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Anticancer effects of a newly-synthesized benzoxazole-derived 5-amino-2-[P-bromophenyl]-benzoxazole in breast cancer cell lines

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Abstract

We investigated the anticancer potential of 5-amino-2-[p-bromophenyl]-benzoxazole [BB] which is a new benzoxazole derivative in different breast cancer cell lines. BB was applied to estrogen receptor positive [ER+] MCF-7 and receptor negative [ER-] MDA-MB cell lines and its effects were determined by histopathological examinations, viability test [MTT], immunocytochemistry assays [Tunnel, VEGF and eNOS]. Moreover, its effects on apoptozis-related proteins such as Apaf-1, cytochrome C, caspase-3, bcl-2 and NF-κB were examined by western blot. Histopathological examinations showed that BB killed many cells for both cancer cell line while the cells lost their adhesion in MCF-7 and lost their adhesion and epiteloid morphology in MDA-MB. MTT analyses demonstrated that BB caused a clear dose depended toxic effect for both cell types. BB application resulted in an increase labeled apoptotic cells after Tunnel staining which was more obvious for MDA-MB compared to that of MCF-7. VEGF staining was decreased after BB application in both cell lines. The decrease of VEGF staining was clearer for MDA-MB compared to that of MCF-7. The status of oxidative stress was shown by eNOS immunocytochemistry which was increase after BB application in both cell lines. The levels of Apaf-1 and bcl-2 were found to be similar while caspase 9 and NF-κB levels were decreased compared to the control group after BB application for both cell lines. On the other hand, cytochrome C levels were slightly increased in MCF-7 cells while this increase was found very obvious in MDA-MB cell lines in BB groups. In conclusion, BB which is a new benzoxazole derivative have shown significant anticancer effects by increasing apoptosis and decreasing angiogenesis in MCF-7 or MDA-MB breast cancer cell lines. These effects of BB seem to be more prominent for [ER-] MDA-MB cell lines which mimic more aggressive type of breast cancer. These results suggest that BB may be useful to treat [ER-] breast cancers and may be added to treatment protocols.

Keywords: Benzoxazole derivative, breast cancer, apoptosis, angiogenesis

Introduction

Cancer is a disease that causes the uncontrolled proliferation of cells and sometimes fatal results, the most common of which is breast cancer, some types of breast cancer are very resistant to drug treatment and are the second leading cause of female deaths. Estrogen receptor positive [ER+] MCF-7 and negative [ER-] MDA-MB cell lines are representative examples of breast cancer in culture where MDA-MB mimics more aggressive type. Oncogenes control cell proliferation, invasion, and angiogenesis [1]. Cancer is a disease in which apoptosis and programmed cell death occur and spread to blood vessels and lymphatic system, and apoptosis is seen to be decreased in some cancer types.

In cytochrome-C, one of the most important proteins effective in the apoptosis pathway, cytosolic apoptotic protease activating factor 1 [Apaf-1] and procaspase-9 come together to form an apoptosome with the activation of release from mitochondria into the cytosol in mitochondria [2]. Later in this complex, procaspase-9 is converted to caspase-9 by activating caspase-3 and -7 [3]. The Bcl-2 gene exerts its antiapoptotic effect by inhibiting the release

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of cytochrome C [5]. A decrease in Bcl-2 leads to apoptosis, while an increase stops apoptosis [4]. One of the most important factors with known angiogenic and apoptopic activity in cancer is Nuclear Factor kappa B [NF-kB] [6].

On the other hand, certain heterocylic compounds have been observed to have inhibiting effects on angiogenesis and activating effects on apoptosis. Benzoxazoles are natural nucleotides and the most common heterocyclic compound, with diverse biological activities including antimicrobial [7], antiviral [8], topoisomerase I and II inhibitors [9], and antitumor activities [10].

Recently, we have described the synthesis of some 2,5-disubstituted-benzoxazole derivatives and their in vitro antimicrobial activity against some Gram-positive and Gram-negative bacteria and the fungus Candida albicans and antitumoral activity [11,12]. Especially 2-[p-nitrophenyl]-5- ethylsulphonyl-benzoxazole was found very potent compound for antitumoral activity [12]. Thus, we have synthesized a new benzoxazole derivative which is 5-amino-2-[p-bromophenyl]-benzoxazole [BB] and demonstrated its biological activity [13].

The aim of this study was to investigate the anticancer potential of this new benzoxazole derivative [BB] by using MTT test and histopathological examination in different breast cancer cell lines. Moreover, its effects on apoptozis-related proteins such as Apaf-1, cytochrome C, caspase-3, bcl-2 and NF-κB were examined by western blot.

Materials and Methods

Chemicals and Reagents

Chemicals and solvents were purchased from Sigma-Aldrich Co. and Fisher Scientific. Chloroform was used as the mobile phase.

General procedure for the preparation of 5-amino-2-[p-bromophenyl]-benzoxazole [BB]

5-amino-2-[p-bromophenyl]-benzoxazole was synthesized by heating 0.02 moles of 2,4-diamino-phenol. After mixing 0.02 mol p-bromo benzoic acid with ₂HCl in 25g of polyphosphoric acid [PPA] and at 210ºC for about 2.5 hours as given in Figure 1, the solution poured on ice was neutralized with 10% NaOH. The solution was washed with water and dissolved in ethanol and crystallized [13]. The structure of the target benzoxazole derivative was confirmed by IR, 1H-NMR, Mass and elemental analysis.

Reaction time: 2.5 h. Reaction temperature: 210°C. C13H9N2OBr, Yield: 64.83%. Mp: 200°C.

IR [cm-1]: 3445-3212, 3100-3080, 1605, 1517, 1499, 1457, 1250-1060, 900-709 ^{1}H NMR [400 MHz, d-DMSO, [δ ppm] J=Hz]: 8.078-8.058 [d, ^{2}H, J=8.0 Hz], 7.650-7.629 [d, ^{2}H, J=8.4 Hz], 7.350-7.328 [d, ^{1}H, J=8.8 Hz], 7.031-7.027 [d, ^{1}H, J=1.6 Hz], 6.732-6.705 [dd, ^{1}H, J=8.8 Hz, J'=2.0 Hz], 3.733 [s, ^{2}H]. MS [70 eV] m/z: 289.5 [M+H], 291.5 [M+2+H]

Cell Culture

MCF-7 and MDA-MB breast cancer cell lines were seeded in medium containing glutamine and 1 penicillin/streptomycin. Cell lines were then passaged. These cells were plated and FBS was added. And it was expected to grow. The morphology of the cells was then observed under the microscope [14].

MTT Assay

Mitochondrial functions of MCF-7 and MDA-MB cells is a colorimetric method that shows the presence of living cells. MTT is measured spectrophotometrically between 570 and 690 nm.

Immunocytochemistry Assay

MCF-7 and MDA-MB cells were fixed with 4% paraformaldehyde in PBS and then washed with 0.1% Triton X-100 in PBS tween [PBST]. Cells were then incubated with biotinylated IgG for 30 minutes, then washed in PBS and then with streptavidin-peroxidase conjugate and PBS. Each sample was incubated with a solution containing 50 µl of diaminobenzidine, the samples were then visualized using an invert-fluorescent-phase microscope. Images were computerized for further analysis of the h-score, which is the result of multiplying the percentage of cells with staining intensity.

Terminal deoxynucleotidyl transferase dUTP nick end labeling [TUNEL] Assay

Colorimetric system kit was used for TUNEL. Cells were treated 2 times with paraformaldehyde, incubated with proteinase K and washed. These cells were then incubated with terminal deoxynucleotidyl transferase. A Ka. Cells washed with buffer solution after treatment were stained with diaminobenzidine [DAB].

Western blot analysis

Protein concentrations of the collected supernatants were determined after the cells were centrifuged. These proteins were separated by gel electrophoresis and transferred to the membrane. Anti-mouse cytochrome c and antimouse monoclonal IgG AIF as primary antibody and alkaline phosphatase conjugated anti-mouse antibody as secondary antibody and β-actin.

Results

In this project, the effect of a benzoxazole derivative [BB] on the selected cell lines which are ER [+] MCF- 7 or ER [-] MDA-MB breast cancer cells was investigated. The behavior of the cells for both cell line were analysed for morphology by phase contrast microscope. There was bipolar morphology for the MCF-7 where as the cells of MDA-MB were found more adhesive with epiteloid shape (Figure 2). Application of BB killed many cells and harmed to the most of the cells for both cancer cell line. The cells lost their adhesion in MCF-7 and lost their adhesion and epiteloid morphology in MDA-MB (Figure 2). The effects of BB were also investigated for both cell lines at IC50 level. The cytotoxic effect of BB was analysed by MTT which showed clear dose depended toxic effect for both cell type with IC50 28 nM for MCF-7 and 22 nM for MDA-MB (Figure 3).

There were specific stainings for VEGF (Figure 4), eNOS (Figure 5) and TUNEL (Figure 6) compared to their negative control. There was decrease in VEGF staining after BB application in both cell lines. The decrease of VEGF staining was clearer for MDA-MB compared to that of MCF-7 (Figure 4, Table 1). The status of oxidative stress was shown by eNOS immunocytochemistry which was increase after BB application in both cell lines (Figure 5, Table 1). TUNEL staining for the detection of apoptosis was altered after the application of BB. Labeled apoptotic cells were clearly increased after the toxic effect of BB for both cell lines.

It was more obvious for MDA-MB compared to that of MCF-7 (Figure 6, Table 1).

NF-κB and proteins relating to apoptosis such as APAF-1, cytochrome C, caspase-3, bcl-2 were assayed in both breast cancer cell lines by using western blot to investigate the potential anticancer effects of BB (Figure 7-11). The levels of Apaf-1 (Figure 7) and bcl-2 (Figure 8) were found to be similar to the control group compared to BB application for both cell lines. The levels of caspase 9 and NF-κB were decreased in both MCF-7 and MDA-MB cell lines in BB group compared to that of control (Figure 9 and 10). Cytochrome C levels in BB groups were slightly increased in MCF-7 cells while this increase was found very obvious in MDA-MB cell lines compared to the control group (Figure 11).

Table 1. H-score analyses of GB on MCF-7 and MDA-MB cells

Group	TUNEL (mean±SD)	eNOS (mean±SD)	VEGF (mean±SD)
MCF7	19.33±6.11	165.67±20.03	207.33±16.62
MCF7+GB	64.00±8.00***	230.00±13.11**	126.33±15.04***
MDA-MB	20.00±7.21	116.00±19.07	165.33±11.67
MDA-MB+GB	91.33±7.02***	187.33±10.06**	84.00±12.00***

(p<0.01), *(P<0.001), Significantly different from control after 5-amino-2-(p-bromophenyl)-benzoxazole (GB) application

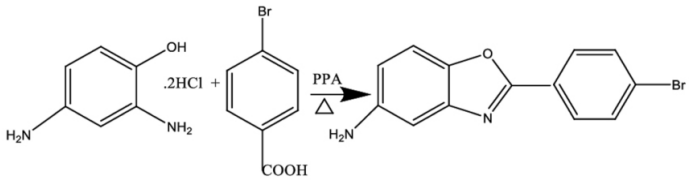

Figure 1. Synthetic pathway of the 5-amino-2-(p-bromophenyl-) benzoxazole (GB)

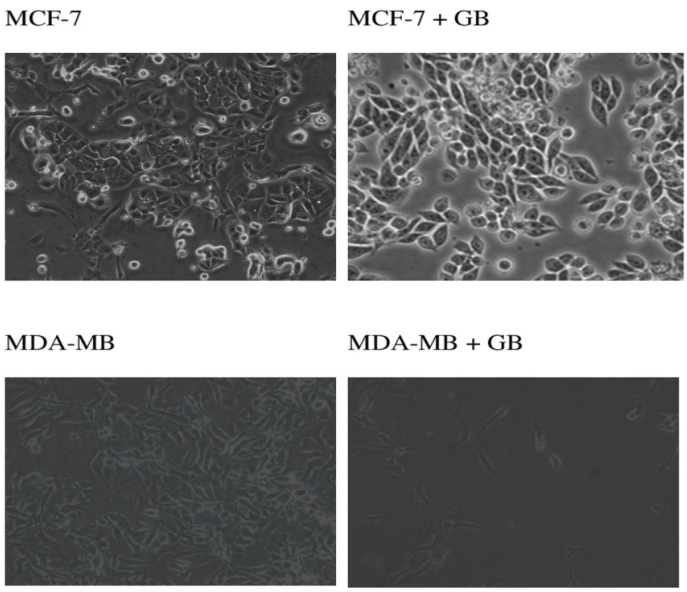

Figure 2. Phase contrast images of the toxic effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on MCF-7 and MDA-MB cells. X 200

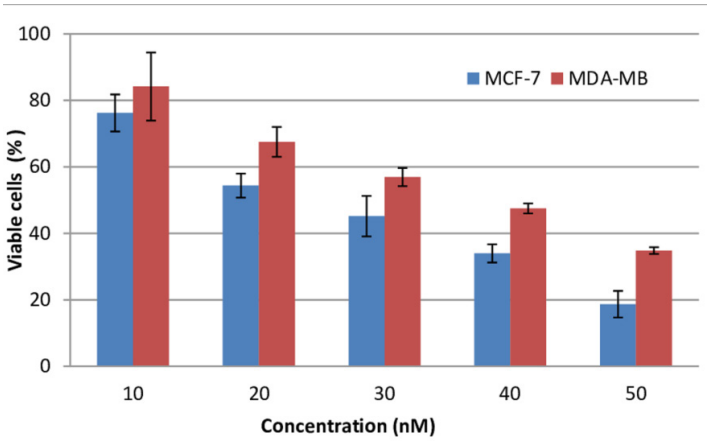

Figure 3. The toxic effect of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on MCF-7 and MDA-MB cells in MTT analysis based on formazone formation reaction, as a percentage of live cells

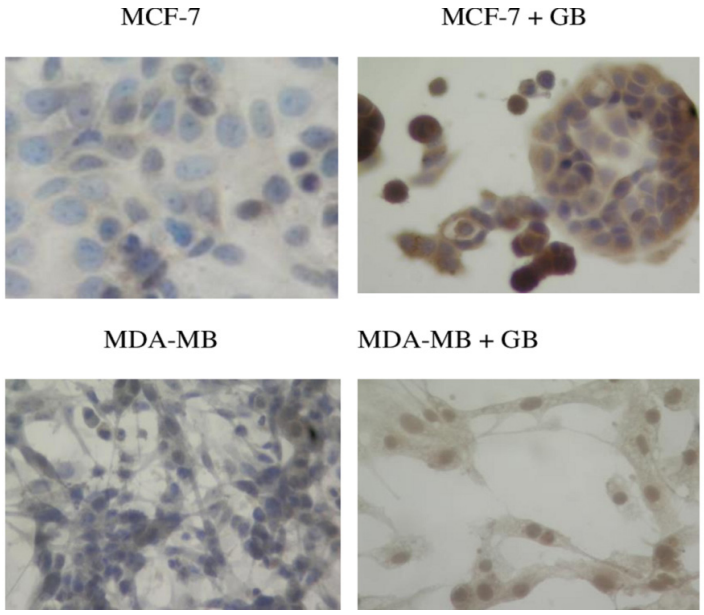

Figure 4. The effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on VEGF immunostaining MCF-7 and MDA-MB cells. X 200

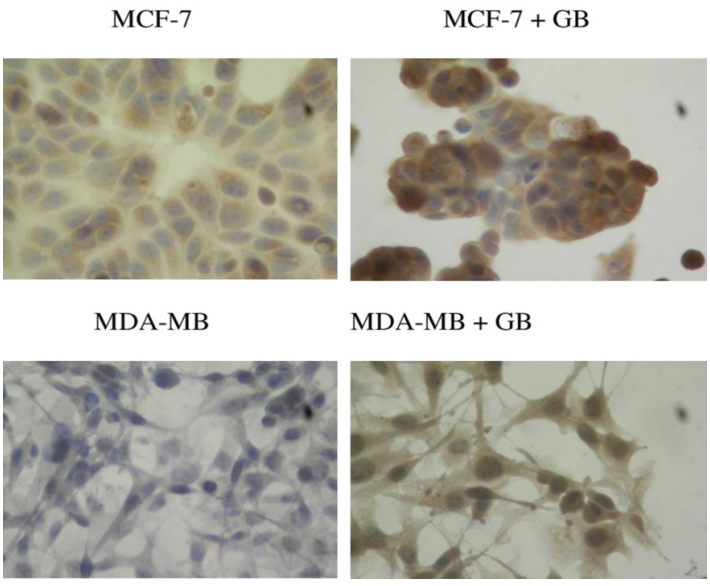

Figure 5. The effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on eNOS immunostaining MCF-7 and MDA-MB cells. X 200

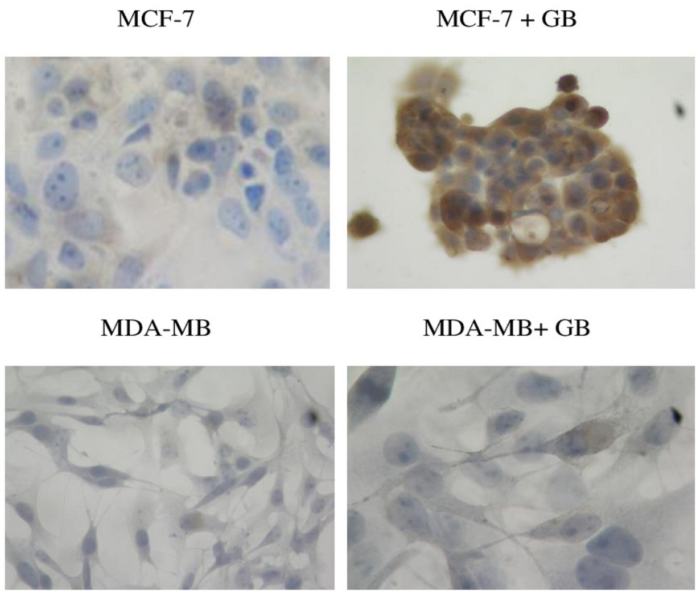

Figure 6. The effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on TUNEL immunostaining MCF-7 and MDA-MB cells. X 200

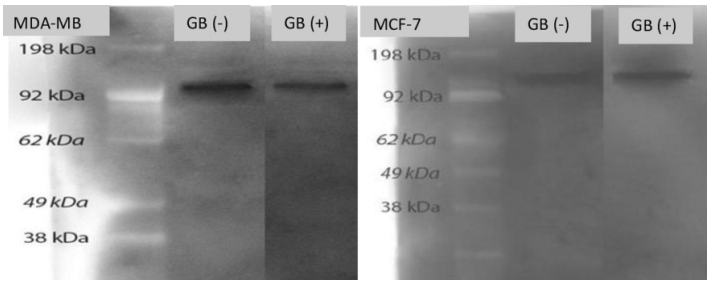

Figure 7. Effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on Apaf-1 levels in MDA-MB and MCF-7 cell lines

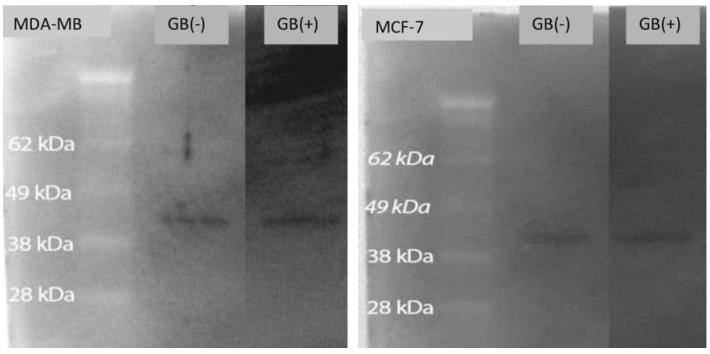

Figure 8. Effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on BCL-2 levels in MDA-MB and MCF-7 cell lines

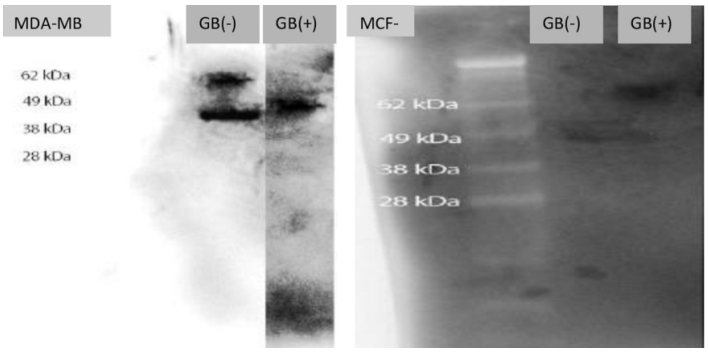

Figure 9. Effects of GB on caspase levels in MDA-MB and MCF-7 cell lines

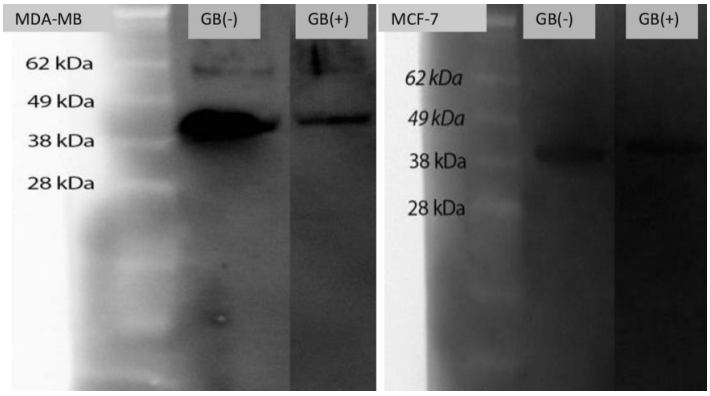

Figure 10. Effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on NF-κB levels in MDA-MB and MCF-7 cell lines

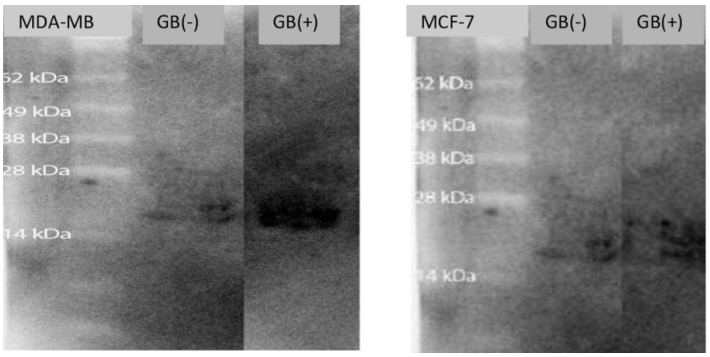

Figure 11. Effects of 5-amino-2-(p-bromophenyl)-benzoxazole (GB) on Cytochrome C levels in MDA-MB and MCF-7 cell lines

Discussion

Treatment of the breast cancer is a major problem in women health and metastasis is responsible for the majority of cancer related death [5]. Breast cancers with the ER [-] compared with the ER [+] cases have been shown to be associated with worse prognostic markers: p53 expression, basal cytokeratin expression, decreased E-cadherin expression, and negative expression of the androgen receptor [15].

In our study, we examinated the anticancer potential of a newly synthesized benzoxazole derivative [BB] on ER [+] MCF-7 or ER [-] MDA-MB breast cancer cell lines by using MTT test, immunocytochemistry assays and determination of apoptosis related proteins APAF-1, cytochrome C, caspase-3 and bcl-2 with NF-κB. It was first established in 1958 that the benzoxazole ring had an antitumoral effect [16]. Between then and the 1990s, effective antitumoral activity was established in benzoxazole compounds carrying bis[2-hydroxiethy]amino group, N-[[p-bis[2-hydroxiethyl]amino]phenyl] formimidoil structure in the second position, which allowed researchers to concentrate on this ring system for its antitumoral effect [17-19]. Sato et al. reported a benzoxazole compound [AJI9561] which was isolated from Streptomyces sp. AC9561, showed mild antitumoral activity [18]. Lage et al. investigated the potential efficiacy of some previously synthesized 2,5-disubstituted-benzoxazole derivatives in various human cancer cell lines [19]. In another study, the monosubstituted benzoxazole compounds bearing pyrazolone ring showed moderate activity against both MCF-7 and A549 cell lines with half maximal inhibitory concentrations of 17.03 and 15.47 μM,

respectively [20]. 2-Arylamino-7-phenylbenzoxazole compound bearing with benzimidazole ring gave submicromolar inhibition of the human cancer cell line MDA-MB-231 on low bind plates [EC50=0.58 μM] [21]. Since the early twentieth century a large of number benzoxazole derivatives were studied and constituted part of the important structures in medicinal chemistry for drug design especially because of varied biological activities that refer to antimicrobial [21,12], antiviral [22], antitumor [10]. Based on these informations, in this study, a new benzoxazole derivative [BB] we have recently synthesized and demonstrated its biological activity was tested for its antitumoral activity in different human breast cancer cell lines. The cytotoxic effect of BB was analysed by using MTT and it showed clear dose depended toxic effect for both MCF-7 and MDA-MB cell lines with IC50 values as 22 nM and 28 nM, respectively.

Apoptosis, which was found to be involved in the development and destruction of cancer cells in the early 1970s, was reported to be programmed cell death that activates intracellular and extracellular signals [23, 24]. Adams and Cory stated that there is a strong relationship between apoptosis and cancer pathogenesis [25]. Takayama et al. showed that there was an escape from excessive proliferation and apoptotic cell death in tumor cells [26]. The study of Watanapocacin et al. on colon adenocarcinoma cell line COLO 205 showed that increasing apoptosis mediated by mitochondria and death receptors inhibits the development of these cells [27]. In our study, TUNEL immunohistochemical staining was used for the detection of apoptosis. Apoptotic cells were clearly increased after the application of BB for both cell lines. Increase in apoptosis was found more obvious for MDA-MB compared to that of MCF-7 cells.

Apoptosis is a pathway regulated by caspase and bcl-2 family proteins [28]. It can increase the expression of anti-apoptotic Bcl-2 members or inactivate the expression of Apaf-1 while decreasing the expression of proapoptotic Bcl-2 members in cancer cells [29]. Since Bcl-2 is located outside the mitochondrial membrane and keeps cytochrome-C inside the mitochondria, it is like the protector of the system. Watson et al. reported that the expression levels of the antiapoptotic Bcl-2 molecule increased in the early stages of colon cancer [30]. After cytochrome C, a protein belonging to the electron transport chain located in the inner membrane of the mitochondria, is released from the mitochondria to the cytosol, Apaf-1 binds to form the procaspase-9 apoptosome [31,32]. Cytochrome-C and Apaf-1 facilitate the degradation of procaspase-9 and the formation of active caspase-9 in the presence of ATP [9]. Wright et al. have demonstrated that Apaf-1 and caspase-9 induced by cytochrome-C are necessary for the intrinsic pathway of apoptosis [33]. In our study, BB application did not change the Apaf-1 and bcl-2 levels in both cell lines while decreased the levels of caspase 9 compared with control. On the other hand, cytochrome C levels in BB groups were increased obviously in MDA-MB but slightly in MCF-7 cell lines compared to that of control. Since the cytochrome C is involved in both intrinsic and extrinsic pathways of apoptosis, these results suggest that BB induced the apoptosis by using extrinsic pathway.

NF-kappa B [NF-κB, Nuclear Factor kappa B] is a transcription factor found in all cell types and found inactive in the cytoplasm. The activated NF-kB pathway can stimulate cell proliferation, apoptosis, angiogenesis, and the ability of cells to invade and metastasize [34,35]. Also, altering NF-kB expression can alter the proliferation and apoptosis ability of tumor cells [35]. On the other hand, reactive oxygen species [ROS] have been shown to interact with NF-κB signaling. The level of NF-κB activity is regulated by the levels of ROS that can be activated or inhibited [36]. In our experiment, it was demonstrated by immunohistochemical staining of eNOS that the use of BB increases the oxidative stress already existing at the basal level and enriches the environment in terms of free radicals. Previous studies suggest that environmental stress reduces the progression of cancer cells and drives cells to death [37]. Therefore, we speculated that BB-induced apoptosis might be related to the NF-κB signaling pathway by reducing the NF-κB levels because of increased oxidative stress. Consistent with our findings, there are articles illustrating that the inhibition of the NF-κB signaling pathway could induce breast cancer cells' apoptosis [32].

VEGF is a mitogenic and antiapoptotic growth factor with proangiogenic activity. It increases vascular permeability and promotes cell migration [38,39]. We also investigated the effect of the heterocyclic compound [BB] on VEGF staining in breast cancer cell lines which is important for the nutrition and invasion of tumor cells. We showed that BB application caused the decrease in VEGF staining in both cell lines which was more evident for MDA-MB compared to that of MCF-7 cells.

Conclusion

In conclusion, BB which is a new benzoxazole derivative have shown significant anticancer effects by increasing apoptosis and decreasing angiogenesis in MCF-7 or MDA-MB breast cancer cell lines. These effects of BB seem to be as a result of decreasing the levels NF-κB in response to increased oxidative stress which activated the extrinsic pathway of apoptosis. Since the differences between BB and control groups are more prominent in MDA-MB compared to MCF-7 cells for morphological findings, Tunnel and VEGF staining, and cytochrome C levels, BB seems to be more effective with regards to its anticancer activity in MDA-MB than MCF-7 breast cancer cell lines. As the [ER-] MDA-MB cell lines mimics more aggressive type of breast cancer which is much more difficult to treat, our results suggest that BB may be useful to treat [ER-] breast cancers and may be added to treatment protocols. Patent application has been done by the Turkish applicant in Turkey.

Conflict of interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical approval
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