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Medicinal Chemistry & Drug Discovery

Synthesis, Biological Evaluation and *In Silico* Studies of Some 2-Substituted Benzoxazole Derivatives as Potential Anticancer Agents to Breast Cancer

Burak Kuzu,[a, b] Ceylan Hepokur,[c] Mehmet Abdullah Alagoz,[d] Serdar Burmaoglu,[e] and Oztekin Algul*[a, f]

In an attempt to develop potent and selective anticancer agents, some 5- or 6- and N-substituted benzoxazol-2-carboxamide derivatives were designed, synthesized, and evaluated for their cyclooxygenase inhibitory, antioxidant, and anti-proliferative activity against MCF-7 and MDA-MB-231 cell lines. Among them 5-OMe, N-piperidine substituted (compound **30**), 5-OMe, N-4-methylpiperidine substituted (compound **31**) and 5-Cl, N-piperidine substituted (compound **34**) benzoxazole 2-carboxamide compounds have a moderate inhibitory effect in COX-1 and COX-2 enzymes. Anti-proliferative studies show that compound **30** (IC_{50} = 5.35 μM) and compound **31** (IC_{50} = 5.82 μM) have similar activity to reference drug 5-FU (IC_{50} = 3.95 μM) on MCF-7 cell but they have lower toxic effect for healthy WI-38 cell line. For the MCF-7 cell line, compounds **30** and **31** show approximately 1.5 times higher selectivity compared to the 5-FU control. Among the synthesized compounds **30**, **31**, and **34** had the best anti-proliferative effect and were used to perform flow cytometry and cell cycle analysis on MCF-7 cell line. To predict the binding modes and pharmacokinetic parameters of all compounds, *in silico* studies were carried out. These compounds may shed light on cancer treatment and cancer research.

Introduction

According to World Health Organization in 2020, cancer is reported to be the second leading cause of death globally, which was estimated at 10 million deaths.[1] Among cancer types, breast cancer (30%) is the most common type in women. Currently, in the United States, about 13% of women are at risk of breast cancer.[2] The American Cancer Society estimated that in the United States, about 281,550 new cases of invasive breast cancer in women will be diagnosed and about 43,600 women will die for 2021.[3]

Chemotherapy is used in almost all stages of cancer, but the growing incidence of drug resistance to chemotherapeutic agents presents a serious medical problem. Chemotherapy can also cause necrotic death of cancer cells and surrounding tissues, which can result to increased inflammation in patients with breast cancer.[4] Inflammation might have distinct effects on cancers and treatment outcomes.[5] Chronic inflammation, as indicated by clinical studies, increases the risk of both treatment resistance and breast cancer metastasis.[6] Clinically proven approaches in reducing cancer-promoting inflammation for breast cancer have not yet been completely understood. Breast cancer survivors on the other hand have shown to have higher levels of circulating cytokines and receptors such as IL-6,[7] IL-8,[8] IL-10[7] or estrogen receptor[9] than their healthy counterparts.[10] Increased levels of inflammatory markers have been associated with persistent fatigue in breast cancer survivors; therefore, higher pro-inflammatory cytokine levels may be a primary cause of fatigue in these patients.[11]

In addition, clinical studies indicated that there is a relationship between breast cancer and oxidative stress, which plays a

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major role in the pathogenesis of breast cancer.[12] Balance between the levels of reactive oxygen species and antioxidant status are observed in the cells of healthy individuals. However, many factors such as; diet, weight, stress, overproduction of estrogen can disrupt this balance and contribute to breast cancer development. According to the results of a study, the blood samples were analyzed and it was found that the risk of developing breast cancer decreased with an increase in the level of the total antioxidant status (TAS).[13] Similar clinical studies show that TAS and total oxidant status (TOS) levels may act as potential biomarkers[14,15] which can help assess the risk of breast cancer development.[16]

Studies with animal models have also shown that chemotherapy-induced occurrence of tumor necrosis factor-alpha (TNF-α) led to increased oxidative stress and brain inflammation.[17–19] Chemotherapy may result in cancer cell death through necrosis, which is a pro-inflammatory form of cell death.[20] Therefore, new classes of chemotherapeutic agents with anti-inflammatory effects in treating breast cancer need to be developed. In this study, the development of new anti-proliferative and anti-inflammatory chemotherapeutic agents and their cyclooxygenase (COX) inhibitions are reported.

It was observed in a previous study that thalidomide-like compounds effective in regulating the production of TNF-α, a procytokine responsible for inflammation, have immunomodulatory[21] and anti-proliferative effects in breast cancer cell line MCF-7 and prostate cancer cell line DU-145. Proteins that exhibit key roles in immune cell activation, inflammation, and cognitive function in the brain are TNF-α and COX-2.[22] Based on the recent advances, TNF-α and COX's are observed to play a vital role in the pathogenesis of many different types of cancer such as breast cancer.[23]

Till date, COX-1 and COX-2 enzymes are known to be well defined. Although COX-1 was reported to be constitutive in many organs or tissues, COX-2 can be induced by various stimulating agents. However, molecular biological studies show many exceptions to this simple paradigm. COX-2 has been overstimulated in various tumors and has a role in carcinogenesis and angiogenesis.[24,25] The effects of COX-2 inhibitors, such as celecoxib, rofecoxib, and sulindac, in chemoprevention of various cancers have been investigated.[26,27] Similar to COX-2, experimental results showed a possible involvement of COX-1 in pain and cancer development, thereby providing a rationale for selective COX-1 inhibitor development.[28] Therefore, COX enzymes are thought to be a promising therapeutic target for cancer.

Well-established studies depict thalidomide's effectiveness as an antitumor agent,[29–31] which led the research to focus on this scaffold. Thalidomide's effectiveness in treating certain kinds of cancers may be attributed to its TNF-α production-inhibiting and anti-angiogenic activities.[32] Furthermore, it was recently found that thalidomide directly inhibits COX-1/COX-2 with comparable efficacy to the representative drug, aspirin.[33] Figure 1 shows thalidomide's structural modification by adding an amino group at the 4-position of the phthaloyl ring that formed pomalidomide, a TNF-α inhibitor and interleukin-2 (IL-2) stimulator that is more potent than thalidomide and lenalidomide,[34] and displays antiangiogenic activity.[35]

Thalidomide was selected in this work as a lead compound for the synthesis of new anti-breast-cancer agents[36] with anti-inflammatory activities and antioxidant effects.[37] Our synthesized compounds were designed to have thalidomide's main essential pharmacophoric features and its analogs. Thalidomide can be a guide for our structural design because it has the common side effects of cytotoxic anticancer agents that were not reported previously.[38] Different mechanisms were reported as anti-cancer drug,[39] having anti-angiogenic activity by VEGF and bFGF-2 inhibition,[40] and inhibition of cells preventing signals that stimulate survival, growth, and development of drug resistance of tumor cells.[41] It is interesting that in order to produce lenalidomide with higher anti-cancer activity, thalidomide's teratogenicity was reduced via structural modification.[42]

The anti-inflammatory effect of thalidomide is associated with suppression of cytokine expression and the anti-oncogenic effect with inhibition of angiogenesis. The mechanism of action of thalidomide appears to be multifaceted, but is not fully understood. NF-kB transcription factor is a key regulator of inflammatory genes such as TNF-α and interleukin-8. Thalidomide has been found to exhibit anti-inflammatory properties by blocking NF-kB activation through a mechanism involving inhibition of IkB kinase activity.[43] Also, a recent study found that Cereblon (CRBN) is a primary target of thalidomide teratogenicity. Because overexpression of the thalidomide-insensitive form of CRBN rescued the effects of thalidomide largely.[44] The new studies on this prove that CRBN is a direct protein target for the immunomodulatory and antiproliferative activities of thalidomide derivatives. It was discovered that thalidomide derivatives bind to CRBN, which is determined as

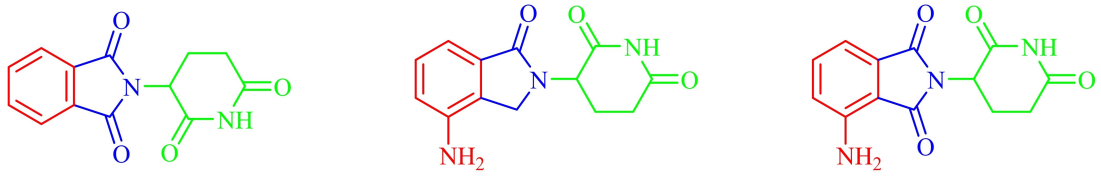


Figure 1. Structures of thalidomide, lenalidomide, and pomalidomide.

the direct target of thalidomide, and inhibits the growth of breast cancer cell lines MDA-MB-231 and MDA-MB-468 cells.[45]

For several years, research groups have made several efforts to design and synthesize new compounds with potential anti-cancer and anti-inflammatory effects.[46–48] Extension for such efforts was carried out in this work. Four common essential pharmacophoric features were found when studying the structure-activity relationship of thalidomide analogs. These features include: (i) hydrophobic domain (benzene ring), (ii) five-membered ring (heterocyclic planar group), (iii) spacer, and (iv) glutarimide moiety (alicyclic amide group; Figure 2). Briefly replacing glutarimide moiety of thalidomide with alicyclic amide derivative and five-membered ring with oxazole ring resulted in significant TNF-α inhibition and anti-inflammatory activity.[49] Other modifications were carried out by substituting the phenyl ring in order to produce new compounds with promising biological activity. In this way, candidate molecules for breast cancer were designed, and it was observed that anti-inflammatory and anti-oxidant properties of the thalidomide structure will not only help increase the quality of life in the treatment process but also help in reducing the side effects caused by breast cancer.

Benzoxazoles are an essential part of many pharmacologically and biologically active drugs. Among the different aromatic heterocyclic compounds, benzoxazole is of great importance due to its outstanding pharmacological activities such as antibiotic, antifungal, antiviral, antitumor, anti-ulcer, antibacterial, anti-inflammatory, anti-tuberculosis and analgesic.[50–52] It is widely used in research as starting material for the production of bioactive structures and is also found in the chemical structures of pharmaceutical drugs such as flunoxaprofen and has great therapeutic importance.[53,54]

Based on the anticancer and anti-inflammatory properties of thalidomide and benzoxazole derivatives, we imitate phthalimide which is planar with the benzene ring in the thalidomide molecule, with the oxazole ring. In addition, we designed the glutarimide group in the thalidomide molecule by forming 2-keto external amide bonded to oxazole. As shown in Figure 2, new therapeutic molecules, similar to thalidomide, were designed for breast cancer, which includes benzoxazole ring. Anti-breast cancer properties of benzoxazole derivatives on MCF-7 and MDA-MB-231 cell lines were examined *in vitro*, and the mechanistic studies were also demonstrated to understand their functional role in a particular pathway. Also, antioxidant and COXs inhibitory effects of synthesized benzoxazole derivatives were investigated *in vitro* in this study. In addition, we investigated the binding properties of benzoxazole compounds to thalidomide direct targets, NF-kB and CRBN proteins by docking studies.

Results and Discussion

Chemistry. A series of novel benzoxazol-2-keto(alicyclic amine substituted)methanone derivatives were synthesized by the reaction of the N-alicyclic secondary amine analogs with the intermediates obtained from the reaction of 2-aminophenol derivatives and diethyl oxalate. In this study, diethyl oxalate was used as a starting material to react with 2-aminophenol

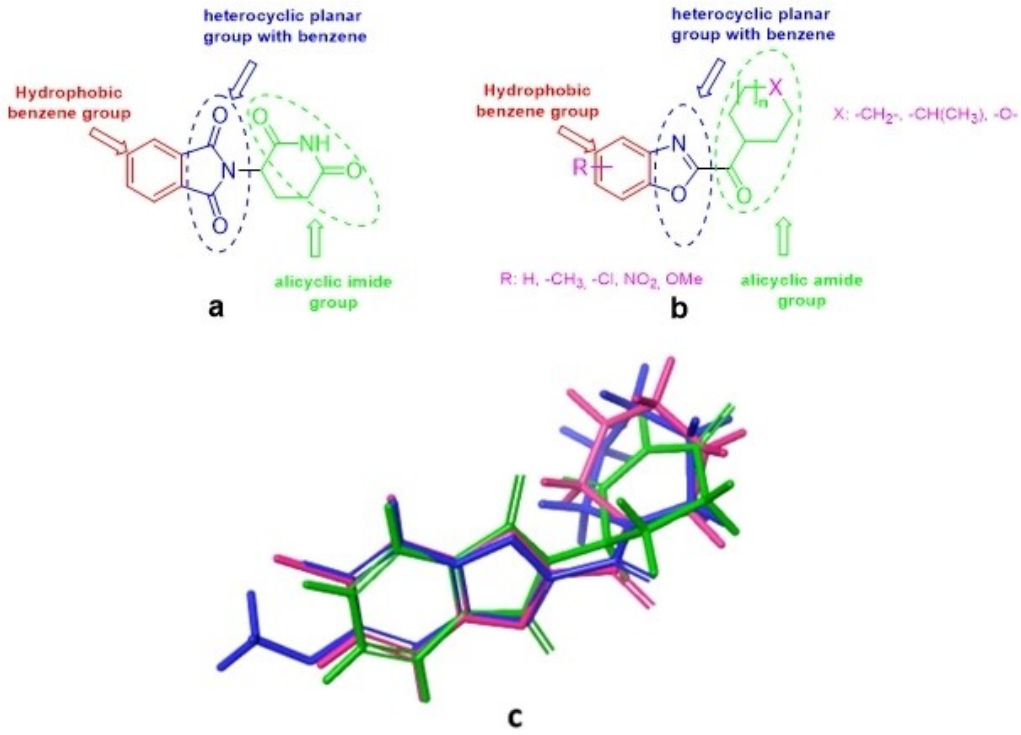
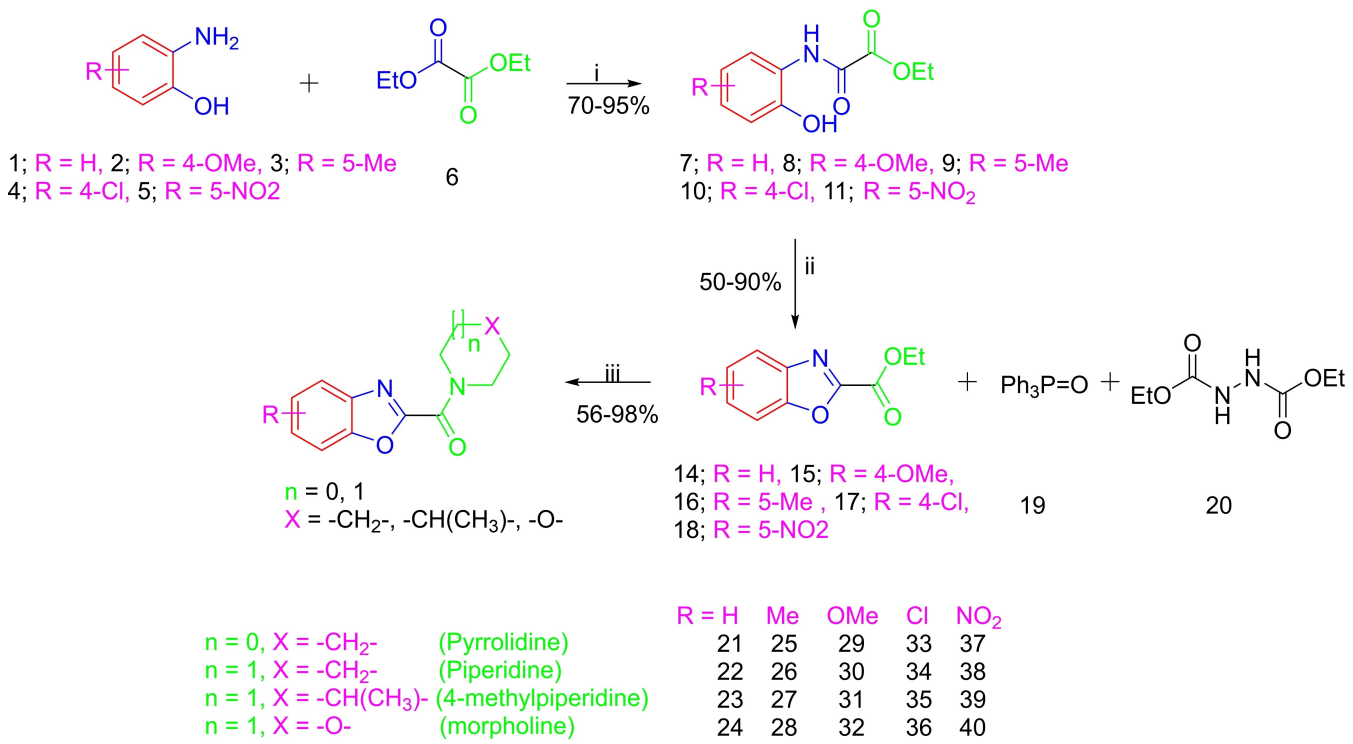


Figure 2. a: Thalidomide, b: designed compounds, c: 3D shape similarity analysis of thalidomide (green), compound **30** (blue) and compound **34** (pink) overlapping.



Scheme 1. Synthesis method of benzoxazole derivatives (**21**-**40**). Reagents and conditions: (i) 10 mL EtOH, reflux, 6 h; (ii) 1.5 eq DEAD, 1.3 eq PPh_3, 10 mL THF room temperature, 0.5 h; (iii) 1.2 eq. Pyrrolidine/Piperidine/4-methypiperidine/morpholine in dry THF, 50–60 °C, 0.5–2 h.

derivatives (**1**–**5**) as shown in Scheme 1. Under reflux conditions, substituted 2-aminophenols (**1**–**5**) underwent a substitution reaction with diethyl oxalate (**6**), affording the corresponding amino-oxo-acetate intermediates (**7**–**11**) with 70–95 % yields. The intermediates were cyclized with triphenylphosphine (PPh_3; **12**) and diethylazodicarboxylate (DEAD; **13**) to obtain benzoxazole-2-ethyl-carboxylates (**14**–**18**) with 50–90 % yields and by-products (**19** and **20**).[55] The amidation of compounds **14**–**18** was carried out using pyrrolidine, piperidine, 4-methylpiperidine, and morpholine in the presence of tetrahydrofuran (THF) to afford the benzoxazole alicyclic amide derivatives, which were corresponding final products (**21**–**40**) in 56–98 % yields. The proposed structures of the compounds were in agreement with the ^{1}H NMR, ^{13}C NMR, and elemental analyses.

In the characterization step, it was observed that some of the compounds had more peaks than expected in the high field ranges of the ^{1}H and ^{13}C-NMR spectrum. This can be explained by the presence and stability of resonance forms, either the amide group itself or iminium, as a result of the presence of tertiary amine rings (Figure 3).

Due to the presence of these two different resonance forms, the NMR device can detect both forms as separate molecules and cause more peaks to be observed.[56] This can be seen similarly in N,N-dimethyl formamide (DMF).[57] A similar situation was observed in oxalamide derivatives compounds that we synthesized with cyclic amines in our previous study.[55]

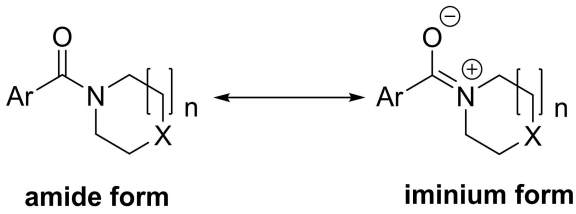


Figure 3. Resonance structures of the amide group.

Cycloxygenase Assay

COX enzymatic routes have been widely accepted inflammatory progression in mammalian cells. Compounds that exhibit anti-inflammatory activity, especially with COX-2 inhibition, are more desirable in delivering therapeutic outcomes for inflammation, pain, cancer, and neurological diseases.[58]

Table 1 presents COX-1 and COX-2 screening assays of the compounds at 1 mmol/L (25 ng/mL). Among the tested compounds, compounds **30**, **31** and **34** had highest inhibition toward both COX-1 and COX-2 enzymes. Compounds **30**, **31** and **34** were found to have 41.52 %, 34.62 %, 54.25 % inhibitory effects for COX-1, and also 50.26 %, 26.35 %, 21.52 % inhibition effects for COX-2, respectively. The other compounds also showed enzyme inhibitions of < 32 %. All compounds exhibited significantly lower inhibition of COX-1 and COX-2 compared with standard drugs, whereas DuP-697 and SC-560 ($p < 0.05$) showed 100 % inhibition for COX-1 and COX-2, respectively.

Table 1. The molecular structures and TAS-TOS, and COX-1 and COX-2 test results.

Compounds	R	n	X	% COX-1 inhibition	% COX-2 inhibition	TAS (mmol Trolox Equiv./L)	TOS (μmol H_2O_2 Equiv./L)
21	H	0	-CH_2-	25.62	23.15	0.39 ± 0.05	1.48 ± 0.65
22	H	1	–CH_2–	0	0	0.38 ± 0.01	2.81 ± 0.96
23	H	1	–CH–CH_3	0	0	0.38 ± 0.03	2.96 ± 0.75
24	H	1	–O–	0	3.21	1.88 ± 0.08	2.52 ± 1.63
25	6-CH_3	0	–CH_2–	4.63	0	1.24 ± 0.06	2.85 ± 0.95
26	6-CH_3	1	–CH_2–	26.35	13.54	0.88 ± 0.09	3.10 ± 0.74
27	6-CH_3	1	–CH–CH_3	21.35	12.54	0.53 ± 0.04	2.22 ± 0.66
28	6-CH_3	1	–O–	2.53	0	0.47 ± 0.06	2.42 ± 0.89
29	5-OCH_3	0	–CH_2–	2.35	0	0.20 ± 0.03	0.94 ± 0.74
30	5-OCH_3	1	–CH_2–	41.52	50.26	0.76 ± 0.03	2.32 ± 0.81
31	5-OCH_3	1	–CH–CH_3	34.62	26.35	0.17 ± 0.02	0.38 ± 0.04
32	5-OCH_3	1	–O–	25.68	12.34	0.52 ± 0.03	2.69 ± 1.05
33	5-Cl	0	–CH_2–	12.65	8.52	2.05 ± 0.01	3.95 ± 0.41
34	5-Cl	1	–CH_2–	54.25	21.52	0.36 ± 0.01	2.64 ± 0.24
35	5-Cl	1	–CH–CH_3	0	2.35	0.13 ± 0.03	0.28 ± 0.62
36	5-Cl	1	–O–	8.56	0	0.40 ± 0.04	2.53 ± 0.84
37	6-NO_2	0	–CH_2–	32.25	21.42	0.90 ± 0.01	2.54 ± 0.35
38	6-NO_2	1	–CH_2–	4.56	2.62	0.13 ± 0.02	1.65 ± 0.74
39	6-NO_2	1	–CH–CH_3	0	0	0.26 ± 0.01	1.82 ± 0.63
40	6-NO_2	1	–O–	12.56	11.35	0.35 ± 0.02	2.02 ± 1.03
DuP-697				–	100	–	–
SC-560				100	–	–	–

Table 2. IC_{50} values of compounds **21–40** against different breast cancer cells after 24 h.

Compounds	IC_{50} (μM)			Specificity	
	WI-38	MCF-7	MDA-MB-231	MCF-7	MDA-MB-231
21	22.49 ± 2.15	17.73 ± 0.42	10.33 ± 0.64	1.27	2.18
22	19.34 ± 1.62	12.23 ± 0.45	10.47 ± 0.65	1.58	1.85
23	21.39 ± 1.04	12.16 ± 0.85	13.10 ± 1.75	1.76	1.63
24	19.58 ± 0.02	13.44 ± 1.25	9.50 ± 0.67	1.46	2.06
25	16.32 ± 0.85	13.78 ± 0.96	11.25 ± 1.86	1.18	1.45
26	25.90 ± 1.35	9.35 ± 0.62	13.83 ± 0.63	2.77	1.87
27	11.63 ± 0.36	8.92 ± 0.63	10.93 ± 0.87	1.30	1.06
28	16.11 ± 0.47	9.38 ± 0.95	11.03 ± 1.63	1.72	1.46
29	15.30 ± 0.96	12.50 ± 0.47	10.06 ± 1.36	1.22	1.52
30	19.04 ± 1.03	5.35 ± 0.47	11.02 ± 1.62	3.56	1.72
31	19.92 ± 2.01	5.82 ± 0.35	11.20 ± 1.23	3.42	1.78
32	15.77 ± 0.35	8.32 ± 1.35	10.26 ± 0.98	1.90	1.54
33	15.32 ± 1.74	13.19 ± 0.85	12.58 ± 1.67	1.16	1.22
34	13.84 ± 1.54	6.62 ± 0.09	9.13 ± 0.62	2.09	1.52
35	12.83 ± 1.42	12.50 ± 0.54	13.45 ± 1.23	1.03	0.95
36	19.24 ± 0.99	13.06 ± 1.25	12.21 ± 1.45	1.47	1.58
37	14.68 ± 1.36	10.69 ± 0.23	10.01 ± 1.35	1.37	1.47
38	15.15 ± 1.63	8.17 ± 1.64	12.92 ± 0.65	1.85	1.17
39	18.55 ± 1.56	7.19 ± 0.75	11.85 ± 1.96	2.58	1.57
40	18.87 ± 0.74	10.53 ± 0.36	12.46 ± 1.62	1.79	1.51
5-FU	9.64 ± 1.59	3.95 ± 0.21	6.26 ± 0.16	2.44	1.54

Table 3. Apoptotic analysis of compounds **30**, **31**, **34** and 5-FU on MCF-7 and WI-38 cells.

Compounds	MCF-7 cell line					WI-38 cell line (%)			
	Live (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)	Apoptosis /Necrosis	Live	Early Apoptosis	Late Apoptosis	Necrosis
30	21.6	11.6	64.1	2.6	29.25	98.8	0.08	0.04	1.14
31	55.5	31.7	9.8	2.9	14.28	90.0	9.73	0.13	0.10
34	47.8	1.8	21.6	28.8	0.82	80.7	0.00	0.00	19.3
5-FU	20.6	7.5	36.2	35.6	1.28	97.5	0.2	0.9	1.4
Control	90.7	0.14	1.69	7.45	0.25	99.9	0.00	0.00	0.1

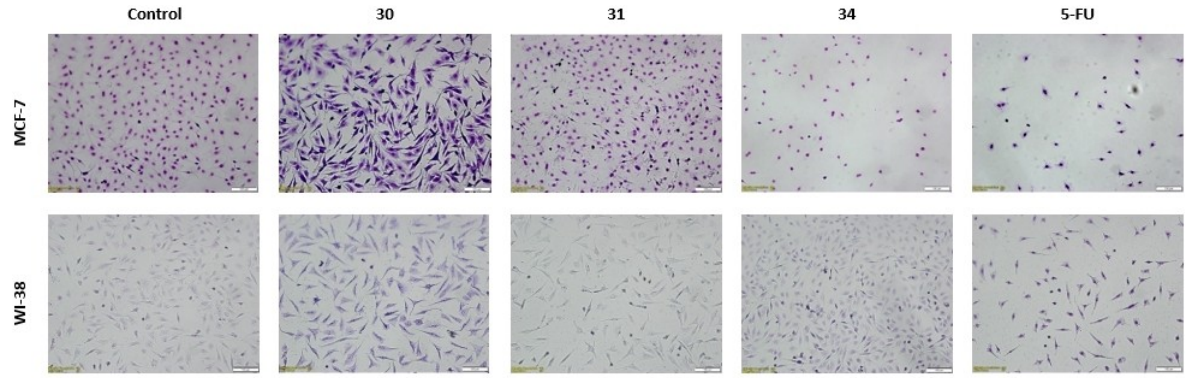


Figure 4. Micrographs showing analysis apoptosis of **30**, **31** and **34** on MCF-7 and WI-38 cell lines using inverted microscope for live cells imaging, and 5-FU drug were used for control (stained with GIEMSA). Colors show dead cells as they go dark purple. **30**, **31**, **34** and 5-FU are showed Giemsa staining in 40x magnification objective.

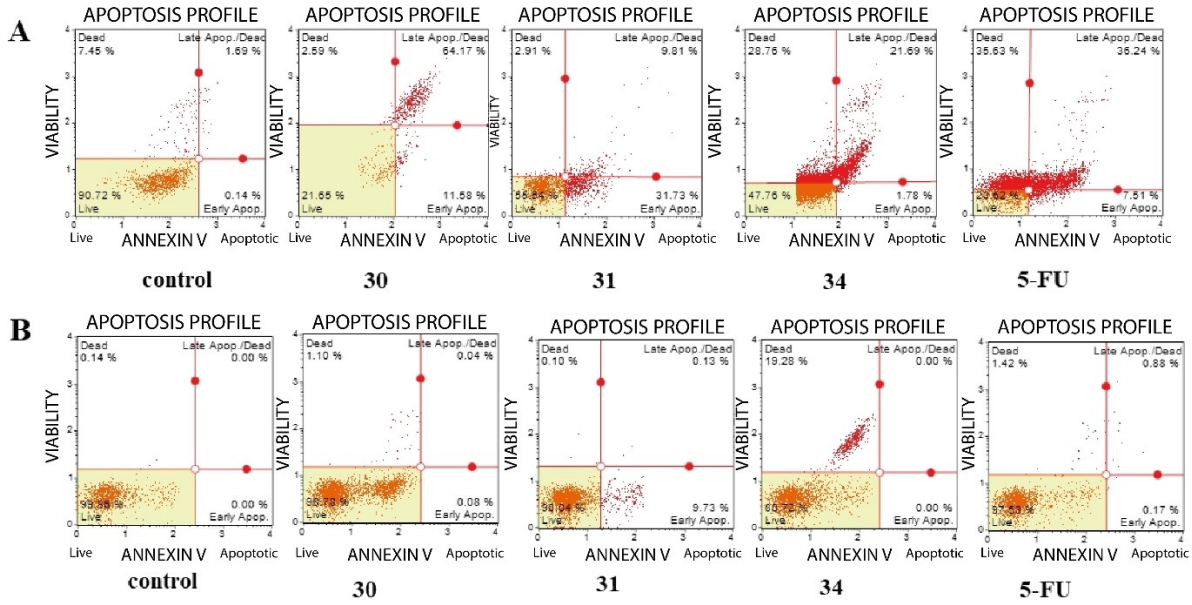


Figure 5. Treatment of MCF-7 (A) and WI-38 (B) cells with compounds **30**, **31**, **34**, and 5-FU for 24 h by quantification of apoptotic cells through flow cytometric analysis.

The inhibitory effect of the compounds on COX enzymes appears to vary depending on the presence of groups in the 5- and 6- position of the benzoxazole structure. Unsubstituted structures **22**, **23**, **24** did not show any effect on COX enzymes, while on compounds **37**, **38**, **40** the presence of methyl or -NO_2 substituents at the 6-position increased the activity. However, generally it is clearly seen that the increased activity is associated with the presence of -OMe and -Cl substituents at the 5 positions of the benzoxazole structure. In addition, among the active structures, COX inhibition was further increased in compounds (such as **30**, **31** and **34**) combined with piperidine and 4-methylpiperidine.

The most potent molecule determined to be effective against COX-2 inhibition and in the MCF-7 breast cancer cell line was compound **30**. In addition, the COX-1 selectivity of compound **34** is quite high (54%), and it is very active molecule in the MCF-7 cell line. Data shown in Table 1 and Table 2 indicate the correlation between the IC_{50} and COX inhibition values found in the cancer cell lines and COXs enzymes.

Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) Designation

The spectrophotometric method, generated by Erel and Kotur-Stevuljevic et al.,[59–61] was used to measure TOS. It was also used to measure TAS using 10 mmol/L 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS) as a chromogen.

The antioxidant properties (TAS and TOS) of molecules were investigated, and Table 1 shows the parameters of oxidative stress for compounds. A general evaluation showed

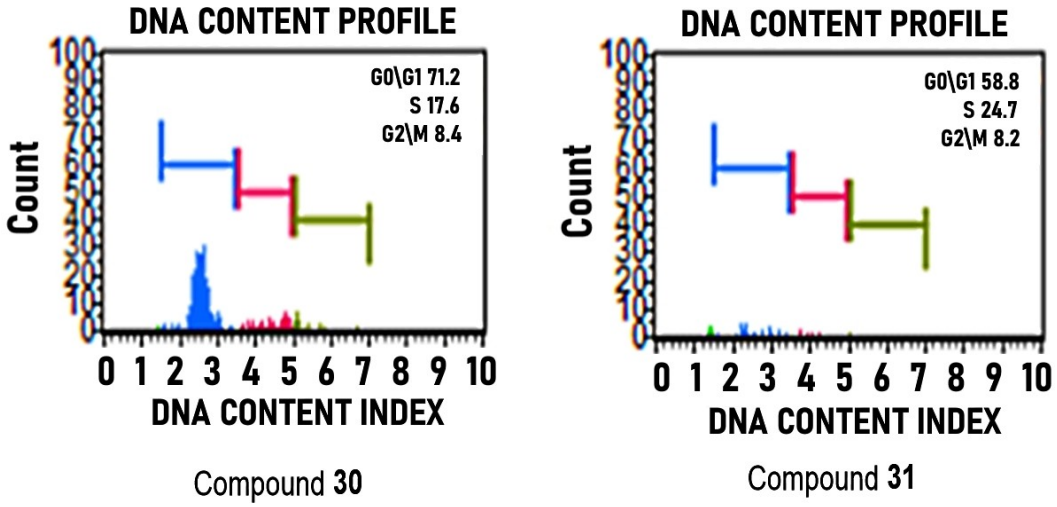


Figure 6. Cell cycle analysis of MCF-7 cells treated with compounds **30** and **31**.

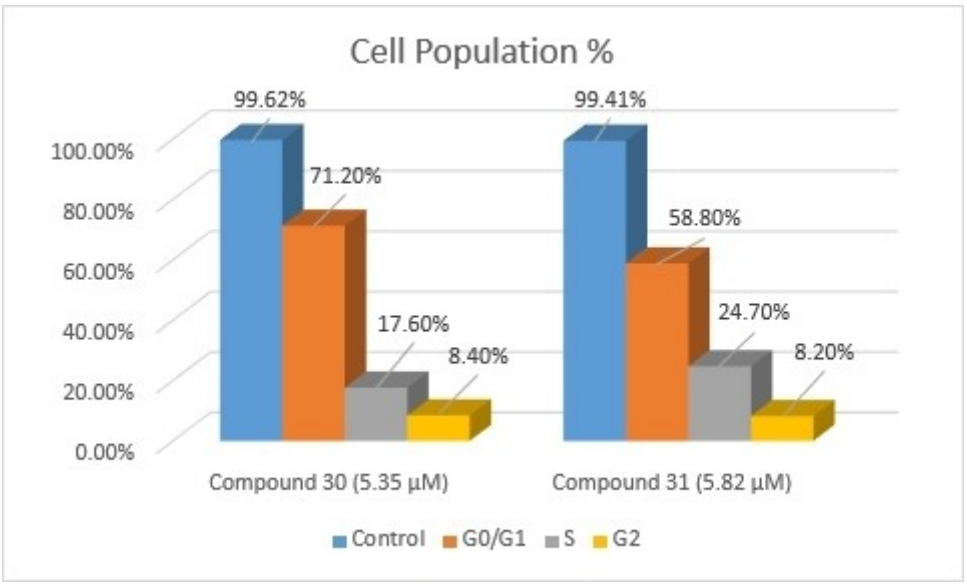


Figure 7. Cell population percentage of MCF-7 cells treated with compounds **30** and **31** in cell cycle.

that TAS and TOS concentrations of compounds **24**, **25** and **33** were significantly higher than other compounds. Together, high levels of TOS and TAS indicate high oxidative stress. Compound **33** has the highest TAS and TOS levels with 2.05 mmol/L and 3.95 µmol/L, respectively (Table 1). Compared with Trolox (a water-soluble analog of vitamin E), compounds **23**, **26**, and **33** had significantly higher TOS concentration. Furthermore, almost all compounds have significantly higher TOS concentrations than TAS.

The organism develops an antioxidant defense system against free radicals that may occur from endogenous or exogenous origin. However, in some cases, as a result of not stopping the increase of free radicals, the oxidative-antioxidative balance is disrupted and oxidative stress occurs. Oxidative stress balance is determined by OSI (oxidative stress index) calculated by TOS and TAS ratio.

In general, based on the results, it is seen that 6-methyl substituted benzoxazole derivatives have higher TAS and TOS values compared to other synthesized compounds. In particular, the molecule **33** was found to have the highest TAS and TOS (2.05 mmol/L and 3.95 µmol/L, respectively) test levels. OSI values approaching zero indicate that the organism's defense system against reactive free radicals is in balance. Among the tested compounds, **24**, **33**, and **35** can be seen to reduce oxidative stress at the highest rate. The OSI values of the remaining compounds are below 0.14, and it can be said that they are molecules with a very high antioxidant effect.

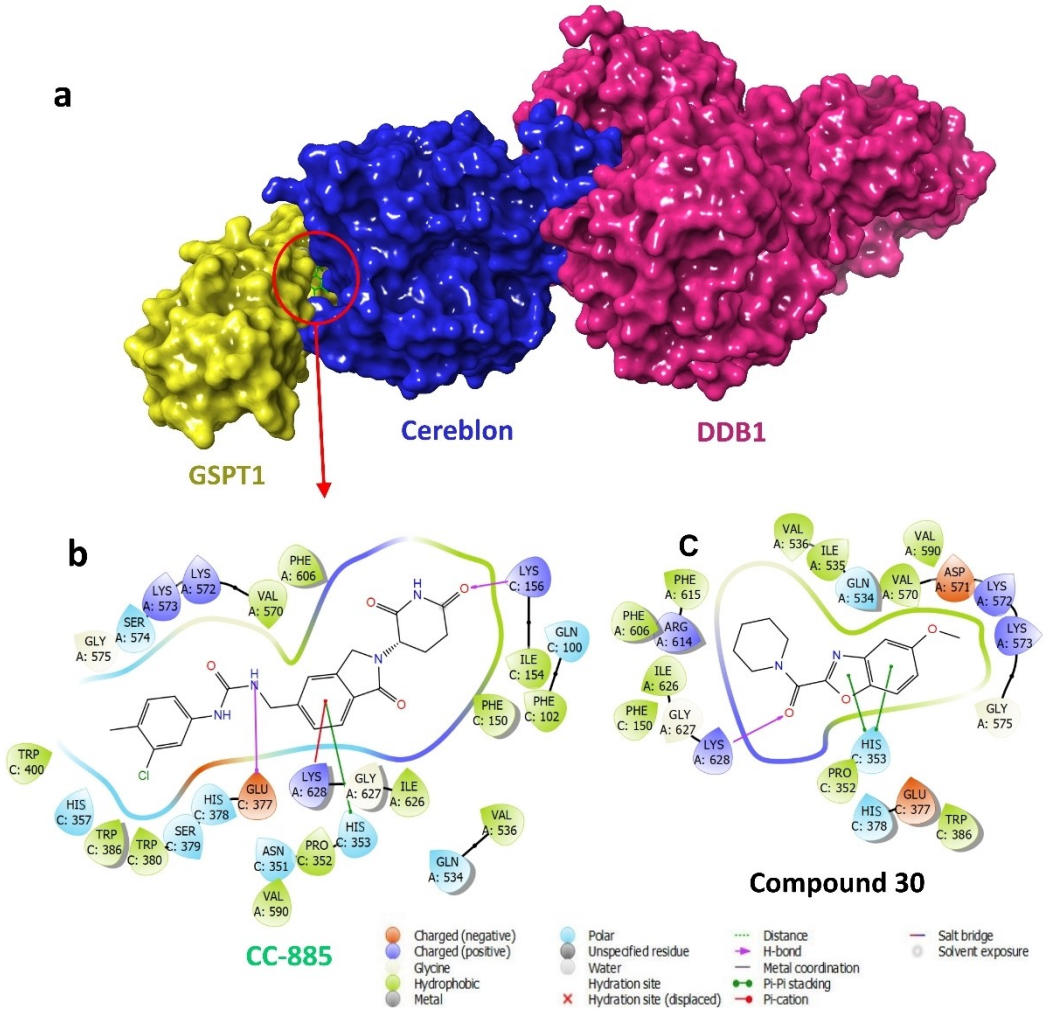


Figure 8. Crystal structure of the DDB1, CRBN, CC-885, GPTS1 complex. a, Surface representation of 5HXB pdb encoded protein. b, 2D interaction of CC-885 with Cereblon and GPTS1. c, 2D interaction of Compound **30** with Cereblon and GPTS1.

In vitro Cytotoxic Activity

The activities of thalidomide and its analogs against MCF-7 and MDA-MB-231 cell lines were investigated in the literature. IC_{50} values of thalidomide in these cell lines were calculated as 360 ± 2 and 413 ± 2 μM respectively. The IC_{50} values of the compounds designed as thalidomide analogs were calculated between 40.3–500 μM in the MCF-7 cell line and 37.2–500 μM in the MDA-MB-231 cell line.[36] In addition, the antitumor drug 5-FU was screened against hormone-dependent MCF-7, hormone-independent MDA-MB 231 cell and it displayed IC_{50} values in the range of 4.7–9.6 μM in the tested cell lines.[62] Therefore, we compared the in vitro cytotoxic effect of our target compounds with 5-FU in the indicated cell lines.

Our synthesized compounds (**21**–**40**) were tested for their antiproliferative activity in MCF-7 and MDA-MB-231 cell lines. Normal cell lines WI-38 (fibroblast cell lines) was used as control as shown in Table 2. Table 2 showed that against MCF-7 and MDA-MB-231, most of this series of compounds have similar cellular activity with IC_{50} values ranging from 5 to 12 μM. In the study of the cytotoxic effect of MCF-7 and MDA-MB-231 cancer cell lines, it can be concluded that the compounds are more sensitive to MCF-7. Among them, compounds **30** ($IC_{50} =$ 5.35 μM), **31** ($IC_{50} = 5.82$ μM) (with a piperidine or 4-Me-piperidine and carbonyl bridge linking group to 5-methoxy-substituted benzoxazole), had the most potent anti-proliferative activity against the growth of MCF-7 cancer cells.

The 4-methylpiperidine moiety of **31** and **39** and piperidine moiety of **30** and **34** contributed to a slight increase in the anti-proliferative activity against MCF-7 cell line as compared with other compounds which have pyrrolidine and morpholine. After examining the molecule's effect on the MCF-7 cells, it was found that all compounds were effective, but the most biologically active molecule were compound **30** ($IC_{50} =$ 5.35 μM), **31** (5.82 ± 0.35 μM) and **34** ($IC_{50} = 6.62$ μM). Although these molecules have comparable activity to the standard drug 5-FU (3.95 ± 0.21 μM), the toxic effects of compound **30** and **31** (19.04 μM and 19.92 μM, respectively) have less than approximately 2 times compared to 5-FU (9.64 μM) on the healthy WI-38 cells. This shows that the compounds **30** and **31** have a

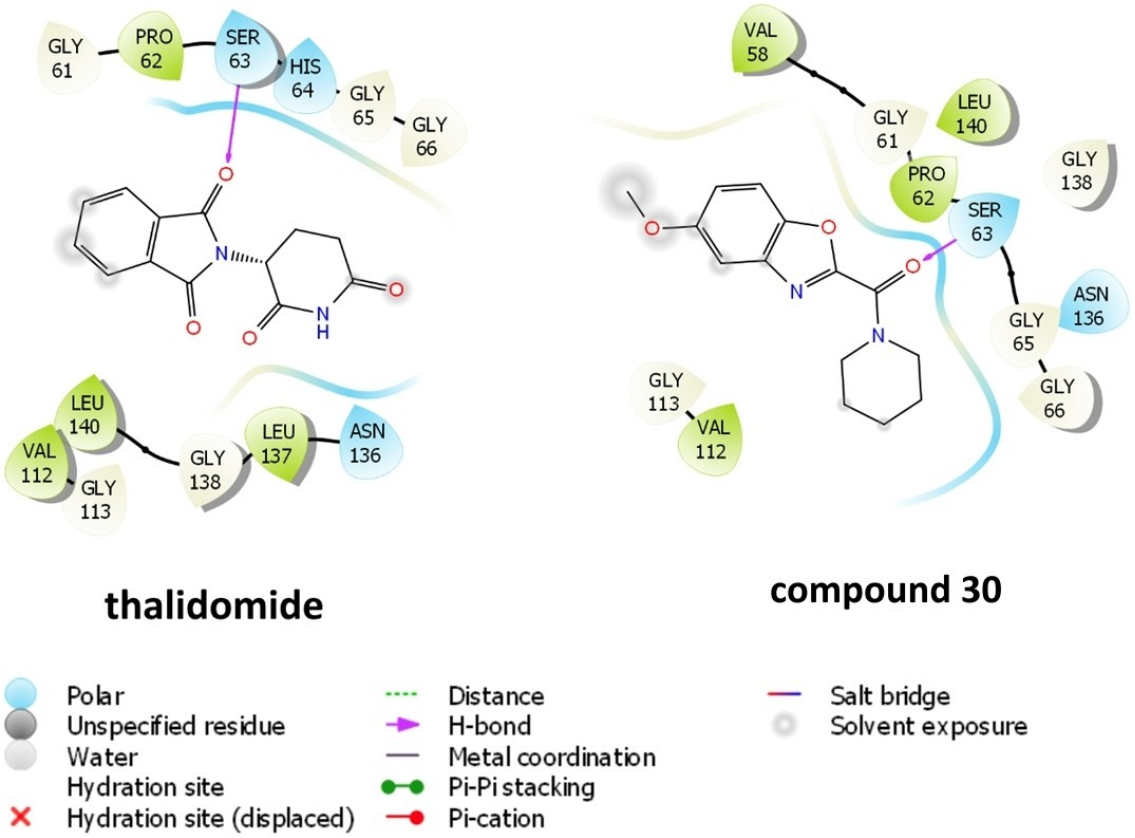


Figure 9. Interaction of Thalidomide and compound **30** with active site of 1NFK.

wider therapeutic index than 5-FU. Moreover, it was found that compound **34** was highly effective with IC_{50} value (6.62 ± 0.09 μM). Furthermore, the specificity of compounds **26**, **30**, **31** and **39** are quite comparable with the **5-FU** on MCF-7 cell lines.

Studying cell morphology and viability and measurement of apoptosis

Based on *in vitro* cytotoxic activity results, compounds **30**, **31** and **34** with the highest efficacy and MCF-7 cancer cell line were selected for further biochemical studies. Morphological changes of selected compounds that treated cancer cells were detected using an inverted microscope. In the study, the morphology and viability of the treated cells were affected minimally even at higher concentration. This behavior is confirmed by the IC_{50} value, where compounds were found to be 0.75 ± 0.15 mg/mL in MCF-7 and MDA-MB-231 cells. Giemsa staining was used to investigate the morphological assay of cell death in order to determine whether the compounds' growth inhibitory activity was related to apoptosis induction. The investigation revealed the changes associated with apoptosis in MCF-7 and WI-38 cells treated with the compounds. In untreated control cells, a uniformly pink fluorescence stain of nucleus was observed (Figure 4).

5-FU has approximately 20% viable cells in the MCF-7 cell line, which is like that of compound **34**. However, cancer drugs cause cell death through apoptosis and not by necrosis. Therefore, compound **34** is more beneficial because it kills cancer cells by causing apoptosis at a higher rate than 5-FU. Furthermore, no toxicity was observed in the healthy WI-38 cell line treated compounds. MCF-7 cells treated with **34** can be said to have a shape similar to the untreated control group. It can also be said that MCF-7 cells treated with the most potent antiproliferative compounds **30** and **31** produced morphological changes similar to cells treated with 5-FU. The results also demonstrated that rate of apoptotic cell death in the tested MCF-7 cells was significantly increased by the synthesized compounds **30** and **31**.

Flow Cytometric Analysis

Efficient compounds **30**, **31**, and **34** were selected to investigate the mechanistic action of cell cycle arrest against breast cancer MCF-7 cells, and cell cycle was evaluated by flow cytometry method. Flow cytometry can determine how many cells are in which division phase. Although there are diploid cells in the G0/G1 phase, the cell's DNA content in the S phase will be between the diploid and tetraploid cells. The shortest phase is the M phase, and the amount of its DNA is the same as the G2 phase. Cells in G2 and mitosis will be tetraploid since they carry 4n amount of DNA. Thus, information about the cell's DNA content is obtained. Flow cytometric analysis of the

cell's DNA content mostly uses fluorescent dyes, which binds to the cell's DNA. The amount of dye bound to DNA is directly proportional to the total amount of DNA in each cell. The most commonly used DNA dyes are propidium iodide, ethidium bromide, and acridine orange.[63]

The effect of compounds **30**, **31**, and **34** on the cell cycle of the MCF-7 cell line was determined by flow cytometry using the DNA content kit. Results of MCF-7 cells treated with IC_{50} dose of compounds **30**, **31**, and **34** for 24 h are shown in Figure 5.

Annexin V-FITC method by flow cytometry was applied to quantitatively determine the apoptotic cells. Compare to 5-FU, which we use as a positive control, it is observed that the apoptosis-necrosis ratio (29.25) is high in MCF-7 cancer cell line of compound **30** and the viability is high in the healthy cell line. In addition, it shows that the apoptosis-necrosis ratio (14.28) in compound **31** is higher than 5-FU. For compound **34**, the apoptosis/necrosis ratio (0.82) was observed to be almost the same as 5-FU (1.28) in Table 3.

The viability rate of compounds **30**, **31**, and **34** in a healthy cell line was also found to be high. These results demonstrated that the compound is targeting the breast cancer cells, with minimum effect on the healthy cells. In addition, it can be said that the addition of electron-releasing R groups such as Cl and 5-OMe to the synthesized compounds containing piperidine and 4-methylpiperidine rings in their main skeleton may increase anti-cancer activity. Among them, derivatives of benzoxazole structure with 5-OMe substituent combined with piperidine and 4-methylpiperidine (compounds **30** and **31**) have the highest apoptosis rate in MCF-7 cancer cell line.

The compounds 30 and 31 induced G0/G1 phase cell cycle arrest in MCF-7 cells

Cell cycle is the process where a series of transient biochemical activities and morphological changes occur in cells that have been stimulated to proliferate.[64] Cell cycle progression is regulated by checkpoints that detect possible errors in DNA synthesis and chromosome separation.[65] One of the target mechanisms accepted in cancer treatment is cell cycle arrest.[66,67]

Efficient compounds **30** and **31** was selected to investigate the mechanistic action of cell cycle arrest against breast cancer MCF-7 cells, and the cell cycle was evaluated by flow cytometry method. MCF-7 cells were treated with compounds **30** and **31** for 24 h, and the untreated cells were used as control. After treatment of compounds **30** and **31**, the procedures used to analyze the cell cycle by flow cytometry (Figure6).

Results of the compound **30** on MCF-7 cells showed 71.2% in the G0/G1, 17.6% in the S, and 8.4% in the G2/M phases. However, as illustrated in Figure 7, MCF-7 cancer cells treated with compound **31** showed 58.8% in the G0/G1, 24.7% in the S, and 8.2 in the G2/M phases. Compared with the control cells, it was observed that MCF-7 cancer cells treated with compounds **30** and **31** significantly increased in the G0/G1 phase, but decreased in the G2/M and S phases (Figure 6). The increased percentage of cells in the G0/G1 phase indicated that compounds **30** and **31** significantly induced G0/G1 phase cell cycle arrest in MCF-7 breast cancer cells.

3D Shape Similarity

3D shape similarity was conducted to describe the scaffold similarity of compounds to thalidomide. Thalidomide selected as query compound. The synthesized compounds were screened against using the untyped atoms methods (pure vdW volume to treat all atoms the same for defining overlaps) Shape Screening module in Maestro.[68] The shape similarity score of the compounds was found between 0.773 and 0.867.

Docking Studies

CRBN was identified as the single target of thalidomide binding. The mechanism of action of thalidomide has been reported to be related to its ability to bind with the CRBN protein located in the E3 ubiquitin ligase complex ($CRL4^{CRBN}$). CRBN, the E3 ligase adapter protein, mediates the induction of neo-substrate proteins by imide compounds such as thalidomide.[44,69] In addition, anticancer effect was observed as a result of degradation of translation termination factor G1 to S phase transition protein 1 (GSPT1), which is in its complex structure with CRBN.[45]

Molecular docking was performed with 5HXB (Cereblon in complex with DDB1, CC-885, and GSPT1) pdb coded protein in order to determine that synthesized compounds can bind with CRBN. For the validation of the docking study, CC-885 (native ligand) contained in the protein was removed, redocked and the RMDS value was calculated as 0.147 Å. Although the docking score of CC-885 molecule is −8,420 kcal / mol, the docking scores of the synthesized compounds (compounds **21**–**40**) are between −7,146 kcal/mol and −8,202 kcal/mol. Compounds **30**, **31** and **34** with the best activity among the compounds also have the highest docking scores (8.202, 8.170, 8.129 kcal/mol, respectively). It has been observed that the binding of CC-885 with residues (GLU337, LYS628 and HIS353) located in the active site of the protein is mediated by the phthalimide ring. It also interacted with the CC-885 glutarimide ring with Cereblon. Compound **30** performed similar interactions with the benzoxazole ring in the structure of the compounds instead of the phthalimide ring (Figure 8). The interactions at the active site of the protein of compound **31** and **34** are similar to that of compound **30**.

The interactions of Compound **30** and CC-885 at the active site of the Cereblon-GPTS1 complex were investigated by molecular docking studies. Similar to CC-885 compound **30** made the pi-pi stacking interaction with HIS353, made hydrogen bonds with LYS628. Although CC-885 made hydrogen bonds with GLU377, compound **30** interacted negatively with GLU377. Also, similar to CC-885 compound **30**, made polar interaction with GLN534, HIS378; charged (positive) interaction with LYS572, LYS573, LYS628; interacted hydrophobically with VAL570, VAL536, VAL590, TRP386, PRO352, PHE150, PHE606, ILE626. These results suggest that compound **30**, like the

thalidomide derivative CC-885, exerts an antiproliferative effect on Cereblon.

Nuclear factor kappa B (NF-κB) in the Rel family is a transcription factor. It plays an important role in maintaining important processes such as differentiation, proliferation and apoptosis in the cell. In addition, it has been reported in the literature that it has an effect on cellular transformation and tumorigenesis. NF-κB exists as a complex p65-p50 heterodimer in the cell.[70]

In docking studies, the p50 subunit of NF-κB protein (PDB ID: 1NFK) was selected as possible the target macromolecule and the anti-proliferative activities against breast cancer cells were evaluated. The ligand interactions at the crystal structure's binding site of the p50-NFkB protein were analyzed. Once the enzyme's active zone was determined, the grid box was created accordingly, and the modeling study was carried out on the area containing these residues. As a result of docking studies in the active region of the protein, the docking score thalidomide was calculated −3.014 kcal/mol whereas the docking scores of the synthesized compounds are between −3,070 and −4,857 kcal/mol (Table S1).

Compounds **30**, **31** and **34** had the highest docking score at −4.857, −4.377, and −4.081 kcal/mol, respectively (Table S1). The compound's interaction with residues in the protein's active site is given in Table 3. The interaction of thalidomide and compound **30** with the active site of 1NFK is also given in Figure 9.

The activities of the synthesized compounds are not higher than 5-FU but higher than thalidomide. However, the compounds **30**, **31** and **34** activities are noteworthy. Compound **30**, which shows the best activity among the synthesized compounds, interacted with the O atom in the carbonyl group with hydrogen bonding with SER63 and interacted with GLY65. Furthermore, thalidomide's failure to H-bond with residues in the active site may have caused lower activity.

The synthesized compounds interacted with SER63, GLY65, PRO62, VAL112, GLY66, GLY113, GLY138, SER72, PHE53, PRO68, PRO62, SER63, GLU73, LYS49, LYS74, LYS77, LYS54 which found at the enzyme's active site however, their activity is thought to be reduced compared with compound **30** due to the inability to interact strongly with SER63, PRO62, VAL112, GLY65, GLY113, GLY138. In addition, it was determined that anticancer activity increased significantly with interactions, especially with SER63 and GLY65. It was found that enzyme interaction with residues in the protein's active site and compound **30** and made hydrogen bonds with SER63 at a distance of 2.30 Å. It is thought that such interaction distances may play an important role in anticancer activity (Table 4).

Docking studies were conducted for COX-1 and COX-2 inhibition, with synthesized compounds and reference molecules SC-560 and DUP 697. All docking results are given in Table S2.

Conclusion

In summary, a series of benzoxazole derivatives similar to thalidomide was synthesized through a simple, applicable, high-efficiency, and fast method. The TAS and TOS values of synthesized derivatives were tested. The molecules were found to have high-capacity antioxidant properties according to the results obtained. Also, the anti-inflammatory activity of **30** and **34** was remarkable. In addition, *in vitro* tests performed on two different breast cancer cell lines showed that compared to the standard drug 5-FU, the molecules had a comparable anticancer effect and lower toxicity on healthy cells, which shows that the selective molecules could be a candidate drug for breast cancer. Moreover, the fact that compound **30** and **31**, the most effective molecules in breast cancer, show approximately 50% inhibition with the COX assay, which indicates a correlation between breast cancer and COX enzymes. As a result, the data obtained show that the molecules are candidate molecules with antioxidant capacity, highly effective for breast cancer cell line, and are an alternative for inflammation.

Table 4. Intermolecular interactions of the most active compounds in the docked complex of p50-NFkB (PDB ID:1NFK).

Compounds	Ligand atoms	Interactions Type	Protein residues	Distance in Å
30	O	H-bound	SER63	2.30
		Hydrophobic	VAL58, PRO62, VAL112, LEU140	
		Polar	ASN136	
		Glycine	GLY61, GLY65, GLY66, GLY113, GLY138	
31	O	H-bound	SER63	2.79
		Hydrophobic	PHE53, PRO68	
		Polar	SER63	
		Charged (-)	Glu73	
		Charged (+)	LYS49, LYS74, LYS77	
34		Hydrophobic	PHE53, PRO68	
		Polar	SER63, SER72	
		Glycine	GLY65, GLY66	
		Charged (-)	Glu73	
		Charged (+)	LYS49, LYS54 LYS74, LYS77	
Thalidomide	O	H-bound	SER63	3.98
		Hydrophobic	PRO62, VAL112, LEU137, LEU140	
		Polar	HIS64, ASN136	
		Charged (+)	GLY61, GLY65, GLY66, GLY113, GLY138	

In our study, IC_{50} values of the compounds which anticancer activities were determined. The cell's apoptotic images were obtained by giving their own IC_{50} values, and its stages were analyzed by flow cytometry. Flow cytometric analysis with IC_{50} dose determined that the cells in the three compounds (**30**, **31** and **34**) increased the early stages of apoptotic death process compared with the control group. To support the results, microscopic analysis revealed that there were apoptotic markers in the cells after treatment with these three compounds. In many sources, cancer cells that grow uncontrollably are also called immortal cells. Normally, a cell that should die does not die, and the further proliferation of this cell initiates cancer. The purpose of cancer treatment is to stimulate the apoptosis of cancerous cells. In this study, three compounds were found to significantly reduce the survival rates of cells in MCF-7 cancer cells. We also observed that there were no toxic effects observed on the healthy WI-38 cells. It was also demonstrated that these compounds exhibit this effect through apoptosis stimulation. The effect of these compounds may shed light on future cancer treatment strategies and cancer research.

Supporting Information Summary

The experimental section containing syntheses and detailed characterization of all compounds, the NMR spectra of compounds **7–11**, **14–18** and **21–40** as well as biological analyses and details on molecular docking study can be found in the supporting information.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: anticancer activity · benzoxazole · COX inhibition · cytotoxic activity · molecular docking

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