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Original Article

Smac Mimetic BV6 Increases the Sensitivity of Cancer Cells to Doxorubicin Through Suppression of Apoptosis Inhibitors

Maryam Farajzadeh[1], Mohammad-Salar Hosseini[2], Koosha Koorehpaz[3], Sanam Nami[1], Mohammad Hojjat-Farsangi[4], Farhad Jadidi-Niaragh[1,3,5*]

[1]Immunology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Student Research Committee, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Research Center for Integrative Medicine in Aging, Aging Research Institute, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Bioclinicum, Department of Oncology-Pathology, Karolinska Institute, Stockholm, Sweden
[5]Department of Immunology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

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***Corresponding Author:**
Farhad Jadidi-Niaragh,
Email: jadidif@tbzmed.ac.ir

Abstract

Background: Drug resistance poses significant challenges in cancer therapy, often resulting in treatment failure and disease progression. Doxorubicin, a widely used anthracycline, frequently faces resistance mechanisms in cancer cells, reducing its therapeutic efficacy. This study investigated the synergistic impact of doxorubicin and BV6 on the proliferation and apoptosis of cancer cells.

Methods: Doxorubicin was combined with BV6 to enhance apoptosis in murine breast (4T1) and colorectal (CT26) cancer cells. The effects of the combined treatment on cell viability and apoptosis were assessed using the MTT assay and apoptosis assays. Additionally, reverse transcription polymerase chain reaction (RT-PCR) was used to assess the impact of co-treatment on apoptosis gene expression. Data were analyzed using GraphPad Prism 6, with a *P* value of less than 0.05 considered significant.

Results: The findings showed that combining doxorubicin and BV6 results in significantly higher cytotoxicity and synergistically enhances apoptosis. Co-treatment with doxorubicin and BV6 induced cell death through decreased expression of anti-apoptotic factors and elevated expression of pro-apoptotic factors, improving the cancer cell death process.

Conclusion: The cancer cells' susceptibility to doxorubicin is enhanced by BV6. Further studies should assess the applicability and effectiveness of these interventions in vivo.

Keywords: Anthracycline, Apoptosis, Doxorubicin, Inhibitor of apoptosis protein, Smac protein

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Introduction

Cytotoxic chemotherapy agents have significantly contributed to cancer treatment; however, cancer remains a leading cause of mortality worldwide.[1-3] These agents are classified into various groups based on different factors such as their mechanism of action and chemical structure, including alkylating agents, anthracyclines, alkaloids, antimetabolites, topoisomerase inhibitors, antitumor antibiotics, and the like.[4-6] Chemotherapeutic agents often face barriers to effectively enter malignant cells, primarily due to p-glycoprotein and intracellular efflux.[7] Consequently, drug resistance is a key drawback to successful cancer treatment, often leading to disease progression and relapse.[8,9] Cancer cells can become resistant to treatment through several different mechanisms, including mutations, alternative signaling cascades, enhanced DNA repair, and the overexpression of drug efflux pumps.[10] These adaptive responses enhance the proliferation and survival of cancer cells, even in the presence of chemotherapeutic agents.

Combination therapy strategies, which involve using multiple agents with different mechanisms of action, are crucial for overcoming drug resistance. By simultaneously targeting various pathways and cellular processes, combination therapy reduces the likelihood of drug resistance in cancer cells.[11] This approach not only enhances treatment efficacy by maximizing cancer cell death but also minimizes the risk of resistance, leading to better clinical outcomes and prolonged survival for patients. Extensive efforts have been made to develop

treatment strategies with potent toxicity to enhance the efficacy of chemotherapy. The primary objective in cancer treatment is to efficiently deliver precise amounts of drugs to tumor sites while reducing damage to healthy cells.[12] Nevertheless, the intricate tumor microenvironment (TME) frequently undermines the efficacy of antitumor treatments owing to various factors such as low pH, hypoxia, and overexpression of glutathione caused by abnormal metabolism in malignant cells. Consequently, the TME becomes an attractive target for enhanced anticancer response in multimodal therapeutic approaches.[13]

Doxorubicin, a cytotoxic anthracycline, is a well-known chemotherapeutic agent due to its effectiveness in treating various cancers.[14] Its antitumor activities are primarily mediated through interference with DNA, topoisomerase II inhibition, and reactive oxygen species production.[15] These actions lead to DNA damage, disruption of DNA replication and transcription, and induction of oxidative stress, ultimately triggering apoptosis in cancer cells. Apoptosis is a programmed key mechanism through which doxorubicin exerts its cytotoxic effects, leading to cell death via intrinsic and extrinsic apoptotic pathways.[16] On the other hand, second mitochondria-derived activators of caspase (Smac) mimetics such as BV6 represent a promising class of therapeutic agents designed to counteract the anti-apoptotic effects of inhibitors of apoptosis proteins (IAPs) [17]. By mimicking the natural pro-apoptotic mitochondrial protein Smac/direct inhibitor of apoptosis-binding protein with low pI (DIABLO), BV6 binds to IAPs and disrupts their interaction with caspases, thereby promoting apoptosis in cancer cells.[18]

While doxorubicin induces DNA damage and oxidative stress, BV6 can amplify the apoptotic signaling by neutralizing IAPs, ensuring that cancer cells cannot evade programmed cell death.[19,20] This dual approach can effectively sensitize resistant cancerous cells to doxorubicin, ultimately overcoming resistance and leading to more effective and durable treatment outcomes. Therefore, this study aimed to evaluate the impact of doxorubicin and BV6 co-treatment on tumor cell growth and progression.

Methods

Reagents and Cell Lines

Cell lines for colon cancer (CT26) and breast cancer (4T1) were obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). To cultivate both cell lines, RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and penicillin-streptomycin (100 units/mL and 100 mg/mL) was used at 37 ˚C with 5% CO_2. MTT reagent was acquired from Merck (Mannheim, Germany).

Cytotoxicity Assay

The MTT assay was used to evaluate the impact of BV6 and doxorubicin on cell survival. To administer this assay, 1.5×10^4 cells from each cell line were seeded in 96-well plates. After 24 hours, various treatments were introduced into the wells, including BV6, doxorubicin, and BV6 + doxorubicin. Dimethyl sulfoxide (DMSO) served as the positive control, while untreated cells acted as negative controls. After the incubation period, each well received 100 µL of MTT (0.5 mg/mL in phosphate-buffered saline) after the supernatant was removed. Following incubating for four hours at 37 ˚C and 5% CO2, the plates were placed in an incubator. To dissolve the formazan crystals, 100 µL of DMSO was then added. After another four-hour incubation, the supernatant was aspirated, and 150 µL of dimethyl sulfoxide was added, followed by an additional incubation period of 30 minutes. Subsequently, an enzyme-linked immunosorbent assay (ELISA) reader was used to measure the absorbance of each well at 545 and 630 nm wavelengths.[21]

Extraction of RNA and Real-Time Reverse Transcription Polymerase Chain Reaction

Following the administration of various therapeutic groups, RNA extraction kits (BioFact, Korea) were used to perform the RNA extraction process in accordance with the manufacturer's guidelines. Subsequently, total RNA was employed to synthesize complementary DNA (cDNA) using a cDNA synthesis kit (BioFact, Korea). The real-time reverse transcription-polymerase chain reaction (RT-PCR) assay utilized a Light-Cycler 480 RT-PCR system (Roche) and SYBR Green Master Mix (Thermo Fisher Scientific) to amplify and assess the expression of target genes. The thermocycling parameters for RT-PCR were established as follows: an initial denaturation step at 95 ˚C for one minute, followed by 40 cycles of amplification. Each cycle comprised a denaturation phase at 95 ˚C for 15 seconds, an annealing phase at 58 ˚C for 30 seconds, and an elongation phase at 72 ˚C for 35 seconds. Furthermore, the Livak method ($2^{-\Delta\Delta CT}$) was employed to indicate the comparative transcript levels for desired genes. The transcript level of β-actin, the housekeeping gene, was also determined. The primers employed in this study are detailed in Table 1.[22]

Table 1. The Utilized Primer Sequences

Gene	Forward	Reverse
BCL2	5′- GGCTGGGGATGACTTCTCTC -3′	5′- ACAATCCTCCCCCAGTTCAC -3′
β-actin	5′- GGTCATCACTATTGGCAACG -3′	5′- ACGGATGTCAACGTCACACT -3′
BIM	5′- GAGATACGGATTGCACAGGA -3′	5′- ATTTGAGGGTGGTCTTCAGC -3′

Note. Bcl-2: B-cell lymphoma; BIM: Bcl-2 interacting mediator.

Apoptosis Assay

Cell Death Detection ELISA kit (Sigma, USA) was used to evaluate cell apoptosis within cells (3×10^4) seeded in 48-well plates. After culturing for 24 hours, cells were subjected to different treatment groups for 48 hours and removed from the plate, with washing performed twice during that time. After an hour of lysis buffer treatment, the cell pellet underwent another round of centrifugation (1200 rpm for 10 minutes). The ELISA kit was utilized to perform an apoptosis assay on the cell lysate. Absorbance at 405 nm was measured to determine the enrichment of mono- and oligo nucleosomes in the cytoplasm of apoptotic cells.

Statistical Analysis

GraphPad Prism 6 was used to statistically analyze the data, and the results were reported as mean ± standard deviation (SD). Statistical significance was determined at a P value < 0.05.

Results

Cancer Cell Viability

Treatment of cells with either BV6 or doxorubicin markedly increased cell death in both cell lines. Meanwhile, the combination of BV6 and doxorubicin resulted in the highest recorded toxicity (Figure 1).

BV6 and Doxorubicin-Induced Apoptosis Levels

An ELISA-based apoptosis assay was conducted, and the results revealed that while doxorubicin and BV6 each act as apoptosis inducers, their combination exhibits a significantly higher apoptotic effect in both cell lines (Figure 2).

Expression of Apoptosis-Related Genes

The effects of doxorubicin and BV6 on the expression of apoptosis-regulating genes, including the pro-apoptotic Bcl-2 interacting mediator (BIM) and anti-apoptotic B-cell lymphoma 2 (BCL2), were investigated. Treatment with doxorubicin and BV6 resulted in the overexpression of BIM and the underexpression of BCL2, leading to an increased tendency for apoptosis (Figure 3).

Discussion

Drug resistance remains a dominating challenge in conventional cancer therapy. Drug resistance in cancer cells is associated with various factors, including genetic and epigenetic alterations, tumor heterogeneity, and complex interactions between tumor cells and the TME.[23] Cancer cells can evade immune surveillance through multiple strategies, including the upregulation of immune checkpoint proteins, the release of cytokines that inhibit the immune system, and the stimulation of myeloid-derived suppressor cells and regulatory T cells. These adaptations not only compromise the efficacy of immunotherapies but also contribute to the dynamic and evolving nature of drug resistance. Recent studies have focused on targeting drug resistance mechanisms to elevate the effectiveness and capacity of anticancer treatments.[24]

A crucial method by which doxorubicin exerts its cytotoxic effects is apoptosis or programmed cell death. As the chief regulators of cell death, IAPs inhibit apoptosis by directly binding to and inhibiting caspases. Smac mimetics such as BV6 are a class of small molecules designed to antagonize IAPs, thereby promoting apoptosis in cancer cells. These agents mimic the function of Smac/DIABLO, binding to IAPs, displacing caspases, and neutralizing their anti-apoptotic effects. In addition to overcoming apoptosis resistance and inducing apoptosis, BV6 also hinders angiogenesis and metastasis in cancer cells.[25]

Czaplinski et al investigated the impacts of VCR/BV6 cotreatment, demonstrating that this combination leads to the phosphorylation of BCL-2 during mitotic arrest, facilitates the activation of BAX (Bcl-2-associated X protein) and BAK, and reduces mitochondrial membrane potential (MMP).[26] Marschall and Fulda indicated that BV6 and TMZ act synergistically to increase the transcriptional levels of pro-apoptotic proteins from the B-cell lymphoma 2 family, particularly Bax and Puma (a modulator of apoptosis regulated by p53).[27] The current study utilized BV6 and doxorubicin to treat murine breast and colorectal cancer cells, revealing that BV6 and doxorubicin significantly affected cancer cell viability and growth with a synergistic effect. These agents effectively

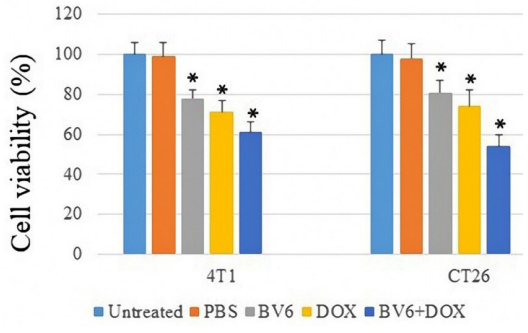

Figure 1. Cell Viability After Treatment with BV6 and Doxorubicin. Note. * P value < 0.05. Data were recorded after 24 hours of incubation. Bar charts indicate mean cellular viability ± SD.

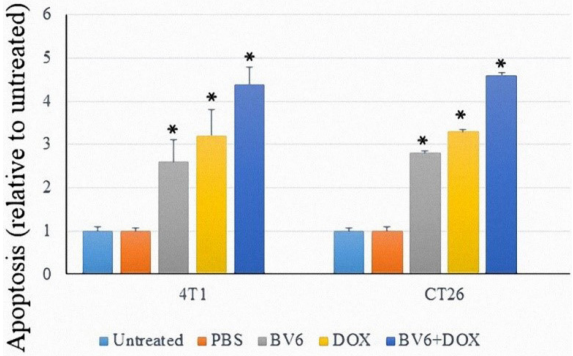

Figure 2. BV6 and Doxorubicin-Induced Apoptosis Levels. Note. * P value < 0.05. Doxorubicin and BV6 induce apoptosis in treated cells. Bar charts show mean ± SD of apoptosis levels relative to the untreated group.

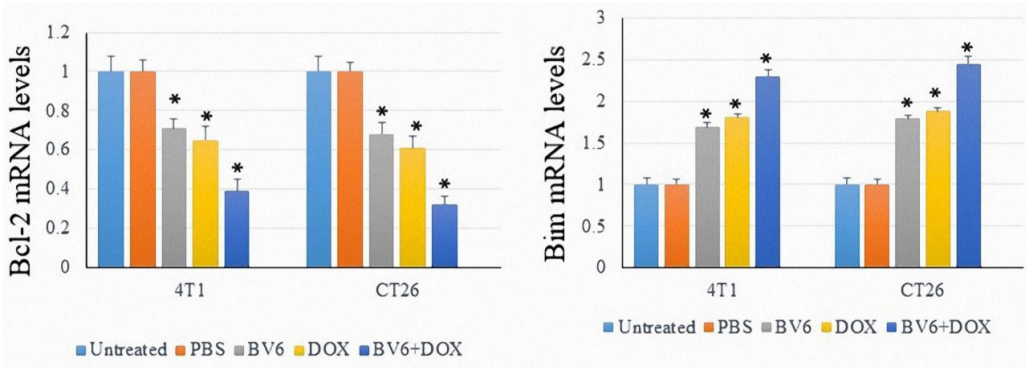

Figure 3. The mRNA Levels of Bcl-2 and BIM as Anti-Apoptotic and Pro-Apoptotic Factors Relative to Untreated Group. Note. Bcl-2 : B-cell Lymphoma 2; BIM: Bcl-2 interacting mediator; * *P* value < 0.05. The vertical axis demonstrates a fold change

modulated the expression of apoptosis-associated genes, leading to metastasis suppression and apoptosis initiation.

Inducing apoptosis is highlighted as a critical and effective strategy for developing anticancer agents.[28] Recent reports have focused on the potential capacity of doxorubicin-based combination therapies. A study by Vu et al demonstrated the extent to which doxorubicin selectively inhibits cell growth and proliferation through its Bcl-2-targeting inhibitory properties.[29] Another study by Park et al demonstrated that the cytotoxic effects resulting from the combination of doxorubicin were linked to the activation and mitochondrial accumulation of BIM, which is a pro-apoptotic protein belonging to the Bcl-2 family.[30] Yang et al confirmed that the combined use of mitochondria-targeted doxorubicin and the Bcl-2 function-converting peptide N9 led to considerable mitochondrial impairment, yielding promising results in the inhibition of both primary and metastatic breast cancer.[31] Hseu et al also demonstrated the apoptosis-inducing impact of doxorubicin in gastric cancer by regulating apoptosis-related genes.[32] Another investigation conducted by Sadeghi-Aliabadi et al revealed that doxorubicin decreases cell development, reproduction, and infiltration by triggering apoptosis.[33] Strong evidence of doxorubicin-related pro-apoptotic effects exists among several solid tumors, including breast cancer, hepatocellular carcinoma, and colorectal cancer cells.[34-36]

Several studies have suggested the synergistic effects of different anticancer agents in conjunction with doxorubicin. For instance, a recent study has proposed the combination of doxorubicin with hydralazine as a potential breast cancer inhibitory treatment.[37] Salzillo et al recently conducted a study revealing that chlorogenic acid significantly boosts the anticancer effectiveness of doxorubicin against osteosarcoma cancer cells through the induction of apoptosis.[38] Doxorubicin has also demonstrated enhanced apoptotic effects in preclinical studies combined with oncostatic agents such as melatonin.[39] Similar synergistic effects have been observed with doxorubicin and other cytotoxic chemotherapy agents.[40]

Considering the apoptosis-promoting features of Smac mimetics and their ability to overcome apoptosis resistance, studies have hypothesized potential additive advantages of co-treatment with cytotoxic anticancer agents.[41] A recent cell study demonstrated how the Smac mimetic SBP-0636457 enhances cell death by inducing tumor necrosis factor α (TNFα)/tumor necrosis factor receptor (TNFR) and nuclear factor kappa b (NF-κB) signaling pathways.[42] This study also indicated the potential role of combination therapy in inducing the necroptosis process, presenting it as an alternative treatment for apoptosis-insensitive breast cancer. Another recent study reported a synergistic apoptosis-inducing impact of doxorubicin and Smac mimetics as enhanced sensitization of doxorubicin-treated cancer cells to apoptosis was observed through activation of FADD/RIPK1/CYLD/TNF/caspase-8 signaling pathway.[43] Some studies have also suggested the combination of doxorubicin with Apo2 ligand or TNF-related apoptosis-inducing ligand (APO2L/TRAIL) could amplify anticancer effects through the administration of Smac mimetics.[44] Moreover, cancer cell sensitivity to doxorubicin has been associated with the Smac/DIABLO pathway, mediated through Bcl-2 and its pro-apoptotic BH3-only member, BIM,[45,46] thereby suggesting potential synergistic effects, which were confirmed by our study results. Therefore, combining Smac mimetic with cytotoxic agents such as doxorubicin presents a favorable apoptosis-inducing approach, offering great potential for developing efficient and effective anticancer therapies.

Conclusion

The Findings of the current study imply that treatment with BV6 and doxorubicin can potentially increase cancer cell sensitivity and how cancer cells react to doxorubicin. BV6 and doxorubicin can suppress cancer cell growth and aggression by activating the pro-apoptotic pathways and regulating the expression of apoptosis-linked genes. Moreover, the simultaneous administration of BV6 and doxorubicin significantly enhances their anti-proliferative and anti-migratory effects. The results indicate the potential effectiveness of this therapeutic approach in treating solid tumors. Nevertheless, further research is needed to evaluate the success of this strategy in vivo.

Ethics statement
This study was ethically approved by Tabriz University of Medical Sciences (Ethic number: IR.TBZMED.VCR.REC.1399.452).

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Conflict of interests declaration
The authors declare no conflict of interests.

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Data availability statement
The data related to the study are available from the corresponding author upon reasonable request.

Author contributions
Conceptualization: Farhad Jadidi-Niaragh, Mohammad Hojjat-Farsangi, Sanam Nami.
Data curation: Maryam Farajzadeh, Mohammad-Salar Hosseini, Koosha Koorehpaz.
Formal analysis: Maryam Farajzadeh, Mohammad-Salar Hosseini.
Funding acquisition: Sanam Nami.
Investigation: Maryam Farajzadeh, Mohammad-Salar Hosseini, Koosha Koorehpaz.
Methodology: Maryam Farajzadeh, Mohammad-Salar Hosseini.
Project administration: Farhad Jadidi-Niaragh.
Resources: Mohammad Hojjat-Farsangi, Farhad Jadidi-Niaragh.
Software: Maryam Farajzadeh, Mohammad-Salar Hosseini.
Supervision: Farhad Jadidi-Niaragh.
Validation: Mohammad Hojjat-Farsangi, Sanam Nami, Farhad Jadidi-Niaragh.
Visualization: Mohammad Hojjat-Farsangi, Sanam Nami, Farhad Jadidi-Niaragh.
Writing–original draft: Maryam Farajzadeh, Mohammad-Salar Hosseini, Farhad Jadidi-Niaragh.
Writing–review & editing: Maryam Farajzadeh, Mohammad-Salar Hosseini, Mohammad Hojjat-Farsangi, Farhad Jadidi-Niaragh.

Consent for publication
Not applicable.

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