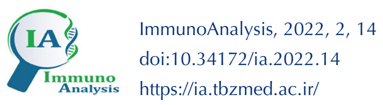
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Original Article

Bcl-2 Inhibition Induces a Synergistic Effect in Combination with Doxorubicin in Chronic Lymphocytic Leukemia

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Abstract

Background: Chronic lymphocytic leukemia (CLL), a common neoplastic disease, is associated with the accumulation of B-lymphocytes in the hematopoietic organs. A main characteristic of CLL cells is the failure to undergo apoptosis, thus resulting in resistance to several chemotherapeutics. Doxorubicin (DOX), an anthracycline widely used for treating various neoplasms including CLL, induces apoptosis via several mechanisms. Despite this, CLL cells become resistant to DOX. A major protein known as B-cell lymphoma-2 (Bcl-2), known to exert direct anti-apoptotic effects on the cell, is reported to be overexpressed in CLL cells.

Methods: We aimed to silence the *Bcl-2* gene by siRNA. Mononuclear cells were isolated from the peripheral blood and bone marrow of eleven untreated CLL patients by Ficoll-Paque. To transfect cells, we used Lipofectamine. Bcl-2 expression was investigated using qRT-PCR. Next, we studied the effect of Bcl-2 silencing in combination with DOX treatment on the viability of cells by an MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay.

Results: Bcl-2 expression was significantly suppressed in CLL cells following siRNA transfection via lipofectamine. Based on the results of MTT, the inhibition of Bcl-2 sensitized CLL cells to DOX treatment. Also, the effect of the treatment was time-dependent.

Conclusion: These findings imply that combination therapy of CLL using anti-Bcl-2 siRNA and DOX can be considered a practical therapeutic approach that should be further evaluated in future studies.

Introduction

Chronic lymphocytic leukemia (CLL), a common leukemia type in the west, is associated with the repletion of CD5⁺, CD19⁺, CD20^{dim}, CD23⁺, and surface immunoglobulin^{dim} B lymphocytes in the peripheral blood (PB), bone marrow (BM), spleen, and lymph nodes. CLL mainly affects men and the elderly (with a median age of 65 at diagnosis).[1] CLL treatment comprises the treatment with several chemotherapeutics or hematopoietic stem cell transplantation (HSCT).[2,3]

Doxorubicin (DOX), also known as Adriamycin, is an anthracycline widely used to treat various neoplasms, including leukemia.[4] It is proven that DOX induces cell death through three distinct mechanisms. First, by incorporation into DNA molecules, which in turn inhibits DNA and RNA synthesis. Second, by inhibiting topoisomerase II, and finally, by damaging cells via free-radical-mediated cell injury.[2,5] Due to the high toxicity of chemotherapy agents and the possibility of the development of drug resistance by CLL cells and relapse, CLL remains an incurable disease that requires new treatment methods.[6,7]

The main reason leading to the development of cancer, relapse, and chemoresistance is the failure to undergo apoptosis.[8] A group of proteins known as the Bcl-2 family strictly regulate cell apoptosis. This protein category possesses both anti and pro-apoptotic properties. A major protein from this family is B-cell lymphoma-2 (Bcl-2), which exerts high anti-apoptotic effects on the cells.[9,10] Bcl-2 inhibits the pro-apoptotic functions of Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist killer (BAK), suppressing apoptosis initiation.[11] It has been well established by several studies that Bcl-2 is upregulated in various neoplasms.[12,13] Based on a survey, CLL cells express high levels of Bcl-2.[14] It has been discovered that in CLL, the overexpression of Bcl-2 results from the lost suppression by miRNA 15/16.[15]

Several specific inhibitors have been designed to inhibit

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Bcl-2 and induce the release of BAK/BAX from Bcl-2 binding.[11] Based on clinical trial results, the inhibition of Bcl-2 by a specific inhibitor called Venetoclax caused a clinical response in 92 out of 116 CLL patients with poor prognoses. Also, 20% of patients achieved complete remission.[16] Although Venetoclax has shown impressive initial clinical activity in various hematological neoplasms, the development of intrinsic or acquired drug resistance is a major impediment to obtaining an efficient and long-lasting treatment with this agent.[11] Therefore, we hypothesized that Bcl-2 inhibition, in combination with a chemotherapy agent, may prevent this resistance as it is proven that Bcl-2 inhibition synergizes with the administration of other approved anti-cancer drugs, including anti-CD20 monoclonal antibodies in CLL patients.[17,18] In acute myeloblastic leukemia (AML), the combinational therapy of Venetoclax and azacytidine induced hopeful clinical results in patients as well.[19] Moreover, based on a study, Bcl-2 upregulation by bladder cancer cells induces DOX resistance. This research group also found that Bcl-2 overexpression inhibited reactive oxygen species (ROS) formation in cancer cells following the treatment with DOX and induced resistance to this chemotherapeutic.[20] Thus, in the following study, Bcl-2 was silenced using siRNA in the primary cells of CLL patients, and the effect of Bcl-2 silencing in combination with DOX treatment on the viability of CLL cells was studied.

Materials and Methods

Materials

Cayman Chemical Company supplied DOX. Human Bcl-2 gene targeting siRNA (Catalog number: sc-29214) was obtained from Santa Cruz Biotechnology, Inc. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit was supplied by American Type Culture Collection (ATCC) and was utilized in accordance with the instructions of the company.

Patient samples

Peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were separated from samples of eleven CLL patients (eight males and three females) using Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) at Shahid Ghazi Hospital, Tabriz. Prior to sample taking, permission was granted by the ethics review board of Tabriz University of Medical Sciences with the ethics code number of IR.TBZMED.REC.1399.204. The demographic data of patients are presented in Table 1. PBMCs and BMMCs from patients were cultivated in RPMI-1640 with 10% FBS.

Cell transfection with siRNA

Primary cells were inoculated at 1×10^4 cells/ well in 96 well plates and were incubated at 37°C, 5% CO_2 for 24 h. Next, 50 pM of siRNA was delivered into the cells using lipofectamine 2000 (Invitrogen) in accordance with the company's directions.

RNA extraction and cDNA synthesis

The total RNA of cells (after 48 hours of incubation with different treatments) was extracted using TRIzol based on the company's directions. Subsequently, 2 µg of Complementary DNA (cDNA) was synthesized using the One-Step SYBR® RT-PCR Kit protocol (Takara Bio Inc., Japan) and stored at −20 °C until use.

Analysis of gene expression

A qRT-PCR test was performed with PrimeScrip Real-time PCR Kit (Takara Bio Inc., Japan) using standard thermal cycling conditions after incubation with different treatments for 24 hours. The protocol provided by the company was followed. Data were normalized to β-actin as an endogenous control. The relative amount of Bcl-2 mRNA level was measured using ΔΔCT Method.

Primer sequences are as follows: *Bcl-2* gene: Forward (F): 5'-ATCGCCCTGTGGATGACTGAGT-3', Reverse (R): 5'- GCCAGGAGAAATCAAACAGAGGC-3',[22] *β-actin*: F: 5'-CACCATTGGCAATGAGCGGTTC-3', R: 5'- AGGTCTTTGCGGATGTCCACGT-3'.[23]

Analysis of cytotoxicity and cell death

First, primary cells were aliquoted to each well at a concentration of 2×10^4 cells/ well. After 24 hours incubation, cells were subjected to various combined treatments of siRNA and DOX and incubated for 24 and 48 hours. The treatments included untreated, Lipofectamine, Bcl-2 siRNA, DOX, lipofectamine-Bcl-2 siRNA, lipofectamine-Bcl-2 siRNA + DOX, and DMSO (0.2%). For the drug, the optimized recommended concentration was used. While for the siRNA, 50 pM was delivered into the cells. Cells were incubated with the treatments for 24 or 48 hours. Subsequently, the medium was replaced by fresh medium enriched with 10 µL of MTT solution and was incubated for 4 hours. Next, 100 µL of DMSO was added to stop the reaction. Plates were analyzed by a plate reader (Synergy 4, BioTec, USA) at 570 nm wavelength. MTT test was repeated three times in 96 well plates for 24 and 48 hours. The following formula was used to calculate the Viability of cells[24]:

$$3)\ \text{Viability} = \frac{(\text{OD treated well}[-\text{blank}])}{(\text{mean OD control well}[-\text{blank}])} \times 100$$

Statistical analysis

Graph Pad Prism V9 was utilized to conduct a one-way ANOVA test on the data to determine whether there were any differences between the groups. $P < 0.05$ was considered significant.

Results

Lipofectamine efficiently transfects cells and suppressed Bcl-2 expression

Following the treatment of cells with various treatment groups, Bcl-2 mRNA levels were determined by qRT-PCR

Table 1. The demographic data of patients[21]

Sample number	CLL #1	CLL #2	CLL #3	CLL #4	CLL #5	CLL #6	CLL #7	CLL #8	CLL #9	CLL #10	CLL #11
Sample type	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM	PB/BM
Disease subtype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype	mature B lymphoid cell phenotype
age	65	54	74	65	81	54	65	55	66	79	80
sex	M	M	M	F	M	M	M	F	F	M	M
WBC	24.27 H	26.03 H	45.01 H	26.83 H	34.45 H	20.19 H	14.21 H	11.15 H	23.33 H	22.02 H	26 H
RBC	2.41 L	0.40 L	3.88 L	4.08	4.72	4.96	5.34	5.49	5.022	1.03 L	0.43 L
Plt	24 L	16 L	326	200	452 H	133	140	269	340	62 L	16 L
Hb (g/dL)	7.5 L	3 L	12.3	11.9 L	12.8	14.1	14.7	13.4	14.9	3.5 L	1.1 L
HCT	23.7% L	4.4% L	35.4% L	40.3%	44.2%	46.4%	47%	45.3%	45.1%	11.2% L	8.9% L
MCV	98.1	110.2 H	91.3	85.3	93.7	93.7	88.1	82.2	86.4	109.6 H	113.4 H
MCH	31.1	28.5	31.7 H	25.1 L	27.1	28.4	27.5	24.4 L	28.5	34.3 H	23.8 L
MCHC	31.7	25.9 L	34.7	29.4 L	29 L	30.3 L	31.2 L	29.7 L	33	31.3 L	24.8 L
RDW	23.6% H	16.6% H	14.1%	13.4%	14.9%	12.6%	13%	12.9%	12.2%	15% H	18%
NEUT	5.8% L	7.7% L	43% H	16.9% L	10.9% L	26.1% L	17.6% L	30.5% L	28.8% L	23% L	9% L
LYMPH	83.6% H	86.3% H	55.9% H	74.6% H	73.7% H	65.3% H	78% H	62% H	63.5% H	69% H	75.3% H
MONO	1.3% L	0.8% L	0.9% L	1.5% L	0.9% L	3% L	1.2% L	3.2% L	2.1% L	3.2% L	1.4% L
CD5	96%	97%	50%	81%	89%	94%	81%	88%	87%	82%	93%
CD19	88%	95%	68%	94%	79%	65%	78%	56%	63%	86%	75%
CD20	83%	50%	70%	60%	79%	71%	70%	51%	54%	58%	74%
CD23	71%	64%	33%	79%	67%	69%	81%	61%	61%	77%	80%
hepatomegaly (mm)	138↑	188↑	125↑	114↑	104↑	112=	130↑	108=	114↑	122↑	188↑
splenomegaly (mm)	176↑	159↑	90↑	84↑	154↑	96↑	66=	85↑	94=	108↑	135↑
lymphadenopathy (long-axis diameter)	12	---	1.5	0.5	---	---	15	---	10	---	17

Abbreviations: CLL, chronic lymphocytic leukemia; PB, peripheral blood; BM, bone marrow; WBC, white blood cell, RBC, red blood cell; PLT, platelet; Hb, hemoglobin; RDW, red blood cell distribution.

to ensure gene silencing efficiency.

Untreated, cells treated with Lipofectamine, scramble, and Bcl-2 siRNA alone did not demonstrate any significant change in the expression of Bcl-2 mRNA. On the other hand, treatment with DOX significantly decreased Bcl-2 mRNA, but not as compared to cells subjected to Lipofectamine-Bcl-2 siRNA. The lowest level of Bcl-2 expression was noted in cells treated with Lipofectamine-Bcl-2 siRNA and DOX. The results of the qRT-PCR test are illustrated in Figure 1.

Bcl-2 silencing by siRNA synergizes with the lethal effect of DOX on leukemic cells

After confirming the silencing of Bcl-2 by siRNA by qRT-PCR, we next investigated the combinational effect of various treatment groups on cell viability via the MTT test. Figure 2 illustrates the findings of the cytotoxicity assay following 24 and 48 hours treatments.

As can be seen, the control groups, including untreated, lipofectamine-treated, and Bcl-2 siRNA-treated cells, did not induce any significant cytotoxic effect on the viability of cells. However, treatment with either DOX or Bcl-2-lipofectamine considerably had a lethal impact on the cells. Next, we evaluated the combinational effect of Bcl-2 siRNA-Lipofectamine + DOX treatment, which was associated with the highest level of apoptosis compared to the other groups. Therefore, Bcl-2 inhibition synergizes with DOX treatment in killing CLL cells.

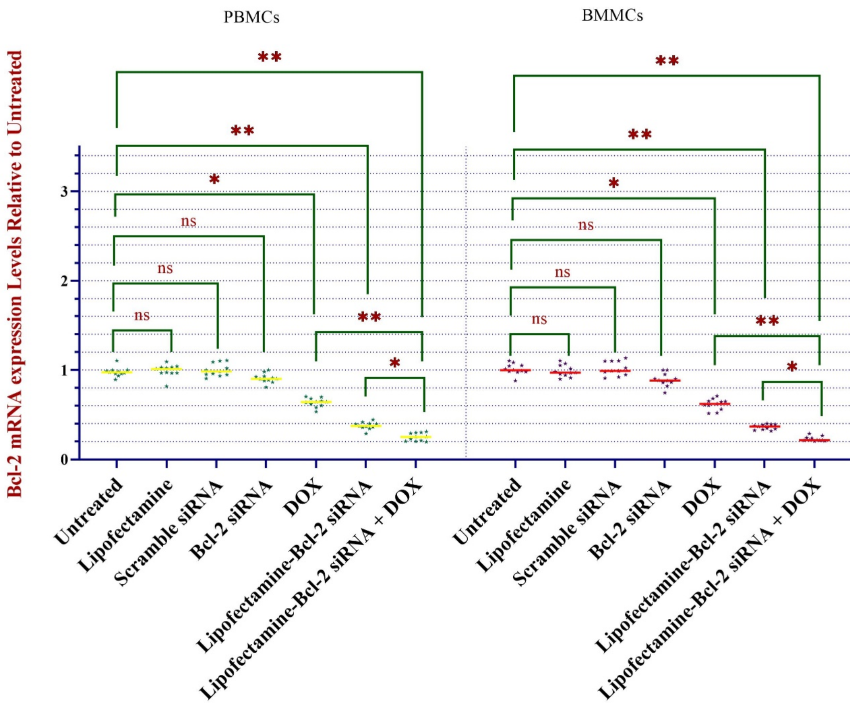

Figure 1. Transfection of leukemic cells with lipofectamine-Bcl-2 siRNA silences the *Bcl-2* gene. Treatment of patient-derived CLL cells purified from the peripheral blood and bone marrow of eleven patients with anti-Bcl-2 siRNA using Lipofectamine leads to the silencing of the *Bcl-2* gene as investigated via the qRT-PCR test. * represents p < 0.05, and ** represents p < 0.01. Abbreviations: PBMC: Peripheral Marrow Mononuclear Cell, BMMC: Bone Marrow Mononuclear Cell, ns: non-significant, DOX: doxorubicin.

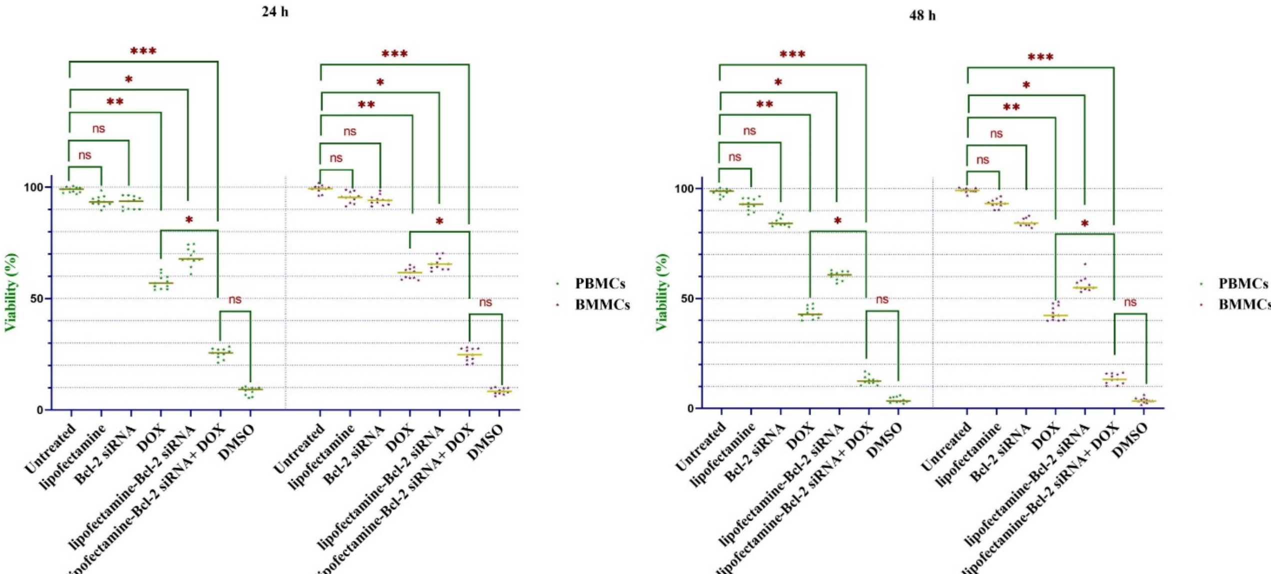

Figure 2. Silencing Bcl-2 sensitizes CLL primary cells to DOX. CLL cells were isolated from the peripheral blood and bone marrow of CLL patients (n = 11) treated with various combinational treatments. Next, the viability of cells was evaluated using an MTT test following 24 and 48 h of incubation. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, bone marrow mononuclear cell; ns, non-significant; DMSO, dimethyl sulfoxide; DOX, doxorubicin.

We also noticed that the effect of the used treatment increased over the next 24 hours treatment. This could be due to the fact that the treatment is time-dependent.

Discussion

CLL is associated with the malignant proliferation and repletion of lymphocytes. CLL with a median age of 65 years happens 85-90% in adults over 50. The exact cause of the disease is unknown.[25,26] DOX, an anthracycline, is used to treat various cancers, including leukemia.[4] In the current study, the combinational effect of Bcl-2 inhibition by siRNA and DOX treatment was evaluated.

Bcl-2, a major anti-apoptotic protein, is known to be upregulated in several studies, including hematological malignancies.[11] Bcl-2 exerts direct suppressing effects on the initiation of apoptosis by inhibiting the pro-apoptotic proteins' function. Bcl-2 overexpression results in the development and resistance to therapy in cancers. Lymphoid malignancies, including CLL, demonstrate Bcl-2 upregulation.[27,28]

In this study, siRNA was used to silence Bcl-2 expression. We used Lipofectamine for siRNA transfection. Based on the results of qRT-PCR, Lipofectamine successfully transfected cells with siRNA. We also evaluated the effect of DOX treatment on the expression of Bcl-2. We found that following DOX treatment, Bcl-2 mRNA levels decreased in the cells. Our result is in line with previous studies reporting that treating cells with DOX decreases the expression of Bcl-2 in malignant cells, including CLL cells.[29,30] As was predicted, cells treated with DOX combined with Bcl-2 siRNA transfection demonstrated the lowest level of Bcl-2 mRNA compared to other groups (Figure 1).

Next, we evaluated the effect of the treatments on cell viability using an MTT test. Bcl-2 inhibition by siRNA, as well as treatment with DOX demonstrated a significant growth inhibition compared to the control groups. The highest level of apoptosis induction was noted in cells treated simultaneously with Lipofectamine-Bcl-2 siRNA + DOX. The results indicate the synergism between the two mentioned treatments.

Several studies report results that are in line with our findings. According to Kang et al, one of the main mechanisms of apoptosis induction by DOX is to induce the generation of ROS. They showed that Bcl-2 upregulation desensitized bladder neoplastic cells to DOX treatment.[30] Another study reports that the enforced expression of Bcl-2 by plasmid transfection in human hepatocellular cancer cells resulted in DOX-resistance.[31] Based on Lu et al, treating drug-resistant triple-negative breast cancer cells with copolymers loaded with DOX and Bcl-2 siRNA, sensitized cells to DOX treatment and exerted further apoptosis.[32] The same results were reported by Chen et al. regarding human ovarian cancer cells.[33] Another study reports that Venetoclax combined with DOX improved apoptosis compared to DOX treatment alone in HCT116 cells (a human colorectal carcinoma cell line).[34] Bcl-2 silencing synergizes with several CLL treatments, including Rituximab[18] and Obinutuzumab (another anti-CD20 monoclonal antibody) as well.[35] A number of studies have reported that Venetoclax monotherapy possesses high anti-cancer effects.[36,37] However, Venetoclax monotherapy seems to fail as complete remission is only seen in 20% of patients. This brings to mind that combinational treatments might be more beneficial. According to the studies mentioned earlier, unlike Venetoclax monotherapy, combining Bcl-2 silencing with chemotherapy agents proves to be effective.

Therefore, not only Bcl-2 silencing synergizes with other chemotherapeutics, but also, silencing its gene is associated with promising results in various neoplasms. The same results were obtained from our study regarding its impact on CLL cells.

Conclusion

Based on the findings of the current research, it is evident that the combinational treatment of DOX + Bcl-2 silencing is more effective than DOX monotherapy in eliminating CLL cells. Bcl-2 inhibition may help overcome DOX resistance and this can be proved by future clinical trials.

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Funding Acquisition: Farhad Jadidi-Niaragh.

Competing Interests

The authors certify that there is no potential conflict of interest in relation to this article.

Ethical Approval

This study was approved by the ethics committee of Tabriz University of Medical Sciences. The ethics code number is IR.TBZMED.REC.1399.063.

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