ImmunoAnalysis, 2022, 2, 9
doi:10.34172/ia.2022.09
https://ia.tbzmed.ac.ir/

Original Research

Combinational Therapy of Acute Lymphoblastic Leukemia with Cyclophosphamide and BV6 Synergistically Induces Apoptosis in Leukemic Cells

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ARTICLE INFO

Article History:
Received: October 21, 2022
Accepted: December 24, 2022
ePublished: December 26, 2022

Keywords:
ALL, Cyclophosphamide, BV6, Leukemia, Chemoresistance

Abstract

***Background*:** Acute lymphoblastic leukemia (ALL), a hematological malignancy, is associated with the accumulation of lymphoblasts in hematopoietic tissues and the peripheral blood (PB). A major obstacle in ALL treatment is the failure to undergo apoptosis, thus resulting in relapses. Cyclophosphamide is an alkylating agent widely used to treat various cancers. BV6 is another agent, under preclinical and clinical trials, which inhibits the function of inhibitors of apoptosis proteins (IAPs) in cells. According to previous studies, IAPs are highly overexpressed in ALL cells. Also, the intrinsic apoptosis pathway is reported to be dysfunctional in ALL cells, making BV6 a new promising option due to its potential to induce extrinsic cell death as well.

Methods: In the current study, the inhibitory effect of the combinational treatment of cyclophosphamide and BV6 was studied on the cell growth of eleven ALL patients using an MTT test. Subsequently, the effect of the treatment was studied on TNF-α secretion as well. Finally, we measured the DNA fragmentation rates of treated cells as well.

Results: The results indicated a synergistic effect between the drugs on the viability of cells. Also, the impact of the cotreatment was increased in a time-dependent manner. We also found that BV6, but not cyclophosphamide, induces TNF-α secretion, activating the extrinsic apoptosis pathway as well.

Conclusion: BV6, by inducing intrinsic and extrinsic pathways, overcomes cyclophosphamide resistance. These results suggest the combinational use of BV6 and cyclophosphamide for the treatment of ALL patients. Inhibition of IAPs helps overcome the failure to undergo apoptosis and can improve the prognosis of ALL patients.

Introduction

Acute lymphoblastic leukemia (ALL), the most common malignancy in children, results from the excess proliferation and accumulation of lymphoblasts in the peripheral blood (PB) and other organs.[1-3] ALL can present with anemia, thrombocytopenia, and leukopenia.[4] The patients may show signs of hepatosplenomegaly and lymphadenopathy.[5,6] Due to the recent advances in chemotherapy, the survival rates for ALL pediatric patients have risen to approximately 80%. On the other hand, the additional 20%, as well as the elderly, experience a more severe disease associated with poor response to therapy, relapse, chemoresistance, and thus, poor prognosis.[7-10] Therefore, ALL patients, adult patients, in particular, require novel therapeutic methods.

Cyclophosphamide, an alkylating agent and highly cytotoxic, is used to treat various malignancies.[11,12] Cyclophosphamide crosslinks DNA strands and inhibits cell cycle progression, followed by the initiation of programmed cell death.[11,13]

A major issue inducing the rescue of tumor cells is the ability to escape cell death. This obstacle can cause resistance to several chemotherapy agents.[14,15] For the initiation of apoptosis, two main mechanisms are recognized. The extrinsic pathway starts with the activation of the tumor necrosis factor-receptor

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(TNF-R) superfamily, and the activation of caspase-8 via a protein named death-inducing signaling complex , which results in the activation of the effector caspases and apoptosis.[16,17] The intrinsic pathway initiates from the release of cytochrome C and second mitochondria-derived activators of caspases (SMAC) from the mitochondria, followed by the formation of a complex between cytochrome C and caspase-9 which then cleaves caspase 3 and the release of SMAC which then inhibits the function of inhibitors of apoptosis proteins (IAPs), that are considered as the main inhibitors of cell death.[18] Through inhibiting caspases, IAPs prevent cell death. It is proven that IAPs are overexpressed in neoplastic cells,[19,20] thus making them an ideal target for cancer therapy. In leukemias, in particular, acute myeloid leukemia (AML) cells overexpress cellular IAP (cIAP-1).[21] In the case of ALL, the overexpression of X-linked inhibitor of apoptosis protein (XIAP) by ALL cells is reported to be correlated with poor prognosis in patients.[22] SMACs act as endogenous antagonists of IAPs.[18] BV6, a bivalent SMAC mimetic (SM), consists of two subunits and possesses a high affinity towards IAPs.[23] BV6 is designed to inhibit cIAP1 and XIAP. According to a study, combinational therapy of BV6 with several chemotherapeutics, including vincristine, dexamethasone, and asparaginase, synergistically decreased tumor relapse and improved the survival of murine ALL models when compared to treatment with each chemotherapeutic alone. The same research also reports that apoptosis induction by BV6 is mediated by both mitochondrial (intrinsic) and death receptor-mediated (extrinsic) pathways.[24] Knowing that leukemic cells cannot execute apoptosis from the intrinsic pathway, BV6 appears to be hopeful.[24] Another study reports that the cotreatment of ALL cells with BV6 and 5-azacytidine (5AC) induced apoptosis and necroptosis in cell.[25] Based on another survey, BV6 cooperates with glucocorticoids in inducing apoptosis in ALL cells as well.[26] Therefore, BV6 seems to form synergism with several anticancer drugs.

As far as we know, no previous research has ever aimed to study the cotreatment effect of cyclophosphamide and BV6 in ALL. Thus, in the present study, we used patients' PB and bone marrow (BM) samples to evaluate the combinational effect of cyclophosphamide and BV6 treatment on ALL cells.

Materials and Methods

Materials

Cyclophosphamide and BV6 were purchased from Selleckchem (UK). The MTT Cell Proliferation Assay Kit was bought from Sigma-Aldrich and was performed by following the directions given by the company.

Patient samples

Based on the Declaration of Helsinki, heparinized PB and BM samples were taken from children diagnosed at Shahid Ghazi hospital. Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were then isolated using Ficoll Paque™ Plus (GE Healthcare, Uppsala, Sweden). Subsequently, cells were cultivated in RPMI-1640 medium enriched with 10% FBS and 2% L-glutamine. Patients' characteristics are demonstrated in Table 1.

Drug dose optimization

To determine the lowest effective dose of each drug, a series of increasing concentrations from 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μM of BV6, and another series of concentrations from 20, 40, 60, 80, 100, and 120 μM for cyclophosphamide were prepared. Next, the cells were incubated with these concentrations for 24 hours. The viability of the cells was then evaluated by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) test, as described later. Thereafter, the optimized concentration (IC_{50}) of the drugs was calculated using GraphPad Prism 9 software.

Analysis of cytotoxicity and cell growth

To assess the viability of cells, an MTT test was performed

Table 1. Patients' characteristics

Patients	Age (y)	Gender	WBC ($x10^3$/mL)	Platelet ($x10^3$/mL)	Hb (g/dL)	LDH Level (Nl: Up to 480 U/L)	Hepatomegaly	Splenomegaly	Lymphadenopathy	C- ALL Ag (CD10) (%)	Subtype
1	3	F	106.3	23	8.2	1801	Yes	Yes	Peripheral	83	Pre B-cell
2	11	M	5.1	39	12.2	1372	Yes	Yes	No	90	Pre B-cell
3	4	M	2.5	52	6.6	395	Yes	Yes	Peripheral	23	Pre B-cell
4	10	M	8	141	10.3	360	Yes	Yes	Peripheral	0	Pre B-cell
5	8	M	10.4	66	8.7	701	No	Yes	No	92	Pre B-cell
6	8	M	4,02	74	9.8	1283	Yes	Yes	No	0	Pre B-cell
7	6	M	9.9	8	2.4	375	NO	Yes	No	79	Pre B-cell
8	11	M	6.5	54	9.8	2437	Yes	No	Peripheral	0	Pre B-cell
9	12	M	74.2	21	9.9	1284	Yes	Yes	Mediastinal	0	T-cell
10	4	M	41.3	53	11.5	1525	Yes	Yes	Mediastinal	0	T-cell
11	9	M	56.4	57	10.1	1367	Yes	Yes	Mediastinal	0	T-cell

Abbreviation: WBC, white blood cell; LDH, lactate dehydrogenase; CALL Ag, Common ALL antigen; Hb, hemoglobin.

according to the protocol given by the company. Primary cells were inoculated in a 96-well plate (2×10^4) and cultivated for a day. Subsequently, cells were treated and incubated for 24 hours or 48 hours with the following mixtures; untreated, BV6 (optimized concentration), cyclophosphamide (optimized concentration), cyclophosphamide + BV6 (optimized concentration), and DMSO (0.2%). Next, the upper medium was replaced with 100 µL of medium containing 10 µL of MTT solution and incubated for 4 hours. Finally, 100 µL of DMSO was added to the well (incubation for 4 hours). Eventually, the absorbance of the wells was measured at 490 nm. The test was carried out three times.

Measurement of apoptosis with enzyme-linked immunosorbent assay (ELISA)

Confluent primary cells were seeded in a 96-well plate, with each well containing 50 µL cell suspension (1×10^5 cells/mL). The following treatments (50 µL) were added to each well: BV6, Cyclophosphamide, BV6 + Cyclophosphamide, and the negative control received no treatment. The plates were then incubated for 24 hours in a humidified 5% CO_2 incubator at 37°C before determining cell death using the Cell Death Detection ELISA kit.

After incubation, the cell suspension was centrifuged (1200 rpm, 10 minutes), and the supernatant was removed. The cell pellet was treated with lysis buffer for 1 h before being centrifuged again (1200 rpm, 10 minutes). The cell lysate was used for apoptosis assay with the ELISA kit. The enrichment of mono- and oligo nucleosomes in the cytoplasm of apoptotic cells was determined based on the absorbance at 405 nm.

Measurement of TNF-α secretion by ALL cells

Primary cells of ALL patients were seeded in 48-well plates. After reaching 80%-90% confluency, cells were washed, followed by administration of media with none, BV6, cyclophosphamide, and cyclophosphamide + BV6. After 12 hours of incubation, the supernatant was harvested and then concentrated to 100 µL using Amicon® Ultra-0.5 Centrifugal Filter 10K Devices (Millipore, Billerica, MA). TNF-α concentrations were determined via QuantiGlo Human TNF-α ELISA kit (R&D Systems, Minneapolis, MN) based on the provided instructions by the company.

Statistical analysis

Data analysis was performed using the GraphPad Prism 9 application. Also, a two-way ANOVA test was used to evaluate the differences between different treatment groups. $P < 0.05$ was considered significant (ns: non-significant, * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$).

Results

Optimized drug dose

Following 24 hours incubation with different drugs, the IC_{50} values of BV6 and cyclophosphamide were measured for PBMCs and BMMCs. The IC_{50} value of BV6 for PBMCs and BMMC was 6 and 5.5 µM, respectively. For the cyclophosphamide, however, the IC_{50} values for PBMCs and BMMCs were 97 and 86 µM.

Cyclophosphamide and BV6 exert synergistic effects on the growth inhibition of leukemic cells

Subsequently, the MTT test was performed to evaluate the effect of the combinational therapy on the growth of cells. The results are presented in Figure 1. No significant change was seen in controls following 24 hours incubation (99.5% of PBMCs and 99% of BMMCs were viable). Both drugs induced significant growth inhibition in patient-derived cells when used alone following 24 hours treatment. BV6 treatment decreased the growth of PBMCs to 65% and BMMCs to 64%. The growth inhibition rates were slightly higher in cells treated with cyclophosphamide when compared to BV6 treatment (46.7% in PBMCs and 47.7%

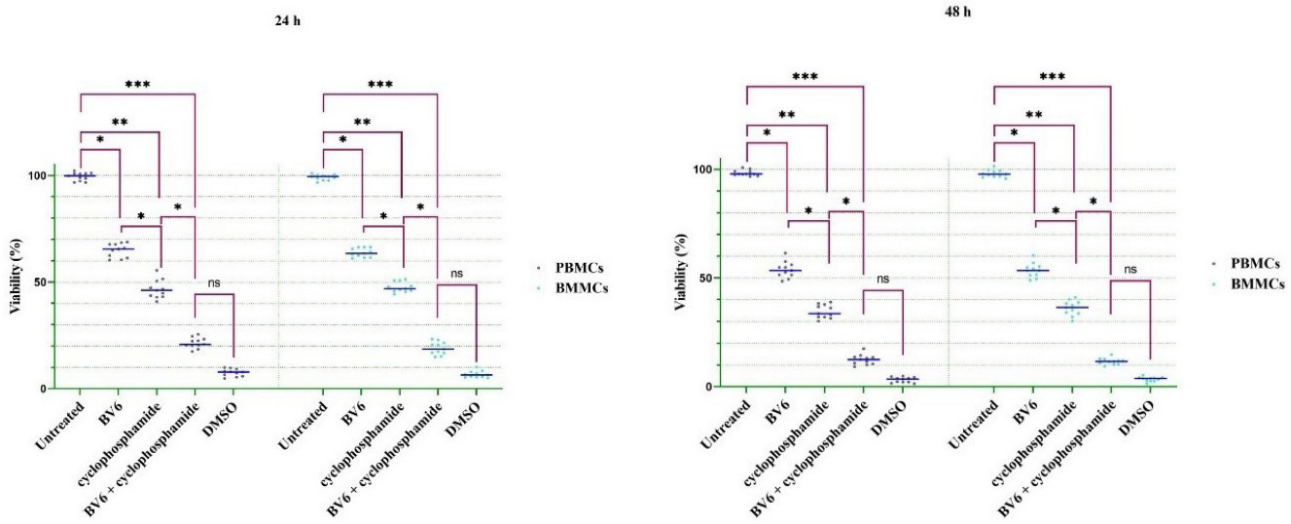

Figure 1. The cytotoxic effect of combination therapy on ALL-derived primary cells. The bar chart demonstrates the effect of BV6 treatment alone and combined with conventional chemotherapeutic cyclophosphamide on 11 ALL patients' derived cells using an MTT test. * Sign represents $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, Bone marrow mononuclear cell; ns, non-significant; DMSO, Dimethyl sulfoxide

in BMMCs were viable). However, treating cells with both drugs exerted the highest inhibitory effect on the growth of cells (21.5% in PBMCs and 18.95% in BMMCs remained viable), which was comparable to treatment with Dimethyl sulfoxide (7.5% viability in PBMCs and 6.70% in BMMCs), indicating that the simultaneous administration of these drugs induces synergistic effects in ALL.

Also, the treatment effect with both drugs increased over time, as the inhibition of cell growth was further increased after 48 hours of treatment (decreased to 12% in PBMCs and 11.5% in BMMCs during this period) compared to 24 hours results.

BV6, in combination with cyclophosphamide, synergistically induced TNF-α secretion by ALL cells

According to the results of the ELISA assay, treatment with BV6 induces high levels of TNF-α secretion by patients' cells. In contrast, cyclophosphamide fails to induce ALL cells to secret this cytokine.

Also, combinational treatment with both drugs did not significantly elevate the secretion level of TNF-α compared to BV6 treatment alone. The results are demonstrated in Figure 2.

DNA fragmentation measurement by ELISA-based assay demonstrated synergism between BV6 and cyclophosphamide in inducing apoptosis

Based on the results achieved from the apoptosis analysis, BV6 or cyclophosphamide treatment alone highly increased the fragmentation of DNA by apoptosis. The apoptotic effect of cyclophosphamide treatment was slightly higher than BV6. The highest levels of apoptosis rates were seen in the group of cells treated with both compounds, indicating the efficiency of co-treatment. The results are illustrated in Figure 3.

Discussion

ALL, a hematological neoplasm, is known by the repletion of lymphoblasts in PB and other organs.[27] ALL treatments usually comprise multidrug chemotherapy regimens. Despite all the advances in ALL management, high rates of disease recurrence are still a major problem indicating the need for novel treatment.[28,29] One of the main reasons contributing to relapse is the failure of ALL cells to undergo apoptosis.[14]

IAPs nowadays are at the center of cancer immunotherapy research, as they are proven to exert direct anti-apoptotic functions in cells. IAPs include cIAP1/2, XIAP, and survivin.[30] The mechanism by which SMs induce cell death depends on the affinity towards IAPs. Following the binding of SMs to cIAP-1/-2, these molecules are autoubiquinated, and eventually degraded by the proteasome. This results in the activation of caspases 8, 3, and 7 activations and finally, intrinsic apoptosis.[23] According to a group of studies, XIAP and survivin (another member of IAPs) are reported to be overexpressed in ALL cells.[22,31] The overexpression of XIAP is associated with poor response to prednisone and more adverse events in patients with T-ALL disease.[22]

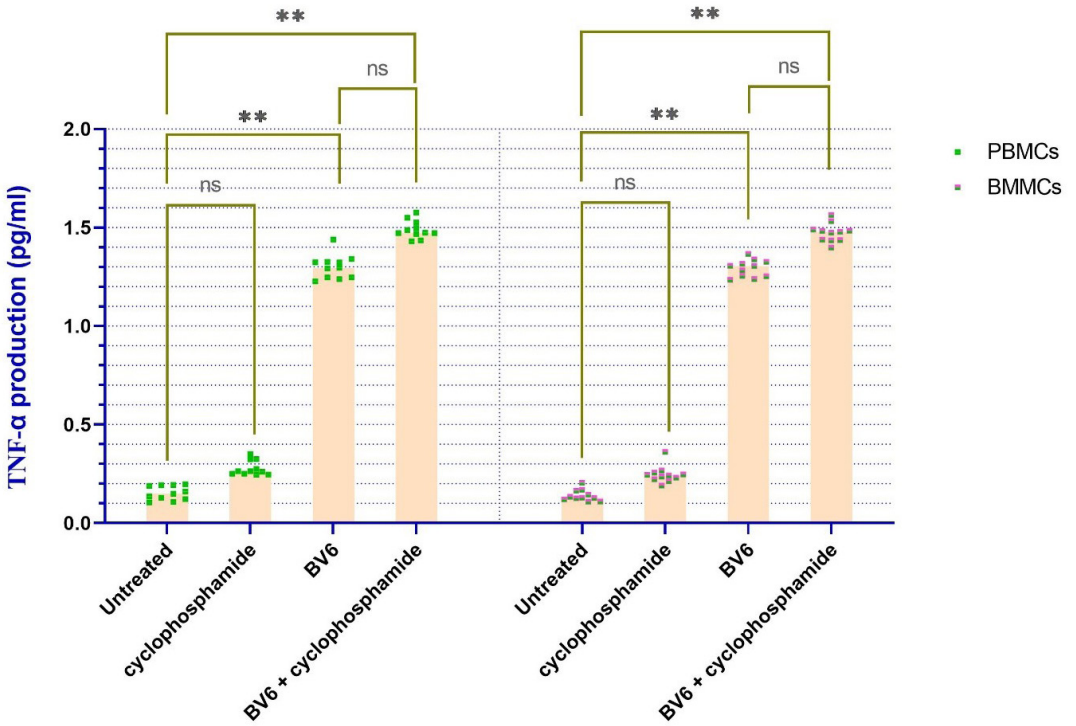

Figure 2. Treatment of ALL leukemic cells with BV6 affects the secretion of TNF-α. To measure the amount of TNF-α production in response to each treatment, culture media were analyzed by ELISA following a 12 h treatment of primary cells with BV6, cyclophosphamide, BV6 + cyclophosphamide, and none (control). * Sign represents $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, Bone marrow mononuclear cell; ns, non-significant

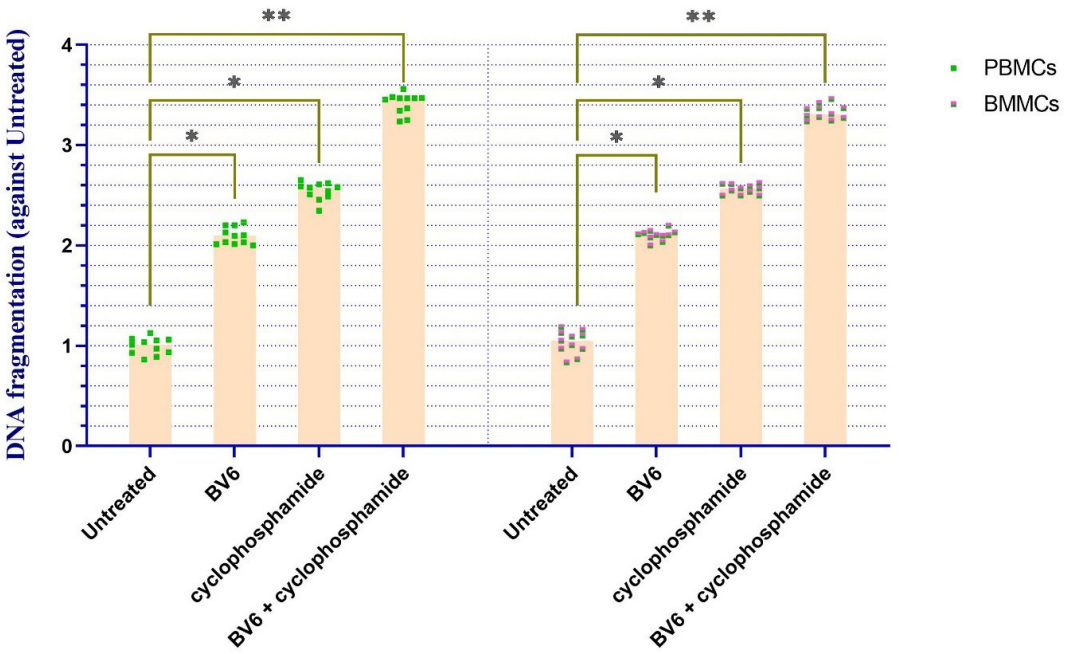

Figure 3. Combination therapy of ALL cells by BV6 and cyclophosphamide induces apoptosis in leukemic cells. The bar chart demonstrates the ratio of DNA fragmentation compared to untreated by ELISA-based apoptosis assay following 24 h treatment with each pharmacological group. * Sign represents $P<0.05$, ** indicates $P<0.01$, and *** indicates $P<0.001$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, Bone marrow mononuclear cell; ns, non-significant

At the same time, survivin overexpression is reported in B-ALL cells and is considerably higher in patients with relapsed disease compared to patients with long-term remission.[31] A group of endogenous molecules known as SMACs antagonizes IAPs functions. Thus, many compounds have been designed to mimic SMAC function to induce cell death.[31,32]

BV6, a recently developed bivalent SM, binds with high affinity to the Baculoviral IAP Repeat (BIR) domain of IAPs, resulting in IAP ubiquitination. Eventually, the IAP is degraded by the proteasome.[30,33] According to previous studies, the loss of cIAP1 results in the activation of NF-κB, which in turn results in TNF-α secretion and the initiation of apoptosis through the binding of TNF to TNF-receptor-1, which results in the activation of caspase-8 and finally apoptosis through the extrinsic pathway as well. Thus, BV6 can induce both extrinsic and intrinsic apoptosis pathways.[30]

Cyclophosphamide, a nitrogen mustard derivative, is a highly DNA alkylating agent.[34,35] Most of the anticancer effects of cyclophosphamide are due to phosphamide metabolites, which crosslink DNA strands resulting in cell apoptosis.[36]

Thus, in the following study, we aimed to evaluate the combinational effect of BV6 and cyclophosphamide on eleven ALL patients' PB and BM samples. According to the results of MTT, treating with each drug inhibited the growth of cells significantly. While the highest level of growth inhibition was seen in the group treated with BV6 and cyclophosphamide, indicating a synergistic effect between the drugs on ALL cells. Also, the impact of both drugs increased over the 48 hours period, meaning the time-dependency of the cotreatment. In addition, it was found that BV6 induces TNF-α secretion from ALL cells, while cyclophosphamide does not possess this property. TNF-α induces extrinsic apoptotic pathway activation by an autocrine/paracrine mechanism. Lacking this property, cyclophosphamide only induces intrinsic apoptosis, while BV6 is effective in the induction of both pathways. Moreover, the results of apoptosis measurement demonstrated that BV6 synergistically induces further apoptosis in combination with cyclophosphamide. Our results are in line with a number of studies suggesting the same mechanism.[17,25,30]

Our results indicate that BV6 significantly sensitized cells to cyclophosphamide. These results align with several other studies evaluating the effect of treatment with various chemotherapeutics in combination with BV6. According to Schirmer et al, BV6 treatment induced both intrinsic and extrinsic apoptosis pathways in B-ALL cell lines. Also, they reported synergism between BV6 and several other chemotherapeutics, including vincristine, dexamethasone, and asparaginase, compared to treatment with each agent alone in ALL murine models.[24] Based on another study, cotreatment of ALL cells with BV6 and 5AC induced apoptosis in cells with caspase activation possibility and necroptosis in apoptosis-resistant ALL cells due to the caspase inhibition.[25] The synergistic effect of BV6 with prednisolone and dexamethasone was also reported by another study. The research reports elevated survival of ALL murine models when treated with the combination of BV6 and glucocorticoids compared to monotherapy, without any further complications.[26]

Conclusion

Based on the results of our study and the previous studies, treatment with BV6 helps to eliminate ALL cells efficiently. Also, BV6 treatment seems to form synergistic effects with other anticancer drugs by further activating apoptotic pathways. Our results can help clear the way for clinical trials aiming to evaluate the impact of this combinational therapy on ALL patients.

Author Contributions

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Writing – original draft: Mohammad Sadeghi.
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Funding Sources

This study was financially supported by the Tabriz University of Medical Sciences grants (65014 and 65018).

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethical Issues

All procedures were conducted in compliance with the ethical principles of Tabriz University of Medical Science, Tabriz, Iran and approved by the regional ethical committee for medical research (Ethical code: IR.TBZMED.REC.1399.202).

Conflict of Interest

The authors declare that they have no competing interests.

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