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Original Research

Blockade of CD73 Increases the Cytotoxic Effects of Fludarabine in Chronic Lymphocytic Leukemia

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Abstract

Background: Ecto-5′-nucleotidase (CD73) is a membrane-bound enzyme that converts adenosine 5′-monophosphate into adenosine. It is proven that the presence of elevated levels of adenosine in the tumor microenvironment induces tumor growth while suppressing immune responses against tumor cells. According to several studies, it is reported that the expression of CD73 is increased in several types of malignancies. In chronic lymphocytic leukemia (CLL), a hematological malignancy characterized by the increased proliferation and accumulation of lymphoid cells in peripheral blood (PB) and lymphoid tissues, CD73 expression is associated with higher-risk disease. Moreover, it is reported that CD73 may contribute to fludarabine (a chemotherapeutic widely used for the treatment of CLL) resistance as well.

Methods: We separated mononuclear cells obtained from PB and bone marrow (BM) from eleven CLL patients. Cells were treated individually or in combination with fludarabine and anti-CD73 siRNA (transfection by lipofectamine), and the effect of treatment on cell survival was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

Results: We found that the highest level of apoptosis induction was observed in cells treated with fludarabine combined with CD73 inhibition, compared to the untreated ($P<0.01$). Our findings are in support of a study reporting the association between CD73 overactivation and fludarabine resistance.

Conclusion: These findings suggest that CD73 inhibition can be a potential target for CLL treatment and can help overcome endurance towards fludarabine and can better the prognosis of CLL-affected individuals.

Introduction

Chronic lymphocytic leukemia (CLL), the most prevalent leukemia in the west, mostly diagnosed around the age of 71, is characterized by the increased proliferation and repletion of lymphocytes in the peripheral blood (PB) and bone marrow (BM) and other hematopoietic organs.[1-3] The malignant cells are mature lymphocytes, mostly CD5 positive, and of B-lineage.[4] The symptoms may include leukocytosis (lymphocytosis), lymphadenopathy, hepatosplenomegaly, BM failure, autoimmune hemolytic anemia, autoimmune thrombocytopenia, and recurrent infections.[5] The precise factor leading to CLL has not yet been discovered. However, it is proven that CLL is highly associated with specific genomic losses and additions, including deletions on chromosome 13 (specifically del (13q14)),[6] 11 (del (11q)), 17 (del (17p)),[7] and trisomy 12.[8]

Several types of drugs, including cytostatic agents (such as alkylating agents, purine analogs, and bendamustine), monoclonal antibodies against CD20, and agents targeting the signaling of B cells, are available for CLL treatment.[1] Consequently, FCR (fludarabine, cyclophosphamide, and rituximab) is the current treatment regimen for younger patients. For the elderly, however, the regimens are different.[9] Fludarabine, a fluorinated adenine analog, acts as a DNA and RNA synthesis inhibitor[10] and is used as the first and second-line treatment for CLL. Despite this, nearly all patients under fludarabine treatment are/become resistant.[11] Therefore, CLL patients require novel

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therapeutic targets.

Ecto-5'-nucleotidase (CD73), a membrane-bound enzyme, that converts extracellular adenosine 5'-monophosphate (AMP) into adenosine[12] is coded by a gene, conserved through evolution, located on the 6q14-21 chromosome.[12,13,14] Excess adenosine concentration in the tumor microenvironment is proven to induce tumor growth, angiogenesis, and metastasis. In addition, it is also associated with suppressed anti-tumor immune responses.[15-18] On the other hand, the presence of adenosine three phosphate (ATP) induces inflammation and immune responses. CD73, along with another protein named nucleoside triphosphate diphosphohydrolase (CD39), which degrades ATP and ADP into AMP, cooperatively degrades ATP into adenosine and suppresses immune responses.[19,20] CD73 is reported to be overexpressed in the cancer microenvironment.[21] Interestingly, CD73 overexpression exerts the same effects on the cancer course as high adenosine concentrations. It is proven that CD73 overexpression induces tumor growth, disease severity, angiogenesis, and tumor spread.[22,23] The inhibition of CD73 is reported to increase anti-tumor immune responses of natural killer cells, cytotoxic T lymphocytes, and the proliferation of $CD4^+$T cells, when co-cultured with the cancer cell, indicating that CD73 inhibition could help potentiate the immune system to efficiently suppress tumor growth.[12,24,25]

In addition, a recent study reports that the expression of CD73 is elevated in CLL patients with splenomegaly compared to patients without this complication. Moreover, it was observed that the overall survival of patients was reversely correlated to CD73 expression levels.[26] Thus, the expression pattern of CD73 possesses prognostic value in CLL. Also, it can be an ideal target for CLL treatment by inducing anti-tumor responses. In addition, a study suggests that adenosine generated by CD73 may outcompete fludarabine over entry to the cell due to the structural similarity of these two molecules, which may result in fludarabine resistance. Therefore, targeting CD73 might also help overcome fludarabine resistance and help reduce the required effective dose of it as well.[27]

In the current study, siRNA was utilized to silence CD73 in the CLL cells of patients. The effect of CD73 silencing was studied in cotreatment with fludarabine.

Materials and Methods

Materials

Fludarabine was purchased from Cayman Chemical Company (IRC No: 7069750324959135). Human CD73 gene targeting siRNA was obtained from Santa Cruz Biotechnology, Inc. The MTT Cell Proliferation Assay Kit was purchased from the American Type Culture Collection (ATCC® 30-1010K) and used as directed by the manufacturer.

Patient samples

According to the Declaration of Helsinki, following the obtainment of permission from the ethics committee of Tabriz University of Medical Sciences, heparinized blood samples were taken from 11 untreated CLL patients at Shahid Ghazi hospital in Tabriz. Next, primary leukemia cells were isolated via Ficoll Paque TM Plus (GE Healthcare, Uppsala, Sweden) from PB and BM from 11 confirmed CLL patient samples. Patients' characteristics are given in Table 1. Patients-derived peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were cultured in RPMI-1640 containing 20% FBS and 2% L-glutamine. Viable cells were enumerated in advance of the downstream analysis.

Cell transfection with siRNA

Patients-derived leukemic cells were seeded at 1×10^4 cells/well in 96 well plates. After 24 hours incubation at 37°C and reaching 70% cell confluency, the cells were transfected with siRNA. Lipofectamine 2000 (Invitrogen) was used to transfect cells with siRNA using the instructions provided by the company. Briefly, CD73 siRNA, control siRNA, and Lipofectamine were diluted at appropriate concentrations provided by the manufacturer with opti-MEM medium, then mixed and incubated for 20 minutes at room temperature. Next, the cells were subjected to the transfection mixtures containing 50 pM of CD73 siRNA for 24 or 48 hours.

Analysis of gene expression

To assess the CD73 expression level in the cells, a qRT-PCR test was used. Total RNA from transfected cells (after 48 hours of incubation) was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the company's instructions.

In short, the total RNA of cells was transcribed into cDNA via the One-Step SYBR® RT-PCR Kit protocol (Takara Bio Inc., Japan). Subsequently, the generated cDNA was used for the qRT-PCR test using the SYBR® Green Real-time PCR master mixture (Takara Bio Inc., Japan).

The reactions were performed using the PRISM 7500 real-time PCR detection system (Life, Carlsbad, CA, USA), and the parameters consist of initial denaturation and activation at 95°C for 30 seconds, followed by 40 cycles for amplification and quantification (each cycle is 5 seconds at 95°C followed by 30 seconds at 60°C). Eventually, the melting curve program was run at the end of each reaction to assess the relative amount of CD73 mRNA by the $2^{-\Delta\Delta CT}$ Method, which normalizes the mRNA level to β-actin mRNA (a constantly expressed gene). The melting curve analysis was also used to confirm specificity. Gene primer sequences are presented in Table 2.

Analysis of cytotoxicity

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used for cytotoxicity assessment of the various combined groups. Patient-derived PBMCs and BMMCs (2×10^5 cells per

Table 1. Patients' characteristics

Characteristics											
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
LYMP	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: Chronic Lymphocytic Leukemia (CLL), Peripheral Blood (PB), Bone Marrow (BM), White Blood Cell (WBC), Red Blood Cell (RBC), PLT (Platelet), Hemoglobin (Hb), Red blood cell Distribution Width (RDW).

Table 2. Primer sequences

Gene	Type	Sequence	Reference
CD73	Forward	5'- GCCTGGGAGCTTACGATTTTG -3'	[28]
	Reverse	5'- TAGTGCCCTGGTACTGGTCG -3'	
β-actin	Forward	5'-GAGACCTTCAACACCCCAGC-3'	[29]
	Reverse	5'- ATGTCACGCACGATTTCCC -3'	

well) were cultivated in 96-well plates in 200 µL of complete culture medium and incubated at 37°C, 5% CO_2 for 24 hours. Subsequently, cells were treated with the following treatments for 24 or 48 hours; untreated, lipofectamine, scramble siRNA (50 pM), CD73 siRNA (50 pM), fludarabine (optimized concentration), lipofectamine-CD73 siRNA (50 pM), Lipofectamine-CD73 siRNA + fludarabine (50 pM of siRNA + optimized concentration of fludarabine), and DMSO (0.2%).

Plates were then centrifuged at 150 × g for 10 minutes, the supernatant was replaced with fresh RPMI and 10 µL of MTT solution (final concentration: 0.5 mg/mL) was added to each well (4 hours incubation). Next, 100 µL of DMSO was added to the wells to dissolve the formazan product. The absorbance of each well was then measured using a plate-reading spectrophotometer (Synergy 4, BioTek, USA). Viability was calculated using the following formula[30]:

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

Statistical analysis

GraphPad Prism V9 software was used to perform a statistical analysis of the data, and a two-way ANOVA test was used to assess the results. Statistical significance was set at $P < 0.05$.

Results

Lipofectamine efficiently transfects cells

Following the cell transfection with siRNA by Lipofectamine, CD73 mRNA expression was analyzed to study the efficiency of gene silencing. The various treatment groups and the changes in CD73 mRNA levels are presented in Figure 1. According to the data of qRT-PCR, treating cells with lipofectamine-CD73 siRNA significantly suppressed CD73 expression in CLL cells compared to the other treatment groups and controls. As seen in Figure 1, fludarabine increases the expression of CD73 in leukemic cells in both PBMCs and BMMCs to some extent. Still, interestingly, the transfection of cells with anti-CD73 siRNA decreases the expression of this factor in leukemic cells. Another point that should be mentioned is the inability of siRNA alone to reduce the expression of the CD73 molecule, which indicates the necessity of a carrier for the effective transfection of cells. In addition, the control groups, including the scramble siRNA and Lipofectamine, did not demonstrate any significant change in CD73 gene expression.

These results showed that CD73 gene silencing by siRNA in leukemic cells derived from PB or BM inhibits the additive effect of fludarabine on CD73 expression, which may prevent the further expansion of leukemic cells.

Inhibition of CD73 expression significantly increases the lethal effect of fludarabine on leukemic cells

After investigating the ability of anti-CD73 siRNA in reducing the expression of this molecule in leukemic cells, the ability of the combined treatment to induce apoptosis in leukemic cells following 24 and 48 hours of incubation, was evaluated.

An MTT assay was performed to assess the result of different treatments on the viability of CLL cells. The results of the MTT cytotoxicity assay are illustrated in Figure 2. As can be seen, while siRNA alone and Lipofectamine did not significantly affect the survival of leukemic cells, treatment of cells with siRNA together with Lipofectamine and especially fludarabine caused

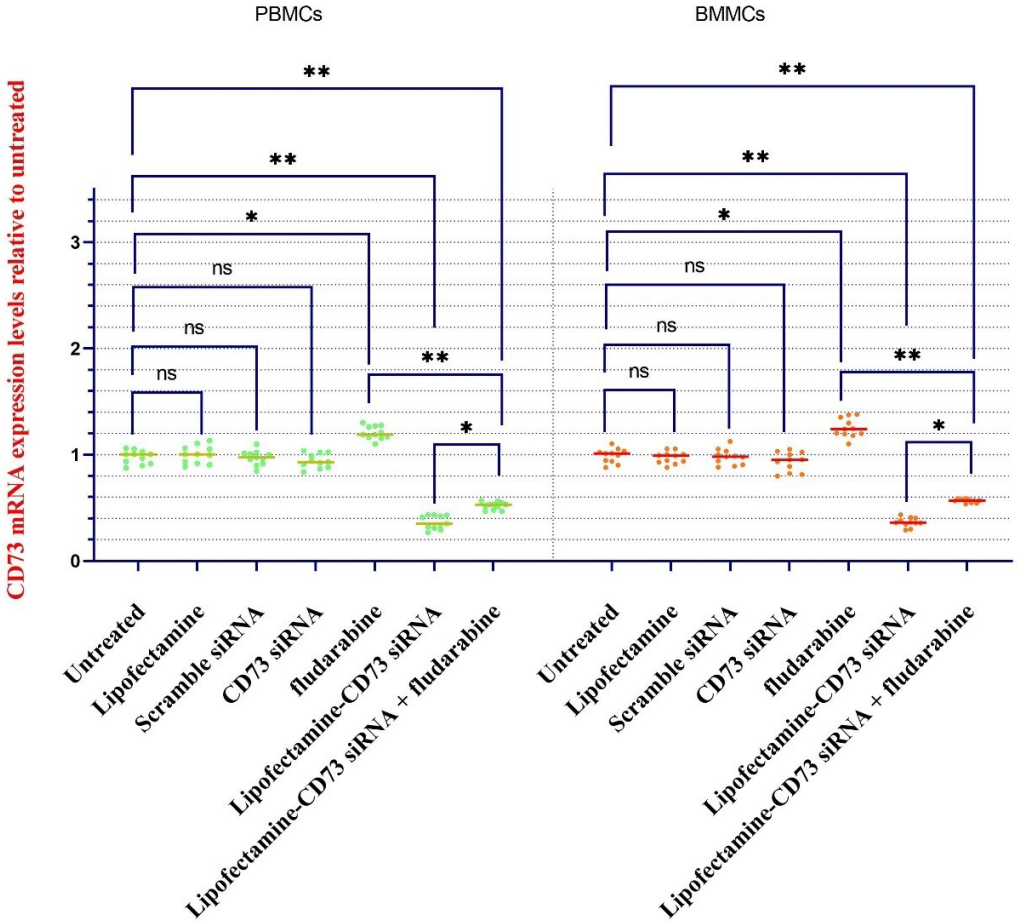

Figure 1. Treatment of leukemic cells with anti-CD73 siRNA suppresses the expression of CD73. siRNA Transfection of mononuclear cells separated from peripheral blood (PB) and bone marrow (BM) of CLL patients (n = 11) via lipofectamine suppressed the expression of CD73 as investigated using qRT-PCR. * represents $P < 0.05$ and ** indicates $P < 0.01$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, bone marrow mononuclear cell; ns, non-significant

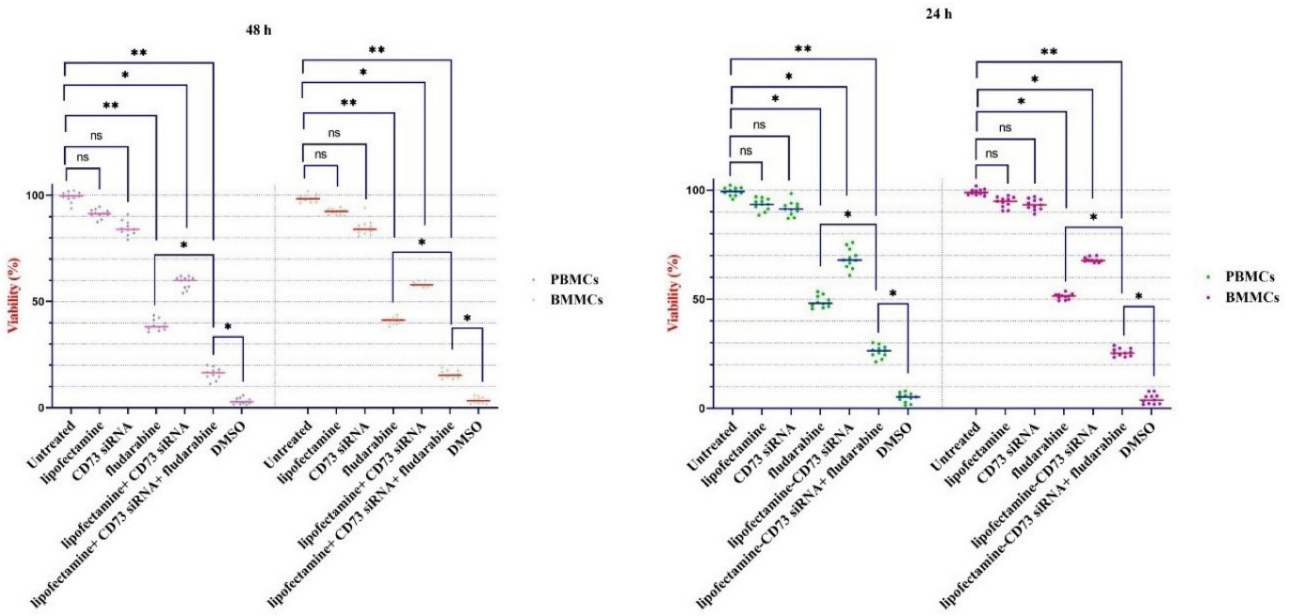

Figure 2. CD73 inhibition increased the toxic effects of fludarabine on leukemic cells. Treatment of mononuclear cells separated from peripheral blood (PB) and bone marrow (BM) of CLL patients (n=11) increased the cytotoxicity of Fludarabine in leukemic cells. * represents $P<0.05$ and ** indicates $P<0.01$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, bone marrow mononuclear cell; ns, non-significant; DMSO, dimethyl sulfoxide

a significant decrease in the survival of leukemic cells derived from PB and BM. Also, the highest cytotoxic effects on leukemic cells were observed when these cells were simultaneously treated with anti-CD73 siRNA and fludarabine.

Another point is that the toxic effects of the treatment increased over time, as the toxic effects after 48 hours were more than 24 hours , indicating the time-dependent effect of the treatment.

These results showed that the combined treatment induced a considerable increase in the death of leukemic cells. The silencing of CD73 could sensitize leukemic cells to programmed cell death induced by fludarabine.

Discussion

CLL is characterized by the malignant proliferation and repletion of $CD5^+$B cells in the blood and hematopoietic tissues.[4,31] The survival of CLL cells is significantly dependent on a supportive microenvironment to provide survival as well as proliferative signals.[4,32-35]

It is proven that extracellular adenosine plays a crucial role in tumor survival and escape from the immune system.[36,37] The presence of adenosine elevated levels in the tumor microenvironment induces tumor development and suppresses anti-tumor immune responses.[15-18]

CD73, a membrane-bound enzyme responsible for the generation of adenosine from AMP, is reported to be overexpressed in tumors.[38] Based on a study, CD73 is overexpressed in CLL cells compared to normal B-lymphocytes, which is correlated with splenomegaly, Beta-2-microglobulin level, and shorter overall survival in patients.[26] Also, according to previous studies, CD73 expression is associated with more severe and earlier disease accompanied by a poor prognosis in CLL.[26,39] Moreover, according to another study, CLL cells in PB express CD73 which is collocated with the expression of markers related to more severe diseases (CD38 and ZAP-70). CD73, by producing adenosine, activates A2A receptors, inhibiting the exodus of CLL cells from the lymph nodes, thus keeping them in a microenvironment ideal for growth.[40-42]

Although it has been established that CD73 expression on CLL leukemic cells increases the survival and expansion of these cells, its exact role in the immunopathogenesis of this disease has not yet been determined. Also, little is known regarding the impact of the expression of this molecule on the response to chemotherapy drugs. According to Nagate et al, CD73 and CD79 are involved in establishing an immunosuppressive microenvironment in adult T-cell leukemia/lymphoma (ATLL), a hematological malignancy with poor prognosis and poor response to chemotherapeutics.[43] Thus, in the current study, the effect of CD73 inhibition in combination with fludarabine was studied in mononuclear cells purified from the PB and BM of CLL patients.

To silence the CD73 gene in leukemic cells we used a Lipofectamine transfection reagent, as siRNA alone has very low penetrating efficacy. To assess the efficacy of transfection and analyze the silencing potential of anti-CD73 siRNA, a qRT-PCR test was performed. The results showed that Lipofectamine efficiently transfects cells with siRNA and reduces CD73 mRNA levels in cells derived from PB and BM. The interesting issue was that fludarabine could increase the expression of the CD73 molecule in leukemic cells. Therefore, it appears that leukemic cells increase the expression of the CD73 molecule on their surface, to compensate for the cytotoxic effects of fludarabine and therefore survive.

After examining the effect of transfection of cells on CD73 expression, the impact of combined treatment on cell survival was evaluated. In the MTT test, CD73 inhibition seems to form a synergism with fludarabine treatment as the highest apoptosis rates were seen in cells with CD73 inhibition and under fludarabine treatment compared to other groups illustrated in Figure 2.

Previous studies have shown that treating fludarabine-incubated cells with adenosine protects cells from apoptosis, by A2A-dependent and independent mechanisms. Moreover, because of the similarity between these two molecules, adenosine may compete with fludarabine over entering cells via the nucleoside transporter. Therefore, this may affect the efficacy of fludarabine treatment as well.[27,44]

Our results support results presented by previously conducted studies on other malignancies as well. According to a study, in mouse models of ovarian cancer, following CD73 inhibition, mice survival was improved.[24] Another study reports that the administration of anti-CD73 antibodies suppresses tumor development in breast cancer mouse models.[45] Allard et al also reported that targeting CD73 showed a synergistic effect with anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) and anti-PD-1 (programmed death-1) monoclonal antibodies in several cancer models, including breast, colon, and prostate cancer.[46] Moreover, CD73 inhibition does not induce autoimmunity, therefore can be a valuable novel method of treatment for patients in need.[21,47] Based on the results of Loi et al, CD73 inhibition proved to synergize with doxorubicin (an anthracycline widely used for cancer treatment) treatment as well. The combinational treatment of doxorubicin and CD73 inhibition resulted in further tumor growth suppression compared to doxorubicin treatment alone in breast cancer mice models.[48] Therefore, considering the results of previous literature, CD73 inhibition not only synergizes with several chemotherapeutics besides fludarabine but also possesses high potential in cancer treatment.

Conclusion

In this study, the effect of CD73 inhibition along with fludarabine treatment on CLL cells was studied. Overall, the results illustrated that this approach increased the apoptosis rates in CLL cells and can be used as a complementary treatment alongside the conventional chemotherapy of fludarabine for CLL. Also, CD73 inhibition seems to help overcome fludarabine resistance and provide new hope for CLL patients.

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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.1399.063).

Conflict of Interest

The authors declare that they have no competing interests.

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