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Anticancer Effect of 1-(Anthracen-10-ylmethyl)-3-(2-cyanobenzyl)-1H-benzo[d]imidazol-3-ium chloride in 2D and 3D Cell Culture Models in Breast Cancer

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ABSTRACT

Triple negative breast cancer (TNBC), an aggressive subgroup of breast cancer that exhibits a highly complex and heterogeneous character has led researchers to seek for new and effective therapeutic agents due to the inadequacy of current treatment options. In the literature, there are benzimidazole derivatives whose antiproliferative effects have been investigated in many *in vivo* and *in vitro* studies. In the present study, a benzimidazolium salt (BS), namely 1-(anthracen-10-ylmethyl)-3-(2-cyanobenzyl)-1*H*-benzo[*d*]imidazol-3-ium chloride was prepared from 2-((1*H*-benzo[*d*]imidazol-1-yl) methyl) benzonitrile (1 mmol) and 10-(chloromethyl) anthracene (1 mmol). In our study, it was aimed to investigate the anticarcinogenic effects of above-mentioned BS, which was previously shown to be more effective than cisplatin (the chemotherapy agent being used in the treatment of TNBC) on 4T1 cells. Cell viability 2-dimensional (2D) and 3-dimensional (3D), colony forming, 3D spheroid formation, wound healing scratch assay and Annexin V/Propidium Iodide (PI) staining analyzes were used to determine cell growth/proliferation, migration and apoptosis, respectively. According to the results, BS significantly reduced viability/proliferation, colony formation and migration in 4T1 cells, even at low doses. It also induced apoptosis. It was concluded that, BS is a potential antiproliferative agent that reduces the carcinogenic properties of 4T1 cells through inhibiting cell proliferation, migration as well as inducing apoptosis. Its mechanism of action should be elucidated with further studies.

Keywords: Apoptosis, Benzimidazole, Migration, TNBC, 4T1

Introduction

Breast cancers, which constitute 25% of cancer cases worldwide, have a very high molecular, clinical and histopathological heterogeneity (1). With microarray studies, breast cancers classified in 5 molecular subgroups (2). Among these subgroups, triple negative breast cancer (TNBC) has a lower 5-year survival rate. Its potential for distant tissue metastasis and its possibility of recurrence is higher than the foregoing subgroups (3). Due to its hormone receptor negative feature, there is not any specific treatment target for TNBC. Although some chemotherapy drugs are being used in the treatment, they have many side effects and resistance development may occur (4). Therefore, new effective agents are needed for the treatment of TNBC.

Compounds including benzimidazole nucleus have very different biological activity properties such as antioxidant (5), antitubercular (6), antimicrobial (7, 8), and anti-cancer (9). Akkoc *et al.* (10) conducted *in vitro* cytotoxic activity studies of a benzimidazolium salt (BS) against human breast cancer (MDA-MB-231), human colon cancer (DLD-1) and human non-cancerous (normal) embryonic kidney (HEK-293T) cell lines using the MTT assay method. This compound was reported to possess high cytotoxic activity against MDA-MB-231 and DLD-1 cell lines with IC_{50} values of 1.26 ± 0.85 μM, 10.98 ± 2.33 μM, respectively. Furthermore, BS was demonstrated to has better anti-proliferative results than cisplatin and busulfan against breast cancer cell line for 72 h. The theoretical calculation studies of BS was performed towards a breast cancer protein, which is a crystal structure of a dimeric caspase-9, ID 2AR9, and colon cancer antigen proteins, ID 2HQ6 by the same research group (8).

There are many benzimidazole derivatives that have been reported to have antiproliferative effects through different cancer signaling pathways in various

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cancers such as colorectal, ovarian, glioma, and breast cancers (11-16). 4T1 metastatic cells, a preclinical mouse model of TNBC, are an important model for testing new agent candidates in the treatment of TNBCs (4).

In this study, we aimed to determine the possible comprehensive therapeutic effects of BS on TBNC by using 4T1 cells though monitoring cell viability, proliferation, migration, colony formation (2D and 3D analysis) and apoptosis for the first time in the literature.

Material and Method

Cell Culture and Drug Treatment: 4T1 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured with Dulbecco Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Capricorn Scientific), 10,000 units/ml Penicillin and 10,000 units/ml Streptomycin (Sigma, P4333) final concentration 1%, at 37 °C in a 5 % CO_2 humidified atmosphere. All studies were done with this complete medium.

BS, 1-(anthracen-10-ylmethyl)-3-(2-cyanobenzyl)-1*H*-benzo[*d*]imidazol-3-ium chloride was prepared from 2-((1*H*-benzo[*d*]imidazol-1-yl) methyl) benzonitrile (1 mmol) and 10-(chloromethyl) anthracen (1 mmol). BS (Molecular weight = 459.97 g/mol) was dissolved in dimethyl sulfoxide (DMSO) with a stock concentration of 100 mM.

Cell Viability Assay: 4T1 cells were seeded in 96-well plates (5000 cells/well). After 24 hours (h), 0.5 μM, 1 μM, 2 μM and 4 μM doses of BS active ingredient were treated. After 48 h, cells were removed with trypsin and centrifuged. 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and the cells were incubated at 37 C° with 5% CO_2 for 4h. Then, the measurements were analyzed in a plate reader at 570 nm wavelength (17).

3D Cell Viability Assay: After 3D cell culture, colonies were dispersed with a pipette. 100 μL of cell mixture was treated into 96 well plates and incubated for 6 h at 5% CO_2, 37 °C. Then, 0.5mg/mL MTT solution was added to the cells. It was incubated for 4 h at 5% CO_2, 37 °C. Then, without discarding the supernatant, 100 μL of DMSO was combined and incubated at 37 °C for 30 minutes. All of the formazan crystals produced by the cells were observed to be dissolved and were read in a plate reader at a 570 nm (18, 19).

3D Hanging Drop Assay: 4T1 cells were added to petri dishes with 30 μL of 1000 cells in droplets with at least 150 drops in each group. The petri dish was then inverted. After 48 h, the cells were taken into medium and cultured for 7 days by treating 0.5 μM, 1 μM, 2 μM and 4 μM doses of BS. Colonies over 50μM in diameter were then counted and analyzed (18, 19).

Colony Formation Assay: 4T1 breast cancer cells were seeded at 1000 cells per well of a 6-well plate. After 48 h, 0.5 μM, 1 μM, 2 μM and 4 μM doses of BS were treated when the cells began to adhere and grow. After 8 days, the medium in the 6-well dish was removed. Then, 2% crystal violet + methanol mixture was added and fixed and stained. After 5 minutes, the dye was removed and washed with PBS. Colonies formed were counted and evaluated (20).

Annexin V/Propidium Iodide (PI) Staining: FITC Annexin V Apoptosis Detection Kit with PI (Biolegend, 640914) was used. 4T1 cells were seeded in each well of 96-well plates (5000 cells/well). After 24 h, 0.5 μM, 1 μM, 2 μM and 4 μM doses of BS were treated. After 48 h, cells were removed with trypsin and centrifuged. Cells were dissolved in 100 μL Annexin binding solution and 5 μL FITC-Annexin V dye and 5 μL propidium iodide were added. It was incubated in the dark for 20 min. Then, 0.4 mL of Annexin V binding buffer was added and read and analyzed in flow cytometry (17).

Wound Healing Scratch Assay: 4T1 cells were seeded in 6-well plates ($10x10^5$ cells/well). After 48 h, approximately the cell density was 70%, plus-shaped wounds were created with a 100 μL pipette tip. Then washing was done with PBS. BS was added in 1 μM and 2 μM doses. Wound widths were measured at 24 h intervals. The study was terminated when the control group was completely closed at 48 h (21, 22).

Statistics: The data were found to be statistically normally distributed according to the Shapiro-Wilk test. All studies were repeated at least 3 times. Standard deviation is given in each study. One way ANOVA test was used for compare more than two groups in statistical analyses. Dunnett was used for posthoc analysis. Those with $p= 0.05$ were considered significant. All graphics and statistical analyzes were done with GraphPad Prism 8. Wound healing assay analyzes were performed with Image-j and flow cytometry analyzes were performed with Flowjo10.

Results

Effect of BS on cell proliferation: The effects of BS on proliferation of 4T1 mouse cancer cell were evaluated by MTT analysis in 2D and 3D models. 2D

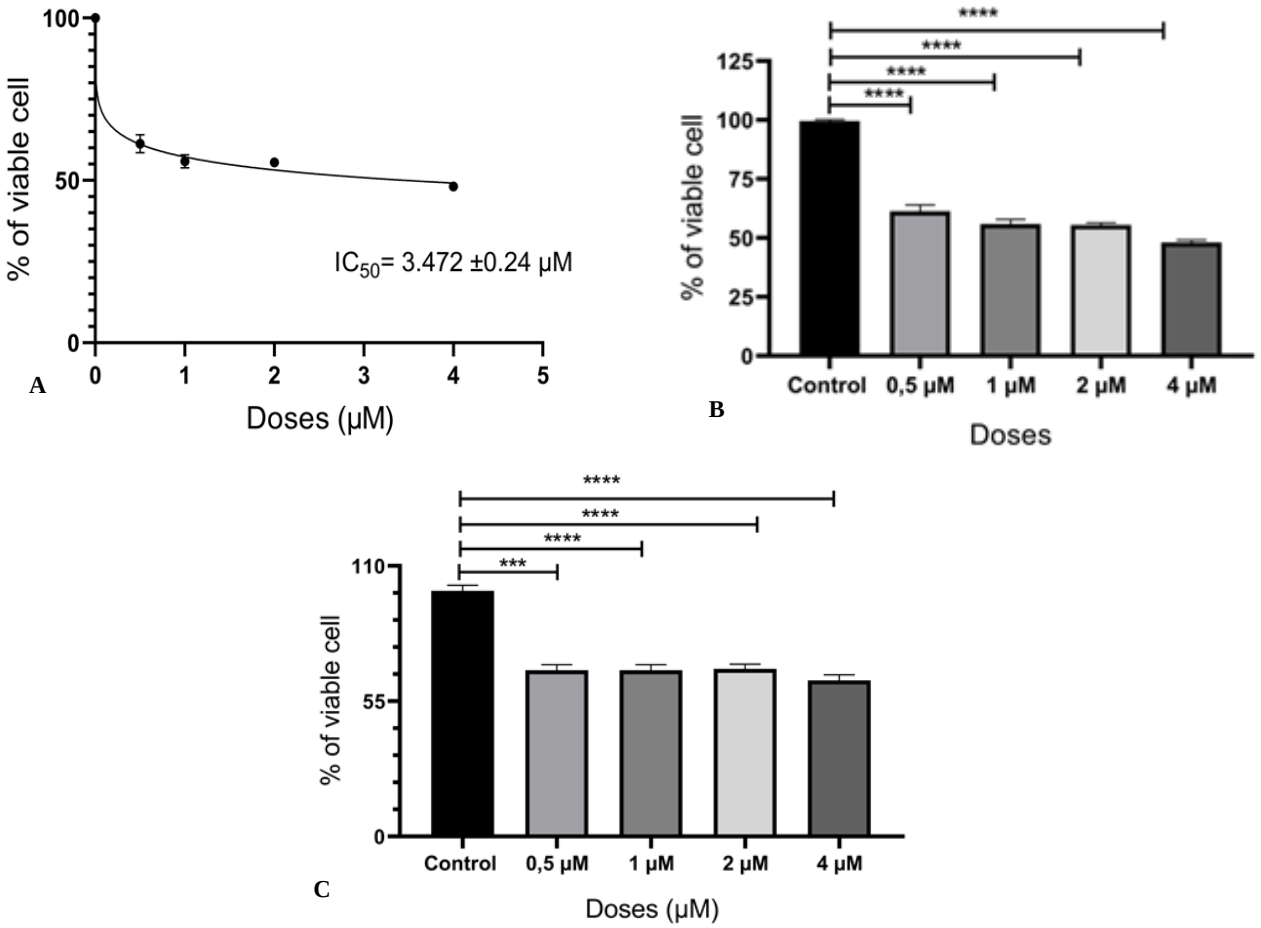

Fig 1. BS inhibits proliferation of 4T1 cells
4t1 cells were treated with BS at different concentrations and times manner. Cell viability/proliferation determined by MTT analysis. a) C_{50} value at 48 h. b) 2D cell viability with MTT analysis. c) 3D cell viability with MTT analysis. Data were presented as mean±S.D, ***p<0.001 and ****p<0.0001

cell viability in 4T1 breast cancer cell proliferation of different doses of BS at 48 h was tested and statistically significant reduction was observed at all doses (0.5 µM, 1 µM, 2 µM and 4 µM) when compared with the control group. Cell viability rates 61.25±1.8 %, 55.91±1.4%, 55.51±1.1% and 48.09±1% in 0.5 µM, 1 µM, 2 µM and 4 µM doses groups, respectively (Fig 1a). It was determined that the IC_{50} value was 3.472 ±0.24µM in the 2D MTT analysis of 4T1 cells (Fig 1b). As a result of cell viability test and time curve analysis, it was found appropriate to use the 48th hour. In 3D cell viability rates 67.60±1.9%, 67.60±1.8%, 68.15±1.8% and 63.50±1.5% in 0.5 µM, 1 µM, 2 µM and 4 µM doses groups, respectively (Fig 1c). In the 3D MTT analysis, it was observed that the minimal doses used in the 2D analysis significantly reduced cell proliferation in 48 h (Fig 1c). It was determined that the IC_{50} value was 122.7 ±5.72µM in the 3D MTT analysis of 4T1 cells (Fig 1d).

Effect of BS on 3D Spheroid Formation and 2D Colony Formation: To determine how BS inhibits the proliferation of 4T1 cells and affects the colony forming potential of the cells, colony formation analysis was performed and colonies formed with crystal violet dye were stained and counted. When the colonies formed were compared to the control, it was observed that a certain decrease occurred and this decrease was determined to be statistically significant. Colony formation rates among the groups were 97.75±1.2%, 82.35±0.6%, 80.85±0.9% and 80.50±1.5% in the in 0.5 µM, 1 µM, 2 µM and 4 µM groups, respectively (Fig 2a). With the performed 3D spheroid analysis, how the decrease in 4T1 cells affected the spheroid form was examined. It was determined that BS significantly reduced the number and size of spheroids, and this decrease was statistically significant (Fig 2b-2c). These results showed that BS inhibited 2D colony formation and 3D spheroid formation of these cells.

Effect of BS on Apoptosis: To test the inhibitory activity of BS in cell viability and colony formation by apoptosis of programmed cell death, Annexin V and PI staining was used in flow cytometry. The early

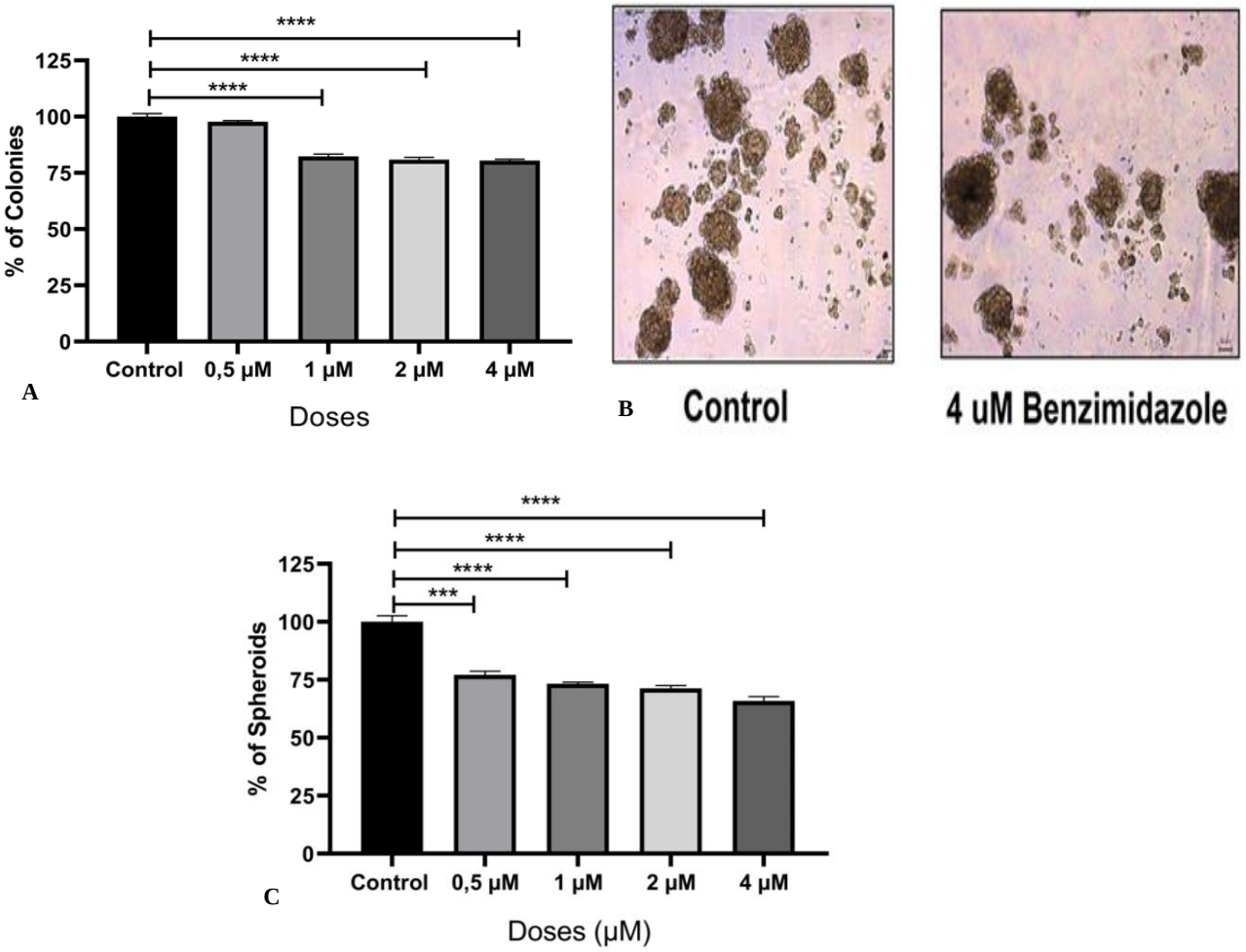

Fig 2. BS inhibits spheroid form by reducing colony formation of 4T1 cells
a)4T1 cells were treated with the indicated doses of BS and evaluated for colony formation by crystal violet staining and the colony was measured densitometrically and image J at the end of the 14 days. b-c) 3D spheroid formation analysis. Data were presented as mean±S.D, ***p<0.001 and ****p<0.0001

apoptosis rate was 0,32 ±0.09% in the control group, 0,49±0.13% in the 0.5 µM group, 0,51±0.15% in the 1 µM group, 0,93 ±0.12% in the 2 µM group and the late apoptosis rate was 5.08±0.27% in the control group, 9.38±0.38% in the 0.5 µM group, 9.59±0.25% in the 1 µM group, 15,2±0.46% in the 2 µM group. The total apoptosis rate was 5.34±0.6% in the control group, 9.77±1.2% in the 0.5 µM group, 10.00±0.03% in the 1 µM group, 12.04±0.52% in the 2 µM group and 15.66±0.74% in the 4 µM group (Fig 3a-b). When the control group and other groups were compared, the increase in apoptosis was statistically significant (Fig 3b).

BS Suppresses Migration of 4T1 Cells: Wound healing scratch assay was performed to determine whether BS had any effect on migration and motility of 4T1 cells. In this assay, a wound was created at the bottom of the plate covered with monolayer cells and the potential of the cells to which the agent was treated to seal this wound was measured. The wound areas in the cells treated with BS did not close compared to the control group (Fig 4a). At 24 h, the wound width was 26.90±4.2% in the control group, 49.2±11.7% at 1 µM and 78.20±15.3% at 2 µM (Fig 4b). Wound width closed in the control group at 48 h, but was 12.70±3.1% and 12.10±3.9% in the 1 µM and 2 µM groups, respectively (Fig 4b). This showed that BS reduced cell migration and motility to a certain extent in 4T1 cells.

Discussion

Female breast cancer has approximately 2.3 million new cases and 11.7% incidence worldwide. It is quite high in female breast cancers with a mortality rate of 6.9% (23). There is a need for new drug candidates and targets for cancer treatment. TNBC is estrogen and progesterone receptor negative, also don't make any or too much of the protein called human epidermal growth factor receptor 2 (HER2).

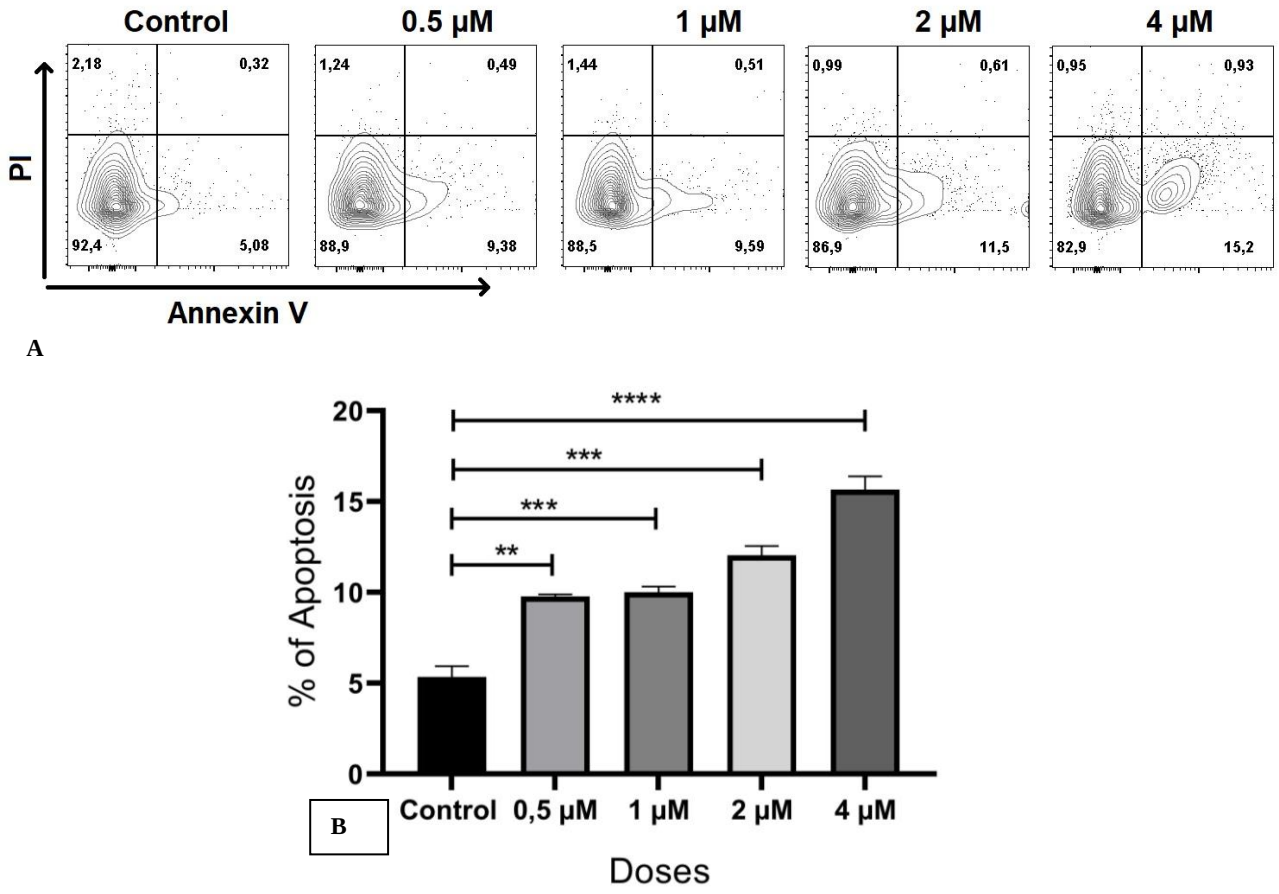

Fig 3. BS induces apoptosis in 4T1 cells
a)Classification of early and late apoptosis in flow cytometry, b)The results showed the percentages of apoptotic cells. The results of apoptotic cell percentages were presented as the mean ± SD from three separate experiments in 4T1 cells, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001

Chemotherapy is used as the standard treatment method due to the aggressive nature of TNBCs and the lack of targeted treatment options. Anthracyclines, taxanes and cyclophosphamide are frequently used in the systemic treatment of TNBC (24). In addition to these chemotherapeutics, alkylating agents that induce deoxyribonucleic acid (DNA) damage, such as cisplatin and carboplatin, are added to treatment regimens (25-27). BS has been demonstrated to have *in vitro* cytotoxic activity against human breast cancer (10). BS has high cytotoxic activity against TNBC(MDA-MB-231) cell lines with IC_{50} values of 1.26 µM for 72 h (8). In this study, proliferation, clonogenicity, migration and apoptosis death pathway from the carcinogenic properties of BS on mouse model cell 4T1 of TNBC were investigated. The effects of BS on the growth/proliferation of 4T1 cells were evaluated by 2D and 3D cell culture. It has been observed that even at low doses are effective in breast cancer. As a result of the comparison of 2D and 3D IC_{50} values, it was seen that BS was more effective in 2D culture. As can be seen in the result of cell viability in 3D culture, the activity progresses linearly above a certain dose. The most important reason for this is that 2D cultures are more affected by drugs from many things, such as the damage of interactions between cells and extracellular environments (30).

It has been reported that sulfonamide derivatives containing benzimidazole in gastric cancers have good selectivity between normal and cancerous cells, inhibit colony formation, and induce apoptosis by stopping the cell cycle (28). Ren et al (29) reported that this compound induces apoptosis and arrests the cell cycle in colorectal carcinoma cells (HCT-16) in their study with a pyrazol-containing benzimidazole derivative. Colony formation is an *in vitro* quantitative technique to study the ability of a single cell to develop into a large colony through clonal expansion. One of the most important issues in cancer is that the cancer metastasizes and forms a colony (31, 32). BS inhibited colony formation 2D cultures are subject to many limiting factors such as disruption of interactions between cellular and extracellular environments, changes in cell morphology. The information obtained in 3D cell culture studies, which can provide a more similar environment *in vivo*

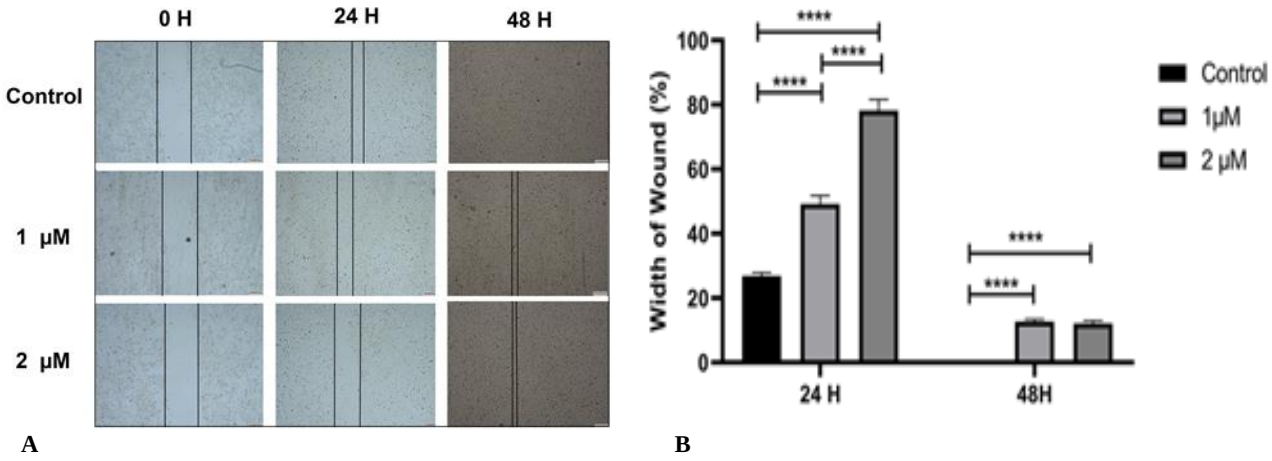

Fig 4. BS suppresses migration and invasion of 4T1 cells to a certain degree
a)A single scratch was made in the center of the confluent cell monolayer and cells were treated with indicated BS doses. The cell migration was monitored for 48 h and visualized by light microscopy. b)The bar graph showed the percentages of the migrating cells to the scratched/wounded area, and the data were expressed as a mean of the percentages of migration ± SD of three independent experiments (a-b), ****p<0.0001

conditions, is more effective (33). We found BS to be quite effective when we tested its effectiveness on 3D spheroids. In our study, when we tested the BS in 3D cell culture, we saw that it was similar to the activity in 2D culture.

Caspase-9 is one of the caspases that initiate apoptosis (34). The theoretical calculation studies of BS were done towards a breast cancer protein, which is a crystal structure of a dimeric caspase-9 (8). The presence of this structural similarity made us think that it may activate caspase 9 and affect apoptosis. We found that apoptosis was triggered at a significant level in breast cancer cells that we analyzed with Annexin-V and PI staining in flowcytometry. It is found that early apoptosis is induced, and the change here is considerable. The difference in late apoptosis rates, however, was not substantial. It is projected that BS will emerge when breast cancer cells are treated for more than 48 hours, causing late apoptosis. More detailed studies are needed to show that its similarity to caspase-9 is effective in initiating apoptosis.

Huang et al (14) reported that the benzimidazole derivative mebendazole inhibited many cancer signaling pathways in ovarian cancer cell lines (OVCAR8 and SKOV3), significantly suppressed proliferation and migration, and induced apoptosis in a study they conducted with cisplatin-resistant cancer cells. Ren et al (16) reported in their study with three benzimidazole derivatives that its derivatives suppress DNA synthesis, migration and invasion in cells and regulate the expression of key epithelial mesenchymal transformation markers. Metastasis is the hallmark of cancer, which is responsible for the most of cancer-related deaths (31). Regarding the findings of our wound healing test, we determined that the effect of BS on metastatic activity was significantly reduced. The decrease in migration indicates that it can be an effective BS against cancer. In the light of the information obtained from the studies we have carried out, more detailed studies should be carried out with BS.

Choi et al. (15) reported that MDA-MB-231 and radiotherapy-resistant MDA-MB-231 (RT-R MDA-MB-231) cells and allograft mice reduced the anticarcinogenic properties of various benzimidazole derivatives and the stem cell markers of these derivatives. Phase II clinical studies of DHW-221 benzimidazole derivative as a dual inhibitor of PI3K/mTOR are ongoing (35). As mentioned above, the anti-cancer properties of benzimidazole derivates have been studied in many studies. Some have also taken part in clinical trials. Consequently, we showed that the BS has cytotoxic effects even at low micromolarity, significantly inhibits proliferation, colony formation and migration, and induces apoptosis in 4T1 cells. More detailed studies on the anticancer properties of BS are needed.

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References

1. Hwang SY, Park S, & Kwon Y (2019) Recent therapeutic trends and promising targets in

triple negative breast cancer. Pharmacology & therapeutics 199:30-57.

2. Waldman FM, et al. (2000) Chromosomal alterations in ductal carcinomas in situ and their in situ recurrences. Journal of the National Cancer Institute 92(4):313-320.
3. Dent R, et al. (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clinical cancer research : an official journal of the American Association for Cancer Research 13(15 Pt 1):4429-4434.
4. Arroyo-Crespo JJ, et al. (2019) Characterization of triple-negative breast cancer preclinical models provides functional evidence of metastatic progression. International journal of cancer 145(8):2267-2281.
5. Aroua LM, et al. (2021) A facile approach synthesis of benzoylaryl benzimidazole as potential alpha-amylase and alpha-glucosidase inhibitor with antioxidant activity. Bioorganic chemistry 114:105073.
6. Subhedar DD, et al. (2021) [DBUH][OAc]-Catalyzed Domino Synthesis of Novel Benzimidazole Incorporated 3,5-Bis (Arylidene)-4-Piperidones as Potential Antitubercular Agents. Polycycl Aromat Comp.
7. Gok Y, Akkoc S, Celikal OO, Ozdemir I, & Gunal S (2019) In vitro antimicrobial studies of naphthalen-1-ylmethyl substituted silver N-heterocyclic carbene complexes. Arab J Chem 12(8):2513-2518.
8. Akkoc S, Tuzun B, Ilhan IO, & Akkurt M (2020) Investigation of structural, spectral, electronic, and biological properties of 1,3-disubstituted benzimidazole derivatives. J Mol Struct 1219.
9. Akkoc S (2021) Design, synthesis, characterization, and in vitro cytotoxic activity evaluation of 1,2-disubstituted benzimidazole compounds. J Phys Org Chem 34(1).
10. Akkoc S, Kayser V, & Ilhan IO (2019) Synthesis and In Vitro Anticancer Evaluation of Some Benzimidazolium Salts. J Heterocyclic Chem 56(10):2934-2944.
11. Kralova V, Hanusova V, Rudolf E, Canova K, & Skalova L (2016) Flubendazole induces mitotic catastrophe and senescence in colon cancer cells in vitro. The Journal of pharmacy and pharmacology 68(2):208-218.
12. Kralova V, et al. (2013) Antiproliferative effect of benzimidazole anthelmintics albendazole, ricobendazole, and flubendazole in intestinal cancer cell lines. Anti-cancer drugs 24(9):911-919.
13. Pinto LC, et al. (2019) Mebendazole induces apoptosis via C-MYC inactivation in malignant ascites cell line (AGP01). Toxicology in vitro : an international journal published in association with BIBRA 60:305-312.
14. Huang L, et al. (2021) Antiparasitic mebendazole (MBZ) effectively overcomes cisplatin resistance in human ovarian cancer cells by inhibiting multiple cancer-associated signaling pathways. Aging 13(13):17407-17427.
15. Choi HS, et al. (2021) Anticancer Effect of Benzimidazole Derivatives, Especially Mebendazole, on Triple-Negative Breast Cancer (TNBC) and Radiotherapy-Resistant TNBC In Vivo and In Vitro. Molecules 26(17).
16. Ren LW, et al. (2022) Benzimidazoles induce concurrent apoptosis and pyroptosis of human glioblastoma cells via arresting cell cycle. Acta pharmacologica Sinica 43(1):194-208.
17. Acikgoz E, Soner BC, Ozdil B, & Guven M (2021) CD133+/CD44+ prostate cancer stem cells exhibit embryo-like behavior patterns. Acta histochemica 123(5):151743.
18. Valcarcel M, et al. (2008) Three-dimensional growth as multicellular spheroid activates the proangiogenic phenotype of colorectal carcinoma cells via LFA-1-dependent VEGF: implications on hepatic micrometastasis. J Transl Med 6.
19. Foty R (2011) A simple hanging drop cell culture protocol for generation of 3D spheroids. Journal of visualized experiments : JoVE (51).
20. Akar S, Altuntaş H, & Hamurcu Z (2022) β-Escin Reduces Cancer Progression in TNBC by Inhibiting Glutamine Metabolism Through Downregulation of c-myc.
21. Izgi K, et al. (2015) Myrtucommulone-A Induces both Extrinsic and Intrinsic Apoptotic Pathways in Cancer Cells. Journal of biochemical and molecular toxicology 29(9):432-439.
22. Sakalar C, et al. (2016) The combination of thymoquinone and paclitaxel shows anti-tumor activity through the interplay with apoptosis network in triple-negative breast cancer. Tumor Biol 37(4):4467-4477.
23. Sung H, et al. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. Ca-Cancer J Clin 71(3):209-249.
24. Smith DW & Fahrney DE (1978) Catalysis by arginine deiminase: evidence for a covalent intermediate. Biochemical and biophysical research communications 83(1):101-106.
25. Bergin ART & Loi S (2019) Triple-negative breast cancer: recent treatment advances. F1000Research 8.
26. von Minckwitz G, et al. (2014) Neoadjuvant carboplatin in patients with triple-negative and

HER2-positive early breast cancer (GeparSixto; GBG 66): a randomised phase 2 trial. The Lancet. Oncology 15(7):747-756.

27. Zhang J, et al. (2015) Chemotherapy of metastatic triple negative breast cancer: Experience of using platinum-based chemotherapy. Oncotarget 6(40):43135-43143.
28. Jian S, et al. (2019) Novel tertiary sulfonamide derivatives containing benzimidazole moiety as potent anti-gastric cancer agents: Design, synthesis and SAR studies. European journal of medicinal chemistry 183:111731.
29. Ren B, Liu RC, Ji K, Tang JJ, & Gao JM (2021) Design, synthesis and in vitro antitumor evaluation of novel pyrazole-benzimidazole derivatives. Bioorganic & medicinal chemistry letters 43:128097.
30. Kapalczynska, M., Kolenda, T., Przybyla, W., Zajaczkowska, M., Teresiak, A., Filas, V. Lamperska, K. (2018). 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. Arch Med Sci, 14(4), 910-919.
31. Fares J, Fares MY, Khachfe HH, Salhab HA, & Fares Y (2020) Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduct Tar 5(1).
32. Rajendran V & Jain MV (2018) In Vitro Tumorigenic Assay: Colony Forming Assay for Cancer Stem Cells. Methods Mol Biol 1692:89-95.
33. 33. Kapalczynska M, et al. (2018) 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. Arch Med Sci 14(4):910-919.
34. Boice A & Bouchier-Hayes L (2020) Targeting apoptotic caspases in cancer. Bba-Mol Cell Res 1867(6).
35. Qin X, et al. (2021) Dual blocking of PI3K and mTOR signaling by DHW-221, a novel benzimidazole derivative, exerts antitumor activity in human non-small cell lung cancer. Clinical and translational medicine 11(9):e514.