7.59 (s, 1H, Ar–H), 7.65 (d, J = 7.90 Hz, 1H, Ar–H), 7.71–7.74 (m, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.15, 51.63, 53. 87, 56.33, 66.56, 66.90, 112.07, 114.09, 121.03, 121.36, 121.86, 123.94, 124.70, 131.54, 136.70, 136.87, 142.23, 150.68, 161.00, 186.29, 194.67. MS (EI) *m/z*: 396 (100%), 309 (24%), 281 (23%), 237 (14%), 151 (29%), 108 (13%), 86 (15%).

*1-[2-(3-Chlorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**5**)*

Yield 83%, m.p. 217°C. IR (KBr, cm^{-1}): ν_{max} 3057 (aromatic C–H), 2974–2912 (aliphatic C–H), 1693 (C=O), 1453 (C=C, C=N), 1277–1116 (C–O, C–N, and C=S), 744 (1,3-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.74 (m, 4H, N(CH_2)$_2$), 3.82 (t, J = 4.62 Hz, 2H, O–CH_2), 4.27 (t, J = 4.72 Hz, 2H, O–CH_2), 6.19 (s, 2H, $COCH_2$), 7.32 (m, 2H, Ar–H), 7.67 (t, J = 7.71 Hz, 2H, Ar–H), 7.73 (d, J = 7.78 Hz, 1H, Ar–H), 7.84 (d, J = 7.50 Hz, 1H, Ar–H), 8.07 (d, J = 7.82 Hz, 1H, Ar–H), 8.18 (s, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.17, 51.71, 53. 86, 66.53, 66.90, 112.12, 121.03, 123.97, 124.73, 128.14, 129.26, 132.30, 135.19, 135.22, 136.67, 137.35, 142.23, 150.68, 185.00, 193.94. MS (EI) *m/z*: 399.5 (100%), 312.5 (23%), 284.5 (26%), 155 (39%) 86 (18%).

*1-[2-(3-Fluorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**6**)*

Yield 87%, m.p. 206°C. IR (KBr, cm^{-1}): ν_{max} 3063 (aromatic C–H), 2981–2862 (aliphatic C–H), 1693 (C=O), 1591–1439 (C=C, C=N), 1275–1116 (C–O, C–N and C=S), 740 (1,3-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.74 (m, 4H, N(CH_2)$_2$), 3.83 (t, J = 4.75 Hz, 2H, O–CH_2), 4.27 (t, J = 4.65 Hz, 2H, O–CH_2), 6.18 (s, 2H, $COCH_2$), 7.29–7.35 (m, 2H, Ar–H), 7.62–7.74 (m, 4H, Ar–H), 7.94–7.99 (m, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.16, 51.75, 53.87, 66.54, 66.89, 112.08, 115.70, 121.04, 123.97, 124.74, 125.75, 133.00, 135.01, 135.46, 136.67, 142.23, 149.50, 150.68, 185.00, 193.94. MS (EI) *m/z*: 384 (100%), 297 (33%), 269 (36%), 268 (15%), 237 (16%), 139 (48%), 86 (23%).

*1-[2-(3-Nitrophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**7**)*

Yield 80%, m.p. 231°C. IR (KBr, cm^{-1}): ν_{max} 3086 (aromatic C–H), 2910–2866 (aliphatic C–H), 1697 (C=O), 1529–1350 (C=C, C=N), 1276–1115 (C–O, C–N and C=S), 742 (1,3-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.73 (m, 4H, N(CH_2)$_2$), 3.81 (t, J = 4.91 Hz, 2H, OCH_2), 4.25 (t, J = 4.94 Hz, 2H, OCH_2), 6.25 (s, 2H, $COCH_2$), 7.29–7.32 (m, 2H, Ar–H), 7.66 (d, J = 6.70 Hz, 1H, Ar–H), 7.72 (d, J = 7.20 Hz, 1H, Ar–H), 7.92 (t, J = 8 Hz, 1H, Ar–H), 8.53–8.58 (m, 2H, Ar–H), 8.80 (s, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.21, 51.91, 53.88, 66.52, 66.93, 112.20, 121.06, 123.94, 124.02, 124.76, 129.61, 132.13, 135.78, 136.68, 142.25, 149.52, 150.44, 186.15, 193.94. MS (EI) *m/z*: 411 (100%), 324 (29%), 296 (20%), 250 (19%), 249 (13%), 130 (14%), 86 (26%).

*1-[2-(4-Methylphenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**8**)*

Yield 86%, m.p. 182°C. IR (KBr, cm^{-1}): ν_{max} 3020 (aromatic C–H), 2976–2864 (aliphatic C–H), 1684 (C=O), 1510–1454 (C=C, C=N), 1279–1109 (C–O, C–N and C=S), 748 (1,4-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.43 (s, 3H, CH_3), 3.69–3.72 (m, 4H, N(CH_2)$_2$), 3.83 (t, J = 4.63 Hz, 2H, O–CH_2), 4.26 (t, J = 4.69 Hz, 2H, O–CH_2), 6.16 (s, 2H, $COCH_2$), 7.29–7.34 (m, 2H, Ar–H), 7.44 (d, J = 8.04 Hz, 2H, Ar–H), 7.66 (d, J = 8 Hz, 1H, Ar–H), 7.73 (d, J = 8 Hz, 1H, Ar–H), 8.03 (d, J = 8.16 Hz, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 21.91, 49.13, 51.34, 53.89, 66.55, 66.88, 112.02, 121.02, 123.90, 124.68, 129.60, 130.82, 133.30, 136.74, 142.22, 146.30, 150.73, 186.34, 194.29. MS (EI) *m/z*: 380 (100%), 293 (16%), 265 (39%), 135 (70%), 91 (26%), 86 (14%).

*1-[2-(4-Methoxyphenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**9**)*

Yield 85%, m.p. 181°C. IR (KBr, cm^{-1}): ν_{max} 3053 (aromatic C–H gerilim bandı), 2914–2860 (aliphatic C–H), 1682 (C=O), 1602–1454 (C=C, C=N), 1230–1030 (C–O, C–N, and C=S), 750 (1,4-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.69–3.71 (m, 5H, N(CH_2)$_2$, O–CH), 3.90 (s, 3H, O–CH_3), 4.25 (m, 3H, O–CH_2), 6.17 (s, 2H, $COCH_2$), 7.15 (d, J = 8.76 Hz, 2H, Ar–H), 7.28–7.34 (m, 2H, Ar–H), 7.65 (d, J = 6.93 Hz, 1H, Ar–H), 7.73 (d, J = 7.73 Hz, 1H, Ar–H), 8.11 (d, J = 8.75 Hz, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.11, 51.05, 53.90, 56.64, 66.55, 66.88, 111.98, 115.44, 120.99, 123.87, 124.66, 128.37, 131.94, 136.75, 142.21, 150.83, 165.46, 186.38, 193.02. MS (EI) *m/z*: 396 (100%), 309, (14%), 281 (30%), 151 (57%), 136 (11%), 121 (11%), 108 (21%), 86 (14%).

*1-[2-(4-Bromophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**10**)*

Yield 81%, m.p. 224°C. IR (KBr, cm^{-1}): ν_{max} 3010 (aromatic C–H gerilim bandı), 2900–2858 (aliphatic C–H), 1685 (C=O), 1510 (C=C, C=N), 1274–1226 (C–O, C–N, and C=S), 742 (1,4-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.72 (m, 4H, N(CH_2)$_2$), 3.82 (t, J = 4.62 Hz, 2H, O–CH_2), 4.26 (t, J = 4.69 Hz, 2H, O–CH_2), 6.16 (s, 2H, $COCH_2$), 7.29–7.34 (m, 2H, Ar–H), 7.66 (d, J = 7.85 Hz, 1H, Ar–H), 7.73 (d, J = 7.80 Hz, 1H, Ar–H), 7.86 (d, J = 8.45 Hz, 2H, Ar–H) 8.06 (d, J = 8.50 Hz, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.16, 51.51, 53.88, 66.54, 66.88, 112.09, 121.03, 123.95, 124.72, 129.71, 131.52, 133.38, 134.57, 136.69, 142.22, 150.53, 186.23, 194.18. MS (EI) *m/z*: 445 (100%), 359 (21%), 331 (34%), 201 (63%), 120 (19%), 86 (19%).

*1-[2-(4-Chlorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**11**)*

Yield 89%, m.p. 218°C. IR (KBr, cm^{-1}): ν_{max} 3020 (aromatic C–H), 2966–2856 (aliphatic C–H), 1685 (C=O), 1589–1398 (C=C, C=N), 1275–1115 (C–O, C–N and C=S), 742 (1,4-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.67–3.73 (m, 4H, N(CH_2)$_2$), 3.83 (t, J = 4.69 Hz, 2H, O–CH_2), 4.27 (t, J = 4.76 Hz, 2H, O–CH_2), 6.17 (s, 2H, $COCH_2$), 7.29–7.35 (m, 2H, Ar–H), 7.66 (d, J = 7.12 Hz, 1H, Ar–H), 7.71–7.74 (m, 3H, Ar–H), 8.15 (d, J = 8.59 Hz, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.16, 51.54, 53.88, 66.54, 66.88, 112.09, 121.03, 123.96, 124.72, 130.41, 131.47, 134.24, 136.69, 140.53, 142.22, 150.54, 186.24, 193.95. MS (EI) *m/z*: 399.5 (100%), 312.5 (23%), 284.5 (41%), 155 (82%), 86 (20%).

*1-[2-(4-Fluorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**12**)*

Yield 86%, m.p. 204°C. IR (KBr, cm^{-1}): ν_{max} 3047 (aromatic C–H), 2918–2856 (aliphatic C–H), 1697 (C=O), 1595–1456 (C=C, C=N),

1276–1105 (C–O, C–N, and C=S), 837 (1,4-disubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.72 (m, 4H, N(CH_2)$_2$), 3.83 (t, J = 4.63 Hz, 2H, O–CH_2), 4.27 (t, J = 4.70 Hz, 2H, O–CH_2), 6.18 (s, 2H, $COCH_2$), 7.29–7.35 (m, 2H, Ar–H), 7.47 (t, J = 8.81 Hz, 2H, Ar–H), 7.67 (d, J = 7.05 Hz, 1H, Ar–H), 7.74 (d, J = 7.09 Hz, 1H, Ar–H), 8.23 (t, J = 7.13 Hz, 2H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 49.15, 51.46, 51.49, 66.55, 66.89, 112.06, 117.22, 121.03, 123.94, 124.71, 132.31, 132.73, 136.71, 142.22, 150.62, 168.13, 186.28, 193.46. MS (EI) *m/z*: 384 (94%), 297 (23%), 269 (48%), 139 (100%), 109 (10%), 95 (14%), 86 (20%).

*1-[2-(2,4-Dimethylphenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**13**)*

Yield 84%, m.p. 170°C. IR (KBr, cm^{-1}): ν_{max} 30536 (aromatic C–H), 2976–2856 (aliphatic C–H), 1693 (C=O), 1612–1429 (C=C, C=N), 1275–985 (C–O, C–N and C=S), 748 (1,2,4-trisubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 2.37 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 3.71–3.75 (m, 4H, N(CH_2)$_2$), 3.86 (t, J = 4.75 Hz, 2H, O–CH_2), 4.28 (t, J = 4.75 Hz, 2H, O–CH_2), 6.05 (s, 2H, $COCH_2$), 7.21 (s, 1H, Ar–H), 7.27–7.35 (m, 3H, Ar–H), 7.73 (t, J = 7.25 Hz, 2H, Ar–H), 8.07 (d, J = 7.5 Hz, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 21.49, 21.61, 48.82, 52.56, 53.60, 66.07, 66.47, 111.39, 120.28, 123.17, 123.96, 127.18, 130.22, 131.95, 133.23, 135.91, 139.12, 141.28, 143.55, 143.63, 185.40, 195.73. MS (EI) *m/z*: 394 (100%), 360 (12%), 307 (43%), 279 (31%), 264 (23%), 260 (10%), 246 (11%).

*1-[2-(3,4-Dimethoxyphenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**14**)*

Yield 83%, m.p. 159°C. IR (KBr, cm^{-1}): ν_{max} 3082 (aromatic C–H), 2935–2850 (aliphatic C–H), 1680 (C=O), 1585–1425 (C=C, C=N), 1251–1018 (C–O, C–N, and C=S), 746 (1,3,4-trisubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.72 (t, J = 4.25 Hz, 4H, N(CH_2)$_2$), 3.82–3.86 (m, 5H, O–CH_2 ve O–CH_3), 3.90 (s, 3H, O–CH_3), 4.27 (t, J = 4.75 Hz, 2H, O–CH_2), 6.14 (s, 2H, $COCH_2$), 7.18 (d, J = 8.5 Hz, 1H, Ar–H), 7.28–7.34 (m, 2H, Ar–H), 7.54 (s, 1H, Ar–H), 7.63 (d, J = 8.25 Hz, 1H, Ar–H), 7.73 (d, J = 7.75 Hz, 1H, Ar–H), 7.84 (dd, J = 2, 8.5 Hz, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 48.84, 50.74, 53.56, 56.16, 56.40, 66.16, 66.52, 110.81, 111.29, 111.65, 120.29, 123.14, 123.65, 123.91, 135.89, 141.37, 149.25, 150.02, 154.46, 185.22, 191.94. MS (EI) *m/z*: 426 (100%), 339 (11%), 311 (30%), 260 (8%), 181 (9%), 151 (9%).

*1-[2-(2,4-Dichlorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**15**)*

Yield 86%, m.p. 190°C. IR (KBr, cm^{-1}): ν_{max} 3043 (aromatic C–H), 2908–2864 (aliphatic C–H), 1716 (C=O), 1579–1411 (C=C, C=N), 1277–981 (C–O, C–N and C=S), 800–739 (1,2,4-trisubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.72 (m, 2H, N–CH_2), 3.77 (t, 2H, J = 4.75 Hz, O–CH_2), 3.81–3.82 (m, 2H, N–CH_2), 4.30 (t, J = 4.75 Hz, 2H, O–CH_2), 6.07 (s, 2H, $COCH_2$), 7.30–7.37 (m, 2H, Ar–H), 7.69–7.75 (m, 3H, Ar–H), 7.87 (s, 1H, Ar–H), 8.09 (d, J = 8.5 Hz, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 48.94, 50.58, 53.36, 66.07, 66.47, 111.44, 120.36, 122.94, 123.35, 124.34, 128.29, 131.12, 132.16, 133.89, 135.70, 138.10, 141.31, 149.38, 184.97, 194.31. MS (EI) *m/z*: 435 (100%), 349 (20%), 321 (21%), 191 (9%).

*1-[2-(2,5-Dichlorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**16**)*

Yield 88%, m.p. 217°C. IR (KBr, cm^{-1}): ν_{max} 3086–3040 (aromatic C–H), 2927–2860 (aliphatic C–H), 1703 (C=O), 1460–1400 (C=C, C=N), 1274–1027 (C–O, C–N and C=S), 842–748 (1,2,5-trisubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.71 (t, J = 4.75 Hz, 2H, N–CH_2), 3.78 (t, J = 5 Hz, 2H, N–CH_2), 3.81 (t, J = 4.5 Hz, 2H, O–CH_2), 4.30 (t, J = 4.75 Hz, 2H, O–CH_2), 6.08 (s, 2H, $COCH_2$), 7.31–7.38 (m, 2H, Ar–H, C_6–H), 7.68–7.77 (m, 4H, Ar–H), 8.20 (s, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 48.92, 53.43, 53.54, 66.04, 66.45, 111.55, 120.33, 123.37, 124.10, 130.00, 130.17, 132.00, 133.16, 133.69, 135.70, 137.00, 138.10, 141.31, 149.40, 184.97, 194.31. MS (EI) *m/z*: 436 (100%), 402 (20%), 349 (44%), 321 (38%).

*1-[2-(3,4-Dichlorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**17**)*

Yield 82%, m.p. 221°C. IR (KBr, cm^{-1}): ν_{max} 3084 (aromatic C–H), 2904–2856 (aliphatic C–H), 1686 (C=O), 1475–1379 (C=C, C=N), 1275–1028 (C–O, C–N, and C=S), 741 (1,3,4-trisubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.71 (t, J = 4.75 Hz, 2H, N–CH_2), 3.74 (t, J = 4.75 Hz, 2H, N–CH_2), 3.82 (t, J = 4.5 Hz, 2H, O–CH_2), 4.27 (t, J = 4.75 Hz, 2H, O–CH_2), 6.18 (s, 2H, $COCH_2$), 7.29–7.34 (m, 2H, Ar–H), 7.66 (d, J = 8 Hz, 1H, Ar–H), 7.73 (d, J = 7.5 Hz, 1H, Ar–H), 7.92 (d, J = 8.5 Hz, 1H, Ar–H), 8.06 (d, J = 8.5 Hz, 1H, Ar–H), 8.39 (s, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 48.88, 51.38, 53.53, 66.12, 66.50, 111.44, 120.31, 123.25, 124.00, 128.73, 130.79, 131.84, 132.54, 134.86, 135.82, 137.55, 141.38, 149.58, 185.04, 192.11. MS (EI) *m/z*: 436 (100%), 349 (28%), 321 (30%), 191 (15%).

*1-[2-(3,4-Difluorophenyl)-2-oxoethyl]-2-[(morpholine-4-yl)-thioxomethyl]benzimidazole (**18**)*

Yield 81%, m.p. 203°C. IR (KBr, cm^{-1}): ν_{max} 3049 (aromatic C–H), 2977–2850 (aliphatic C–H), 1689 (C=O), 1610–1419 (C=C, C=N), 1286–1117 (C–O, C–N and C=S), 739 (1,3,4-trisubstituted benzene). ^{1}H NMR (500 MHz, DMSO-d_6, ppm): δ 3.70–3.74 (m, 4H, N(CH_2)$_2$), 3.82 (t, J = 4.5 Hz, 2H, O–CH_2), 4.27 (t, J = 4.75 Hz, 2H, O–CH_2), 6.17 (s, 2H, $COCH_2$), 7.29–7.34 (m, 2H, Ar–H), 7.63 (d, J = 8 Hz, 1H, Ar–H), 7.70–7.75 (m, 2H, Ar–H, C_7–H), 8.04 (brs, 1H, Ar–H), 8.24 (t, J = 9.25 Hz, 1H, Ar–H). ^{13}C NMR (125 MHz, DMSO-d_6, ppm): δ 48.86, 51.28, 53.55, 66.13, 66.50, 111.38, 118.28, 118.42, 118.76, 118.90, 120.32, 123.24, 124.00, 126.76, 132.13, 135.82, 141.31, 149.40, 184.97, 194.31. MS (EI) *m/z*: 402 (100%), 368 (12%), 315 (54%), 287 (54%), 257 (11%).

Anticancer screening

Cell cultures

A549 (human lung adenocarcinoma cells), C6 (rat glioma cells) and MCF-7 (human breast cancer cells) cell lines were used in the studies. The cells were incubated in 90% RPMI supplemented with 10% fetal bovine serum (Gibco, UK). All media were supplemented with 100 IU/mL penicillin–streptomycin (Gibco, UK) and cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. L-Glutamine (2 mM), sodium pyruvate (1 mM), and insulin (10 mM) were used for A549 and MCF-7 cells. Exponentially growing cells were plated at 2 $\times$ 10^4 cells/mL into 96-well microtiter tissue culture plates (Nunc, Denmark) and incubated for 24 h (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). Stock solutions of compounds were prepared in dimethyl sulfoxide (DMSO) and further dilutions were made with fresh culture medium.

MTT assay

The cytotoxic activities of the tested compounds were determined by cell proliferation analysis using standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [35, 36]. A549, C6 and, MCF-7 cells were cultured in 96-well flat-bottom plates at 37°C for 24 h (2×10^4 cells per well). All of the compounds were dissolved in DMSO individually and added to culture wells at varying concentrations (3.9–500 μg/mL), the highest final DMSO concentration was under 0.1%. After 24 h drug incubation at 37°C, 20 μL MTT solution (5 mg/mL MTT powder in PBS) was added to each well. Then 3 h incubation period was maintained in the same conditions. Purple formazan was generated at the end of the process which is the reduction product of the MTT agent by the mitochondrial dehydrogenase enzyme of intact cells. Formazan crystals were dissolved in 100 μL DMSO and the absorbance was read by ELISA reader (OD_{570nm}). The percentage of viable cells was calculated based on the medium control.

DNA synthesis inhibition assay

This method was performed in the 96-well flat-bottomed microtiter plates by using a 5-bromo-2′-deoxy-uridine (BrdU) colorimetric kit. doxorubicin and cisplatin were used as positive control drugs. A549, C6 and, MCF-7 cells were collected from cell cultures by 0.25% trypsin/EDTA solution and counted in a hemocytometer. Suspensions of cell lines were seeded into 96-well flat-bottomed microtiter plates at a density of 1×10^3 cells/mL. The tumor cell lines were cultured in the presence of various doses of the test compounds or standard drugs. Microtiter plates were incubated at 37°C in a 5% CO_2/95% air humidified atmosphere for 24 h and 48 h. At the end of each day, the cells were labelled with 10 mL BrdU solution for 2 h and then fixed. Anti-BrdU-POD (100 mL) was added and incubated for 90 min. Finally, microtiter plates were washed with phosphate buffered saline (PBS) three times and the cells were incubated with substrate solution until the color was sufficient for photometric detection. Absorbance of the samples was measured with an ELx808-IUBio-Tek apparatus at 492 nm. The growth percentage was evaluated spectrophotometrically versus untreated controls with used cell viability of growth assay. Results for each spectrophotometrical measure were noticed as percent of growth inhibition [37]. All experiments were done in triplicates.

Flow cytometric analysis of apoptosis and necrosis using Annexin V/PI dual staining

All measurements were performed on a FACS-Calibur cytometer (Becton Dickinson USA). Detection of apoptosis was performed using the Annexin V-FITC Apoptosis Detection Kit from BD, Pharmingen, according to the manufacturer's instruction. After induced apoptosis of selected cell lines (C6 and MCF-7) by the addition of IC_{50} concentrations of selected compounds (compounds **8**, **9**, **11**, and **14**) and positive controls including cisplatin (50 mM) (24 h incubation), cells were collected by centrifugation for 5 min. Then cells were washed twice with cold water and resuspended in Annexin V-FITC binding buffer at a concentration of 1×10^6 cells/mL. Cells were stained with 5 mL Annexin V-FITC and incubated in the dark at 25°C for 15 min. Then the cell suspension was centrifugated for 5 min and cells were resuspended in Annexin V-FITC binding buffer. Propidium iodide (5 mL) was added and the tubes were placed on ice and away from light. The fluorescence was measured using a flow cytometer. The results were analyzed by using FCSExpress software and represented as percentage of normal and apoptotic cells at various stages [38]. The percentage of apoptotic cells was calculated from the number of cells in sub-G1 phase, representing fragmented cell vesicles. The four areas in the diagrams stand for necrotic cells (Q1, positive for PI and negative for annexin/FITC, left square on the top), live cells (Q3, negative for annexin and PI, left square at the bottom), late apoptotic or necrotic cells (Q2, positive for annexin and PI, right square on the top) and apoptotic cells (Q4, negative for PI and positive for annexin, right square at the bottom), respectively. The experiment was repeated three times.

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