Synthesis and the effect of a novel benzoxazole compound on breast cancer cell line

Funda Kosova ORCID:0000-0001-8070-5067
Ozlem Temiz Arpaci ORCID:0000-0002-2485-345X
Ercument Olmez ORCID:0000-0003-3535-2471
Ibrahim Tuglu ORCID:0000-0002-0569-8415

1Celal Bayar University School of Health Vocation, Manisa, Turkey
2Ankara University Pharmacy Faculty, Department of Pharmaceutical Chemistry, Ankara, Turkey
3Celal Bayar University Medical Faculty Department of Pharmacology, Manisa, Turkey
4Celal Bayar University Medical Faculty, Department of Histology and Embryology, Manisa, Turkey

Received 03 January 2019; Accepted 30 January 2019

Abstract

Breast cancer today is the most frequent cancer among women, and the second most common cause of cancer deaths among women. The aim of this study was to synthesize a new benzoxazole derivative, scan it for anti-cancer potential by MTT test using different breast cancer cell lines, and examine its effects on NF-κB and apoptosis-related proteins (APAF-1, cytochrome C, caspase-3, bcl-2) by the western blot method. The newly-synthesized benzoxazole compound was applied to breast cancer cell lines (MDA-MB, MCF-7) and its cytotoxicity was measured quantitatively by MTT test. Later, the level of its effects on NF-κB and apoptosis-related proteins (APAF-1, cytochrome C, caspase-3, bcl-2) were examined by the western blot method. In our study, the structure of the synthesized new 5-[4-chlorobutanamido]-2-(p-methylphenyl)benzoxazole was proved by elemental analysis, 1H NMR and mass spectroscopy analysis methods. When the toxic effects of the application of the compound on the cell lines was examined by MTT, it had a greater toxic effect on MCF-7 when compared with MDA-MB, and IC50 levels were lower. When the protein was examined in immunohistochemistry with regard to VEGF, eNOS and TUNEL, it was observed that it caused a reduction in VEGF and an increase in eNOS and TUNEL. In the assay of the proteins by western blot, when benzoxazole compound was added to the MDA and MCF-7 cell line, there was no difference from the control group in Apaf-1 and BCL-2 levels, but a reduction was observed in caspase and Nfkβ levels compared with the control group. When the compound was added to the MDA-MB cell line, an increase was shown in the Cytochrome C level compared to the control group, but no difference was seen in the MCF-7 cell line. It is felt that this synthesized new benzoxazole compound increases apoptosis by reducing the activation of Nfkβ, and in this way has shown an effect of inhibiting tumor growth in cancer treatment. In addition, it is felt that this can provide hope in cancer treatment by the improved phase studies.

Keywords: Benzoxazole compounds, NF-κB, APAF-1, sitokrom C, caspase-3, bcl-2

Introduction

Cancer is a disease which caused by changes in critical genes that control cell proliferation, differentiation and survival. Continuous and uncontrolled proliferation of cells causes cancer [1,2]. Cancer and apoptosis are common in the cell population dynamics [3]. Cancer is one of the most common causes of death in the world. In the last decade there have been great advances in the diagnosis and treatment of these patients. However, the prognosis of some cancers is very poor. Apoptosis and angiogenesis are important factors for growth and differentiation, but are also important for tumor invasion and metastasis. Nuclear Factor kappa B (NF-κB) has been shown to play an important role in both apoptosis and angiogenesis mechanisms. Apoptosis is a controlled and programmed cell death process [4], which is activated by three pathways by intracellular and extracellular signals, regulated by many proteins. In the early 1970s Kerr et al, found that apoptosis was associated with the elimination of potential cancer cells and tumor development [5]. Adams and Cory’s shown that a strong relationship between apoptosis irregularity and cancer pathogenesis [6]. Takayama et al. shown that proliferation of tumor cells and escape of apoptotic cell death are present [7]. Wang et al. stated that apoptotic activity in breast cancer would be initiated by combined treatment regulating caspase cascade [8]. Li et al reported that Bcl-2 was effective in the early diagnosis and treatment of breast cancer [9]. NF-κB is mainly active and located in the nucleus. In some cancers (such as some Hodgkins and diffuse large B-cell lymphoma cells), due to chronic stimulation of the IKK pathway, the IkB genes may be mutated and damaged. Furthermore, the Rel / NF-kB transcription
Cell Culture studies were made by Celal Bayar University medical faculty, department of Histology and Embryology. A human breast cancer cell line MCF-7 and MDA-MB were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in DMEM F-12 medium supplemented with 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin at 37 °C in a 5% CO2 incubator. The morphology of the cells was examined on alternate days using an inverted microscope (CK40-F200, Olympus, Tokyo, Japan) and photographed. All experiments were repeated for three times.

### Benzoxazole compound application

The compound was prepared as a 1 μg/ml stock solution, which was diluted for use to 100, 50, 25, 12.5 and 6.25 μl solutions in dimethylsulfoxide (DMSO). The same volume of DMSO was used as the vehiclecontrol for Heterocyclic compounds experiments at a final concentration of 0.1%.

### MTT Assay

Mitochondrial functions of MCF-7 and MDA-MB cells were determined by MTT as an indicator of viable cells. This method is a colorimetric assay for measuring the reduction of yellow, water soluble MTT dye to a purple formazan product by active mitochondria. MTT stock solution (50 μg MTT + 10 ml PBS) can be stored in the refrigerator. All PBS in solutions was adjusted to pH 7.4. The culture medium was removed from the culture wells, then 200 μl/well MTT solutions (1 ml stock solution of MTT + 9 ml growth medium) was added and the plate was incubated for 3 h under culture conditions. MTT solution was removed, then 200 μl DMSO was added to each well, and spectrophotometric measurements were performed using a microplate reader at 570 nm with a 690 nm reference filter.
Western blot analysis
The Cells were centrifuged at 12,000× g at 4 °C for 15 min and supernatant (cytosolic extract) were used. Protein concentrations were determined by a dye-metal based colorimetric protein assay [10]. Commercially available Pierce 660 nm protein assay reagent (Pierce/Thermo Scientific, Rockford, IL) which is not affected by the levels of the reducing agents. An equal amount (30 μg) of protein was applied to each well and proteins were separated in a 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis proteins were transferred to PVDF membrane. Membrane were blocked with 3% nonfat dry milk. Anti-mouse (Biovision, USA) cytochrome c, and antimouse monoclonal IgG AIF (Santa Cruz Biotechnology, TX, USA) were used as a primary antibody and alkaline phosphatase conjugated anti-mouse (Invitrogen, NY, USA) was used as a secondary antibody. β-actin primary antibody (Genscript, USA) was used in loading control analysis for normalization. The signal was detected on PVDF membrane by BCIP/NBT Substrate (Invitrogen, NY, USA). Membranes were scanned using densitometer (Bio-Rad, GS-800) and signal intensity was determined by Quantity One Software (Bio-Rad, CA, USA) to compare expression levels among groups. Normalized three independent experiment results were done.

Statistical analysis
Statistical significance was determined using the SPSS 15.0. Significance (IC50 levels) was defined as p ≤ 0.05.

Results
In this project, we synthesized a new benzoxazole compound. We looked at purity controls of the compound by thin layer chromatography and melting degrees. We applied MTT test to the benzoxazole compound and we determined the mitochondrial functions of the cells and thus the cell density of the cells as colorimetric. Those with anticancer potential in terms of survival were separated. Then the remaining group was VEGF, eNOS and TUNEL. Here, the IC50 dose was given the most appropriate response. And then, we examined the effects of the compound which is the most suitable for bioactivity on different breast cancer cell line, NF-κB, and apoptosis (APAF-1, cytochrome C, caspase-3, bcl-2) related proteins with western blot method.

As a result, we carried out biological activity studies on the benzoxazole compound. The structure of the synthesized compound was proved by elemental analysis, 1H NMR and Mass spectroscopic analysis methods (Figure 1).

Although MCF-7 cells were adherent, adherent, and islands-shaped semiconfluent, the cells showed apoptotic morphology with P/22 GBMA administration, proliferation stopped and the majority died. MDA-MB cells were found to be proliferated and died similarly after administration (Figure 2).

Compared with MCF-7, MDA-MB cells were found to be more resistant and less dead at the same dilutions, which was significantly more significant (p <0.001). (Table 1).

When benzoxazole compound was added on the MDA and MCF-7 cancer cell line, we found that Apaf-1 level was similar compared to control group (Figure 3).

When the benzoxazole compound was added on the MDA and MCF-7 cancer cell line, we found that Caspase level was decreased compared to control group (Figure 5).

When the compound was added on the MDA cancer cell line, we found that Cytochrome C level was increased but was similar on MCF-7 cancer cell line compared to control group (Figure 6).

When the compound was added on the MDA and MCF-7 cancer cell line, we found that Nfkβ level was decreased compared to control group (Figure 7).

![Figure 2](image1.png)

**Figure 2.** Cells that are semiconfluent and confluent in MCF-7 and MDA-MB breast cancer cell lines.

![Figure 3](image2.png)

**Figure 3.** The effect of Apaf-1 on MDA ve MCF-7 breast cancer line

![Figure 4](image3.png)

**Figure 4.** The effect of BCL-2 on MDA ve MCF-7 breast cancer line
Discussion

Cancer is a disease which appears when cells multiply in an uncontrolled way with sometimes fatal consequences, and which has been much more commonly seen in recent times. Breast cancer today is the most frequent cancer and the second most common cause of cancer deaths among women. Some types of breast cancer are very resistant to drug treatment. Breast cancer today is the most frequent cancer and the second most common cause of cancer deaths among women. Endogenous estrogen excess is also thought to play a significant role [15]. High endogenous estrogen level has been shown to increase the progression of postmenopausal carcinoma in the breast [16,17].

The aim of this study was to synthesize a new benzoxazole derivative which will investigate for anti-cancer potential by MTT test using different breast cancer cell lines. Moreover, The relation of this effect with apoptosis-related proteins such as APAF-1, cytochrome C, caspase-3 and bcl-2 with NF-kβ by the western blot will be examined.

In 1958, benzoxazole ring was found to have antitumoral effects [18]. Bis (2-hydroxyethyl) amino group, N - ((p-bis (2-hydroxyethyl) amino) phenyl) formimidoyl structure [19], (2-(benzodioxan-5-y)l) The presence of effective antitumoral activity in the benzoxazole compounds carrying the cityl group [20] allowed the researchers to concentrate on the antitumoral effect on this ring system. In another study, have been synthesized which 7-substituted-2-phenylbenzoxazole, benzimidazole derivative with 4-substituted-2-phenylbenzoxazole compounds and this compounds have been reported to have cytotoxic activity on mammals with lower DNA binding properties S. typhimurium [21,22]. Sato et al., found that AJI9561 compound obtained from Streptomyces sp. AC9561 and showed that alsoantitumor activity [23]. In another study, the UK-1 compound has a broad spectrum and potent antitumoral activity (IC50 value around 20 nM) on leukemia, lymphoma and some solid tumor cells, [24]. Lage et al., has found that some 2,5-disubstituted carboxazole, substituted benzoxazine and benzamide-derived compounds were to be highly effective against cancer cells, (stomach, breast, pancreas, fibrosarcoma and melanoma) [25]. In the light of this information, in this study, it is aimed to evaluate the antitumoral activities of this study for the first time by synthesizing novel derivative that carry the benzoxazole ring as the main structure.

Apoptosis is a form of programmed cell death characterized by the morphological changes carried out by the caspases and regulated by Bcl ve 2 family proteins [26]. Cancer cells reduce the expression of pro-apoptotic Bcl-2 members; they may increase the expression of anti-apoptotic Bcl-2 members or inactivate Apaf-1 expression [27]. Decrease in Bcl-2 levels leads to apoptosis, while increased in BCL-2 levels protect from death in the cells [28]. Bcl - 2 is like a guard of the mitochondria due to its presence outside the mitochondria and keeping the cytochrome in the mitochondria. Watson et al. states that the expression levels of the anti-apoptotic Bcl-2 was increased in colon cancer [29].

Cytochrome c is a protein belonging to the electron transport chain in the inner membrane of the mitochondria. After the cytochrome c is released from the mitochondria to the cytosol, it binds to the cytosolic apoptotic protease activation factor-1 (Apaf-1) for create apotosm and binds with procaspase-9 for create apoptosm [30,31]. Apaf-1 is the cytosolic protease activation factor. The induction of apaf-1 occurs by the release of cytochrome c from mitochondria, cytochrome c is the activator of Apaf-1 [32]. Wright et al. found that Apaf -1 and caspase-9 were required for cytochrome-c induced apoptosis [33].

Caspases which are important role in apotosis, are cysteine-dependent aspartate protease family. Cytochrome-c and apaf-1 facilitate the destruction of procaspaz-9 and the formation of active caspase-9 in the presence of ATP. Caspase-9 activates caspases, such as caspase-3 and caspase-7, called caspase cascade [24]. Procaspase-9 is involved in apoptosis and caspase-9 becomes activated and than caspase-9 aktivates caspases such as caspase-3 [34].

NF-kappa B (NF-κB, Nuclear Factor kappa B) is a transcription factor. It is inactive in the cytoplasm. Moves to the nucleus when activated. There are 5 types: NF-toB1, NF-elerB2, RelA (p65), RelB and c-Rel.Rel or NF-kappaB (NF-kB) In addition, these transcription factors continuously activate sites of disease including cancer, arthritis, chronic inflammation, asthma, neurodegenerative diseases and heart diseases [10,35]. Many studies have been cross-linked between MAPK p38 and NF-ylB pathways. Gochman et al. clearly demonstrated the involvement of the MAPK p38 pathway.
in IKK activation. IKK inhibitors decrease Irfβα phosphorylation after exposure to peroxynitrite [35].

In this study compared to MDA-MB, MCF-7 cells had a more toxic effect and IC50 levels were lower. In a previous study, a similar compound was used in vivo and in vitro, and its size-reducing effects were determined in both culture medium and tumor tissue [36]. It is understood that increased oxidative stress and apoptosis due to aggressive and invasive in the cancer cell line is induced by this compound and cause more cell death [37,38]. Another effect of BTB was on cell death. The dilution effect of direct toxic effect was replaced by apoptosis in cell death mechanisms at IC50 level. Drifting to apoptotic death was easier with MCF-7, but less with MDA-MB. Previous studies have shown that other similar compounds that support apoptosis and apoptosis are used as caspase and other activators as signaling pathways [37,39].

It has been shown by immunohistochemical staining that the use of the benzoxazole compounds have already increased the oxidative stress present at the basal level and enriched the environment for free radicals. In previous studies, it has been suggested that ambient stress reduces the progression of cancer cells and leads to death of cells [40,41]. In our studies found that were compared with tamoxifen as a positive control, indicating that our findings would have a similar effect. H40 points to the absence of toxic effects of benzoate compounds on normal cells in negative control studies on non-invasive breast cell lines. However, it is to be understood that there is no toxic effect in normal somatic cells and in vivo conditions in the healthier primary culture. In this sense, it is consistent with the findings of our study [39,42,43]. As a result, it is thought that this compound can be used in the treatment of cancer.

In our study, in the determination of the proteins by western blot, we found that when the benzoxazole compound was added to the MDA and MCF-7 cell lines, there was no difference between the control group of Apaf-1 and BCL-2, whereas the level of caspase and Nfkβ decreased compared to the control group. Cytochrome C levels were higher in MDA-MB cells compared to the control group and no difference was found in MCF-7 cell lines.

Consequently, in our study show that the benzoxazole ring system is structural similar to the heterocyclic adenine and guanine bases in the structure of nucleic acids. This suggests that this group of compounds can exhibit many chemotherapeutic effects over different pathways. We have found that this newly obtained heterocyclic compound is more effective in MDA-MB cell lines. We believe that this benzoxazole compound enhances apoptosis by decreasing the activation of Nfkβ and thus, by inhibiting the growth of the tumor in cancer treatments. As an advanced stage of this study, we plan to investigate how the benzoxazole compound can act on proteins in the angiogenic pathway. If we obtain useful results from this study, then we are planning to carry out studies to support our findings in animal cancer models, which is a higher stage of this study. Therefore, the application of heterocyclic compound appears to contribute to the treatment of breast cancer. By the help of heterocyclic compound breast cancer treatment may help survival of cancer patients.

Competing interests
The authors declare that they have no competing interest.

Financial Disclosure
All authors declare no financial support.

Ethical approval
Consent of ethics was approved by the local ethics committee.

References
10. Dr. Thomas Gilmore. NF-kB Transcription Factors Biology Department, Boston University 5 Cummington Mall, Boston, Massachusetts. 2012;02215-2406.


