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ORIGINAL ARTICLE

A benzimidazolium salt induces apoptosis and arrests cells at sub-G1 phase in epithelial ovarian cancer cells

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Abstract

Background Ovarian cancer, also known as a silent killer, is the deadliest gynecological cancer in women worldwide. Epithelial ovarian cancers constitute the majority of ovarian cancers, and diagnosis can be made in advanced stages, which greatly reduces the likelihood of treatment and lowers the survival rate. For the treatment of epithelial ovarian cancers, the search for synthetic agents as well as agents of natural origin continues. The effects of 1-(2-cyanobenzyl)-3-(4-vinylbenzyl)-1*H*-benzo[*d*]imidazole-3-ium chloride (BD), a benzimidazole derivative, were investigated on epithelial ovarian cancer cells.

Methods and results In our study, the effects of BD on proliferation, colony formation, cell death by apoptosis and the cell cycle in A2780 and A2780 Adriamycin (ADR) ovarian cancer cell lines were investigated. Proliferation was examined with cell viability analysis, colony formation and apoptosis with Annexin V staining and cell cycle analyses with PI staining, respectively. As a result of the analyses, BD inhibited cell proliferation and colony formation, induced apoptosis and cell death at 48 h in A2780 and A2780 ADR cells at 10.10 and 10.36 µM concentrations, respectively. In addition, A2780 and A2780ADR cells were arrested in the Sub-G1 phase of the cell cycle.

Conclusions BD suppresses cancer cell progression by showing antiproliferative effects on ovarian cancer cells. Further analyses are required to determine the mechanism of action of this agent and to demonstrate its potential as a suitable candidate for the treatment of epithelial ovarian cancer.

Keywords Apoptosis · Benzimidazole · Cell cycle · Ovarian cancer

Introduction

Ovarian cancer is one of the leading causes of death among gynecological cancers in women worldwide [1]. In general, ovarian cancers develop from germ cells (5–10%), connective tissue in the ovary that secretes stromal or reproductive hormones (10–15%), and most develop from ovarian epithelial cells (> 80%) [2]. The World Health Organization (WHO) classifies epithelial origin cancers (EOC) morphologically according to cell type as serous, mucinous, endometrioid, clear cell, transitional cell Brenner and differentiated type tumors [3]. EOC exhibit highly heterogeneous clinicopathological features, histology and genomic profile. Ovarian cancers that are asymptomatic in the early stages are called *silent killers* because they can only be diagnosed in the advanced stages of the disease [4, 5]. Current treatment options include surgery, platinum-based drugs, paclitaxel and cisplatin-based chemotherapy [6–8]. The detection of the disease in the advanced stages also limits the possibility of treatment and treatment options. The recurrence of the disease increases in parallel to increments in chemotherapy resistance. As the aforementioned drug resistance has become one of the main problems for treatment, instead of drugs (i.e. cisplatin, taxan) currently being used, efforts are needed to research new and effective substances for use in treatment.

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Benzimidazole, formed by benzene and imidazole rings, is a heterocyclic aromatic compound [9]. Benzimidazole, which has a wide range of biological activities, shows antimicrobial, anticarcinogenic, antidiabetic, analgesic, anti-inflammatory, antiviral, antihypertensive and anthelmintic effects [10–14]. This wide range of biological activities can be achieved with various substituents in the benzimidazole core. In a previous study, it was reported that a benzimidazole derivative (BD), written as 1-(2-cyanobenzyl)-3-(4-vinylbenzyl)-1*H*-benzo[*d*]imidazole-3-ium chloride, displayed cytotoxic effects against DLD-1, MDA-MB-231 cancer cell lines while it exhibited no detrimental effect against healthy HEK-293T cells [9]. For this purpose in our study, for the first time in the literature, the effects of this BD on A2780 and A2780ADR ovary cancer cells were investigated.

Materials and methods

Synthesis of organic compound

The known compound, namely 1-(2-cyanobenzyl)-3-(4-vinylbenzyl)-1*H*-benzo[*d*]imidazol-3-ium chloride, BD was prepared according to the literature from 2-((1 H-benzo[*d*] imidazol-1-yl)methyl)benzonitrile (1 g, 1 mmol) and 4-vinylbenzylchloride (0.65 g, 1 mmol) at 80 °C in dimethylformamide (DMF) [9]. The white product was purified by crystallization in ethanol. The open structure of the compound is given in Fig. 1.

Cell culture and conditions

A2780 and A2780ADR cells, which were gifted by Dr. Ibrahim Tekedereli (Inonu University, Turkey), were seeded in DMEM (Capricorn Scientific) medium containing 10% FBS (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml penicillin/streptomycin (Capricorn Scientific). The cells were cultured at 37 °C, 5% CO_2 in a humidified incubator, and when they reached 90% density, they were passaged to ensure the continuity of the culture.

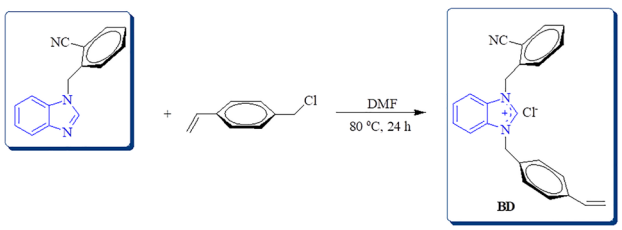

Fig. 1 The structure of BD

Cell viability assay

To determine the effects of BD on the proliferation of A2780 and A2780ADR cells, viability analysis was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Cayman Chemical, 298-93-1). Cells were seeded in 96-well plates at 5000 cells per well and were allowed to incubate for 24 h (h). Cells were treated with BD in the range of 1, 5, 10, 20, 40, 60, and 80 µM over 24, 48 and 72 h at the end of the incubation period. At the end of the treatment periods, MTT was applied and measurements were made with the ELIZA-reader at 560 nm. DMSO (Serva, 67-68-5), the solvent of BD, was used as control.

Colony forming assay

Colony formation assay was performed to determine the effect of BD on the growth and proliferation of A2780 and A2780 ADR cells. Cells were seeded in 6-well plates at 1000 cells per well and were incubated for 24 h. Colony formation was observed for 14 days by applying the determined concentrations of BD at the end of the incubation period. At the end of this period, colonies stained with crystal violet were monitored and counted [15].

Analysis of apoptosis with Annexin V staining

The effect of BD on cell death by apoptosis of A2780 and A2780 ADR cells was analyzed using Annexin V staining. A2780 and A2780 ADR cells were seeded with 300.000 cells in 25 cm^2 flasks and incubated for 24 h. After incubation, cells were treated with BD and incubated for 48 h. Then the cells were harvested and stained in accordance with the FITC Annexin V detection kit (Biolegend, 640,914) procedure according to the manufacturer's instructions and the number of apoptotic cells was determined by flow cytometry [16].

Analysis of cell cycle with PI staining

The effect of BD on the cell cycle in A2780 and A2780 ADR cells was analyzed with propidium iodine (PI) staining. A2780 and A2780 ADR cells were seeded with 300.000 cells in 25 cm^2 flasks and incubated for 24 h. After incubation, cells were treated with BD and allowed to incubate for 48 h. Then the cells were harvested and stained according to the manufacturer's instructions in accordance with the PI (Biolegend, 421,301) staining procedure, and cell cycle stages were determined by flow cytometry [17].

Real-time PCR analysis

Total RNA was isolated from BD-treated A2780 and A2780 ADR cells using trizol reagent. RNA purity and concentration were measured using a Nanodrop One (Thermo NanoDrop Lite) spectrophotometer. Reverse transcription was performed with each reaction containing 1 µg of RNA and iScript™ Advanced cDNA Synthesis Kit (Biorad, 1,725,121). Quantitative real-time PCR (qRT-PCR) was used to investigated apoptosis-related genes (Bax, caspase3, caspase8, bcl2) in a cDNA sample. The study was set up to measure the expression levels of these genes in the cDNA sample by comparing them to a reference gene (β-Actin) using the $2^{-\Delta\Delta Ct}$ method. The same experiment was done 3 times to ensure the validity of the results. The thermal profile for qRT-PCR was set to denature the DNA at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. Primers used for apoptosis genes in RT-PCR analysis are shown in Table 1.

Table 1 Primer sequences

Caspase 3	Forward primer	CCAGTGGAGGCCGACTTCTT
	Reverse primer	AGCATGGCACAAAGCGACTG
Caspase 8	Forward primer	ACACTTGGATGCAGGGGCTT
	Reverse primer	ACTGTGCAGTCATCGTGGGG
Bax	Forward primer	AGAAGCTGAGCGAGTGTCTC
	Reverse primer	CGGAAAAAGACCTCTCGGGG
Bcl-2	Forward primer	GTGTGTGGAGAGCGTCAACC
	Reverse primer	GGGCCGTACAGTTCCACAAAG
β-Actin	Forward primer	CAACCGCGAGAAGATGAC
	Reverse primer	AGGAAGGCTGGAAGAGTG

Statistical analysis

The analyses were performed with the SPSS program. The data are presented as mean ± standard deviation (SD) according to one-way ANOVA and Student's *t*-test. All data are representative of at least three independent experiments performed in triplicate. Results with a p-value of 0.05 or less were considered significant.

Results

BD inhibits cell proliferation and colony formation of A2780 and A2780ADR cells

BD was added to the culture medium at concentrations of 1, 10, 20, 40, 60, and 80 µM for 24, 48 and 72 h. The highest cytotoxicity was achieved at 48 h and, thus the IC_{50} values were calculated at 48 h. BD exhibited cytotoxic effects

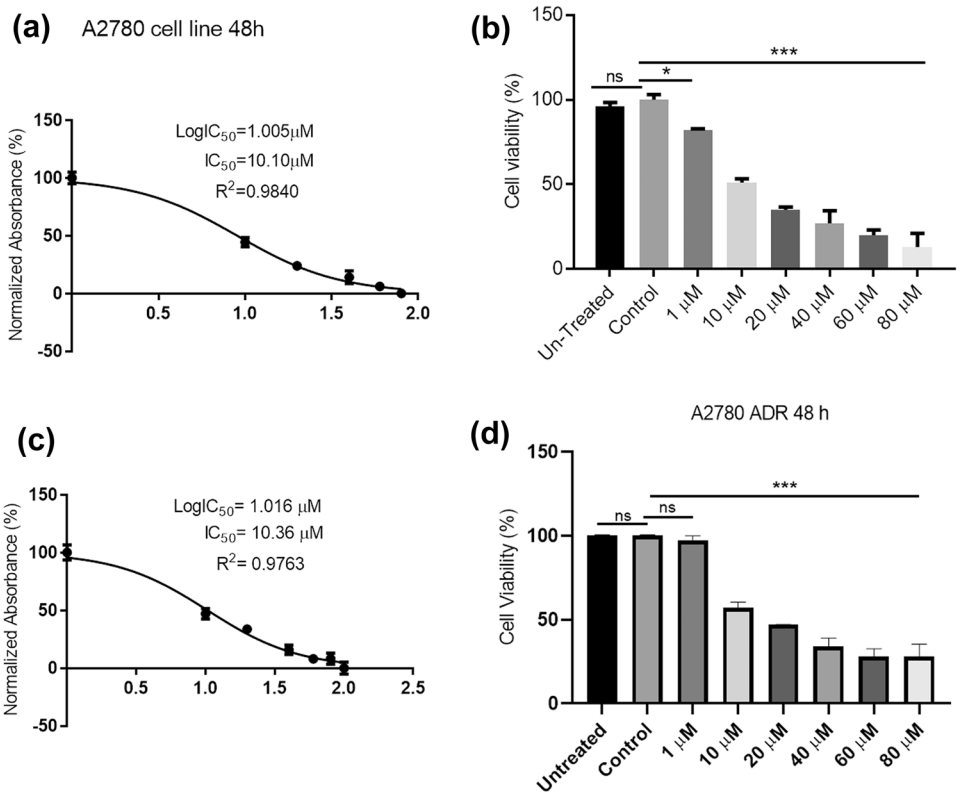

Fig. 2 BD, A2780 and A2780ADR inhibit cell growth in ovarian cancer cells. A2780 and A2780ADR cells were seeded at a density of 3 × 10 3 cells/well into 96-well plates and treated with a series of concentration of BD (1, 10, 20, 40, 60 and 80 µM) or a vehicle only control for 48 h and cell viability was measured by MTT assay, **a** Determination of IC50 values by nonlinear regression analysis in BD-treated A2780 cells, **b** Effect of BD on the viability of A2780 cells, **c** Determination of IC50 values by nonlinear regression analysis in BD-treated A2780ADR cells, **e** Effect of BD on the viability of A2780 cells. The values are the mean ± SD from three independent experiments. ***p < 0.001, **p < 0.01 vs. the control group

against A2780 and A2780ADR cells, with IC_{50} values of 10.10 µM and 10.36 µM, respectively (Fig. 2). With the results obtained after viability analysis, it was concluded that BD inhibited the proliferation of A2780 and A2780ADR cells (Fig. 2).

In order to determine the effects of BD on the growth and proliferation of cells, colony forming potentials were investigated in addition to the viability assay. Colony formation was significantly reduced in BD-treated A2780 and A2780ADR cells compared to the control groups (Fig. 3), and this decrease was statistically significant (p***<0.001).

BD treatment induces apoptosis in Ovarian cancer cells

To determine whether inhibition of cell proliferation by BD treatment of A2780 and A2780ADR cells is associated with induction of apoptosis, apoptosis analysis was performed with flow cytometry using FITC Annexin V. BD induced apoptosis dramatically in both A2780 and A2780ADR cells (***p < 0.001; ***p < 0.001, respectively) (Fig. 4). In addition to Annexin V staining, gene expressions of BAX, Bcl-2, caspase-3 and caspase-8, which are apoptotic proteins, were examined by RT-PCR and any changes in the expressions of these genes were determined. Levels of BAX and caspase-3 expressions increased markedly in BD-treated ovarian cancer cells compared to the control group (*p < 0.05; **p < 0.01; ***p < 0.001) (Fig. 4e and f). Bcl-2 expression was significantly reduced in BD-treated ovarian cancer cells compared to control group for both cell lines (***p < 0.001). In addition, there was no difference between groups in terms of caspase-8 gene expression for both cell lines.

BD causes ovarian cancer cells to arrest in the sub-G1 phase of the cell cycle

Cell cycle analysis was performed using flow cytometry with PI staining to determine whether BD has an effect on the cell

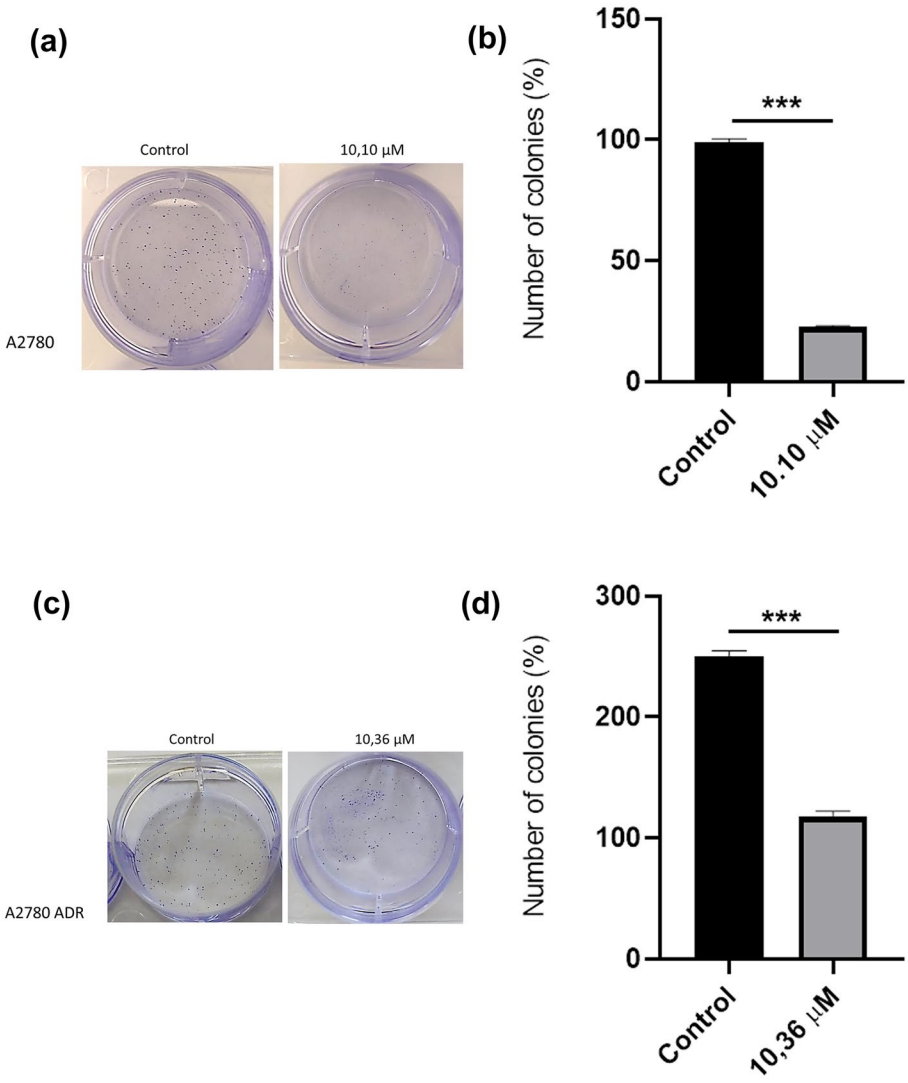

Fig. 3 BD, A2780 and A2780ADR inhibit cell proliferation in ovarian cancer cells and suppress colony formation of cells. A total of 1000 cells were seeded in six-well plates and cultured for 10–14 day. Colony formation was detected. **a** Visualization of colonies by staining with crystal violet of colony formation in A2780 cells after BD treatment, **b** Quantitative analysis of the colony numbers of A2780 cells after BD treatment, **c** Visualization of colonies by staining with crystal violet of colony formation in A2780ADR cells after BD treatment, **d** Quantitative analysis of the colony numbers of A2780ADR cells after BD treatment. The values are the mean ± SD from three independent experiments. ***p < 0.001, **p < 0.01 vs. the control group

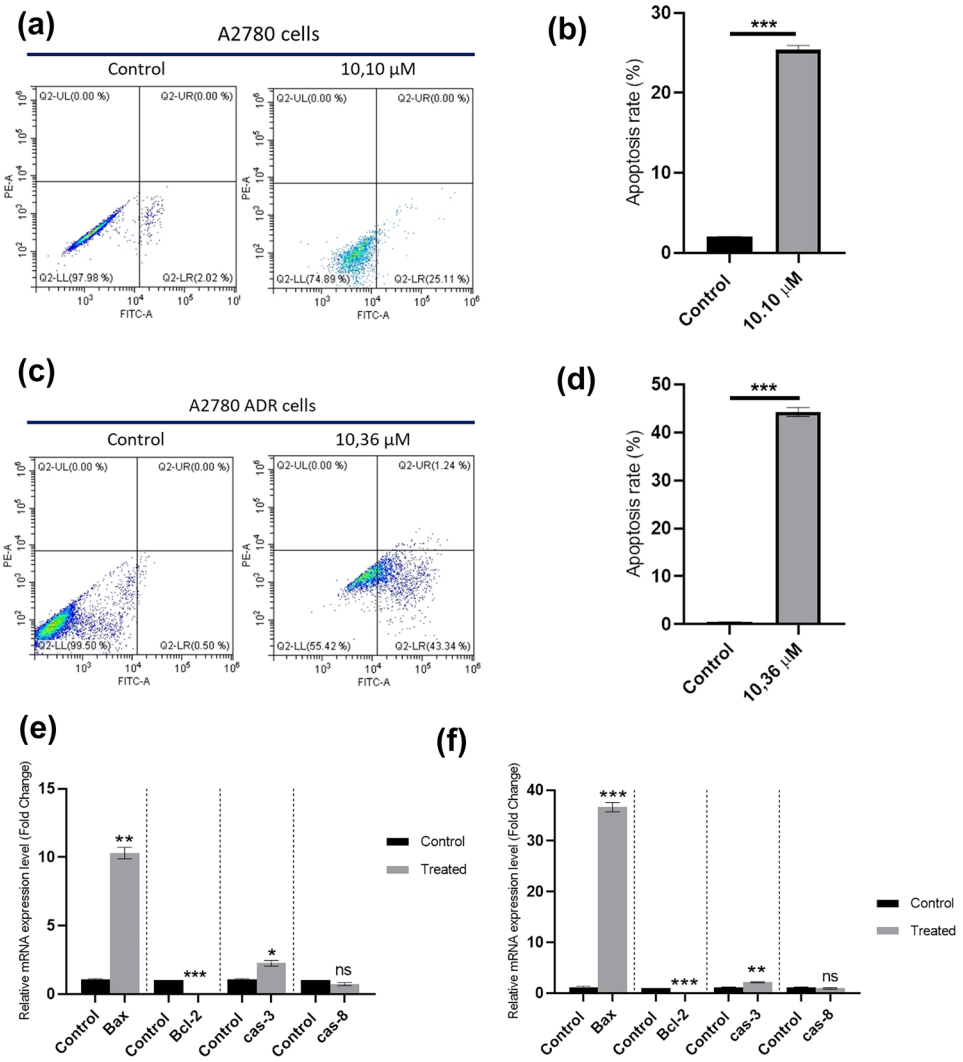

Fig. 4 BD induces apoptosis in A2780 and A2780ADR cells. Cell death by apoptosis in cells treated with BD at concentrations of IC_{50} for 48 h was determined by Flow cytometry using Annexin V/PI staining, **a** Histogram of apoptotic cells in BD treated A2780 cells after measurement, **b** Analysis of apoptotic cells numbers in BD treated A2780 cells compared to control group, **c** Histogram of apoptotic cells in BD treated A2780ADR cells after measurement, **d** Analysis of apoptotic cells numbers in BD treated A2780ADR cells compared to control group. **e**–**f** bax, bcl-2, cas-3 and cas-8 mRNA levels were determined Real Time PCR in A2780 and A2780ADR cells. ***p < 0.001, **p < 0.01, *p < 0.05

cycle. After BD treatment of A2780 and A2780ADR cells at concentrations of 10.10 and 10.36 µm, respectively, for 48 h, FITCH annexin V staining was performed to measure the amount of cells accumulating at checkpoints of the cell cycle. Most of the cells in both BD-treated cell lines significantly (***$p < 0.001$) remained in the sub-G1 stage (Fig. 5). Moreover, a striking accumulation was seen in the G0/G1 phase of the cell cycle in BD-treated A2780ADR cells compared to the control cells.

Discussion

Ovarian cancers, often known as the silent killer, cannot be diagnosed until the disease progresses to stage III or IV in more than 70% of cases due to lack of symptoms in the early stages. Failure to diagnose at these stages reduces treatment success and also decreases survival rates [18]. The side effects of the current chemotherapies and the development of chemotherapy resistance have pushed researchers to find effective new agents for the treatment of ovarian cancer. Benzimidazole, found in bicyclic form in nature, consists of the fusion of benzene to the 4,5 position of the imidazole ring [19, 20]. In some earlier studies, it was reported that benzimidazole and its derivatives possess significant cytotoxic effects against different cancer cell lines including DLD-1 and MDA-MB231 [21–24]. In the present study, we analyzed the activity of BD, written as 1-(2-Cyanobenzyl)-3-(4-vinylbenzyl)-1 H-benzo[d]imidazole-3-ium chloride, as a drug candidate by investigating its anticarcinogenic effects on A2780 and A2780ADR cells in vitro.

Our findings showed that BD had cytotoxic effects on A2780 and A2780ADR cells and significantly reduced cell proliferation as evidenced from the cytotoxic test (Fig. 2). Colony formation analysis is an irreplaceable method that demonstrates the growth potential of cancer stem cells and their ability to form a large colony from one cell [25]. In the current study, antiproliferative and antigrowth

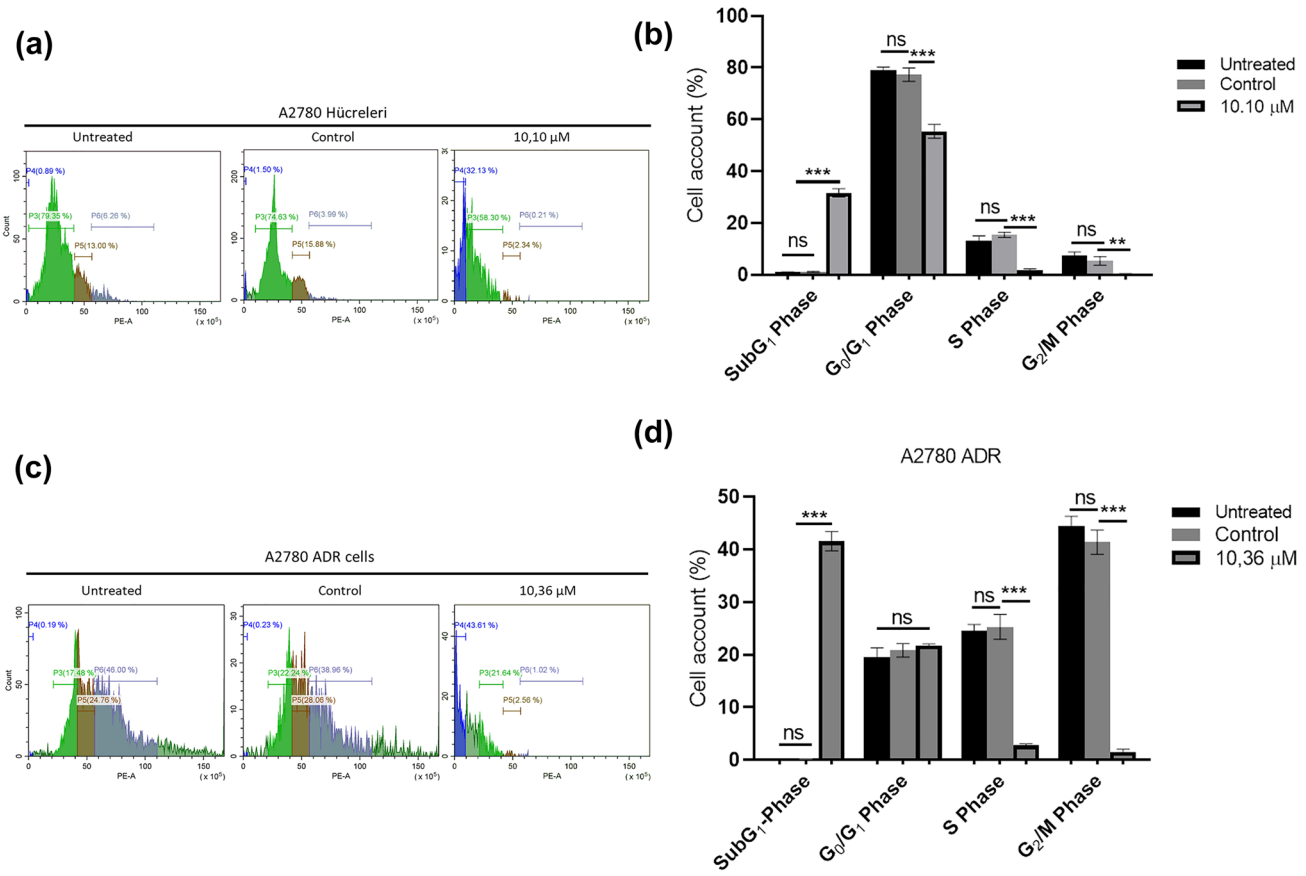

Fig. 5 In BD treated A2780 and A2780ADR cells, cells are arrested in the Sub-G1 phase of the cell cycle. Cell cycle analysis determined by PI staining, **a** Histogram showing the percentages of BD treatment in A2780 cells in cell cycle phases of cells, **b** The percentages results of the cell cycle phase were presented as the mean ± SD from three separate experiments in A2780 cells, **c** Histogram showing the percentages of BD treatment in A2780ADR cells in cell cycle phases of cells, **d** The percentages results of the cell cycle phase were presented as the mean ± SD from three separate experiments in A2780ADR cells. Data were presented as mean ± S.D.; *p < 0.05, **p < 0.01, ***p < 0.001; *ns* non-significant

influences of BD were determined by colony formation analysis of ovarium cancer cells (Fig. 3). Our results are consistent with the findings of some previous studies in which benzimidazole derivatives also displayed cytotoxic effects. In the study by Elayapillai et al. the effect of an antiparasitic drug mebendazole (benzimidazole derivative) on proliferation was investigated via colony formation analysis in ovarian cancer cells. They showed that this benzimidazole derivative inhibited the growth of ovarian cancer cells [26]. Choi et al. [27] investigated the anticarcinogenic effects of six different benzimidazole derivatives for MDA-MB 231 and radiotherapy-resistant MDA-MB 231 breast cancer cells and in experimental animals. In this study, benzimidazole derivatives reduced proliferation of cancer cells and inhibited colony formation [27]. Zhen et al. [28] researched anticarcinogenic effects of broad spectrum anthelmintic flubendazole in vivo and in vitro. Flubendazole showed strong cytotoxic effects in breast cancer cell lines MDA-MB231 and MDA-MB468 and displayed antiproliferative effect with a decrease in colony formation. In that study, the cytotoxic effects of flubendazole were linked to its role in activating autophagic cell death. In line with these studies, in our study, BD showed strong cytotoxic effects on A2780 and A2780ADR cells.

There are many studies in the literature indicating that benzimidazole and its derivatives have anticarcinogenic effects on various cancer cells such as breast cancer, liver cancer, colon cancer and glioblastoma [29–32]. According to some previous literary data, several benzimidazole derivatives exhibit anticarcinogenic properties such as behaving like growth factor receptor inhibitors, inhibiting sirtuins, and acting as Pin1 inhibitor and aromatase inhibitor, which have many roles from epigenetic regulation to regulation of metabolism [33–35].

Chemotherapeutic drugs such as vinblastine, cyclophosphamide, methotrexate, tamoxifen, and paclitaxel cause anticarcinogenic effects by arresting the cells at different stages of the cell cycle in various cancer types [36]. In our study,

a cytotoxic agent BD kept A2780 and A2780ADR cells in sub-G1 stage. Ren et al. (2021) showed that a pyrazole-benzimidazole derivative exhibited strong anticarcinogenic effects in HCT116, MCF-7 and Huh-7 cell lines and moreover it stopped HCT116 cells at the G0/G1 stage of the cell cycle [31]. Ohno et al. (2021) reported that a benzimidazole derivative CCL299 kept hepatocellular carcinoma and Hep-2 cervical carcinoma cells in the G1 stage through upregulation of the p21 level and hence induction of apoptosis [31]. In the trial by Nazreen et al. [32], the influence of different benzimidazole derivatives on cell cycles of MDA-MB231, A549 and SKOV3 cells were investigated. A benzimidazole derivative named compound 13 kept MDA-MB231 and A549 cells in the G1/S phase and SKOV3 cells in the S phase. In the same study, another benzimidazole derivative named compound 10 kept SKOV3 and MDA-MB231 cells in G2/S phase, while keeping A549 cells in G1/G2 phase [37]. Atmaca et al. (2020) reported the effect of a newly-synthesized benzimidazole called compound 5 on different cancer cell lines. Positive correlations between cell cycle arrestment and induction of apoptosis were reported by this study [38]. Similar to the results of our study, another benzimidazole derivative (1-benzyl-2-phenyl-benzimidazole) causes chondrosarcoma cells to be arrested in the Sub-G1 phase in a study by Liu et al. [34]. However, in other studies, cells were arrested in different stages of the cell cycle. The cell cycle analysis in our study showed that the accumulation of ovarian cancer cells in the Sub-G1 stage had no effects on the cell cycle of BD. The reason for this accumulation in both cell lines at this stage may be attributed to apoptotic cell death [39].

In our study, BD was determined to induce apoptosis in A780 and A2780ADR cells. Apoptotic death may be triggered by various stimuli, mainly involving intrinsic and extrinsic pathways [34, 36, 40]. The extrinsic pathway is triggered by the activation of caspase-8 as a result of extrinsic stimuli such as TNF-alpha, TRAIL and FasL stimulating death receptors. The intrinsic pathway is responsible for the expression of the bcl-2 apoptotic protein family with stimuli from chemotherapy drugs and radiation, which have consequences such as DNA damage and oxidative stress. In both pathways, finally caspase 3 activity is triggered and apoptosis occurs [40]. In our trial, the expressions of bax, bcl-2, caspase-8 and caspase-3 were investigated to determine whether BD triggers apoptosis using the intrinsic or extrinsic pathways. The increase in the pro-apoptotic/anti-apoptotic bcl-2 (bax/bcl-2) ratio in cancer cells is an important index for triggered apoptosis [36]. We determined that BD induced apoptosis in ovarian cells by monitoring this index (Fig. 4). Gan et al. reported that B-norcholesteryl benzimidazole induced apoptosis in SKOV3 ovarian cancer cells by Annexin V Fitch staining and western analysis showing apoptotic protein expressions [37]. They stated that B-norcholesteryl benzimidazole induced bax expression and reduced bcl-2 expression; thus, it increased the bax/bcl-2 ratio [37]. Similarly, in our study, we examined BD-induced apoptosis in ovarian cancer cells using Annexin V Fitch staining and determined an increase in bax/bcl-2 ratio. In the study by Liu et al. (2012), researching benzimidazole derivative 1-benzyl-2-phenyl-benzimidazole, this substance induced apoptosis in chondrosarcoma cells through extrinsic pathway initiators caspase-8 and FADD. In our trial, no change was seen in the expression of caspase-8. It may be concluded that BD did not affect death receptors in any way in ovarian cancer cells. Due to these results of our study, it is suggested that BD induced apoptosis in ovarian cancer cells through an intrinsic pathway. Decreases in the proliferation of ovarian cancer cells may have resulted from induction of apoptosis. Similar to the aforementioned studies in which various benzimidazole derivates were researched, in our study BD inhibited the growth and proliferation of ovarian cancer cells and induced apoptosis.

Conclusion

In conclusion, the results of the current trial showed that 1-(2-cyanobenzyl)-3-(4-vinylbenzyl)-1*H*-benzo[*d*]imidazole-3-ium chloride (BD) inhibited the growth and proliferation of ovarian cancer cells and significantly induced apoptosis in these cells for the first time in the literature. Further studies and analyses are needed to further elucidate the potential of this substance as a chemotherapeutic agent for use in the treatment of epithelial ovarian cancers, which constitute an important portion of gynecological cancers.

Limitation

The effect of BD on epithelial ovarian cancers was attempted to be demonstrated in A2780 and A2780ADR cells. In order for the study to be generalizable to all ovarian cancers, it is necessary to study different ovarian cancer cell lines. However, since these cell lines were not available, the study was conducted with the cells we had.

Author contributions All authors contributed to the concept and design of the study. The design and synthesis of the benzimidazolium salt used in the study was carried out by Senem AKKOC. Material preparation, data collection and analysis were carried out by [Sakine AKAR], [Mustafa CAKIR] and [Halil OZKOL]. The first draft of the article was written by [Sakine AKAR], and all authors commented on previous versions of the article. All authors read and approved the final manuscript.

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Data availability Not applicaple.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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