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Original Article

Silencing of mTOR Facilitates the Cyclophosphamide-Mediated Cytotoxicity in Acute Lymphoblastic Leukemia

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Abstract

Background: Acute lymphoblastic leukemia (ALL) is a neoplastic disorder in which lymphoid progenitors of patients proliferate malignantly and accumulate in peripheral blood (PB), bone marrow (BM), and other sites. According to several studies, the mammalian target of rapamycin (mTOR), which mediates several biological processes in cells, including cell survival, autophagy, cell polarization, etc, is highly involved in cancer progression, chemoresistance, and relapse. Several studies have shown that mTOR is hyper-activated in ALL cells. Also, its inhibition is associated with a better response to several anticancer drugs.

Methods: In this study, the effect of mTOR inhibition along with cyclophosphamide treatment (an alkylating agent used for the treatment of several malignancies) was evaluated. In the current research, peripheral blood and bone marrow mononuclear cells from eleven ALL patients were treated with anti-mTOR siRNA (transfection by Lipofectamine) and cyclophosphamide. The efficacy of mTOR inhibition was evaluated by qRT-PCR. Next, the effect of different treatments was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

Results: The findings demonstrated that the mTOR mRNA level was significantly reduced in leukemic cells when treated with mTOR siRNA-lipofectamine. Moreover, silencing mTOR considerably sensitized cells to cyclophosphamide. Cells treated with both cyclophosphamide and mTOR siRNA demonstrated the highest level of apoptosis.

Conclusion: Based on the results achieved from the cytotoxicity test, we found that mTOR inhibition has a synergistic effect with cyclophosphamide treatment in ALL. Therefore, this combination therapy can be a promising approach for treating ALL, which should be examined more in subsequent investigations.

Introduction

Acute lymphoblastic leukemia (ALL) is characterized by the malignant expansion of lymphoblasts in the bone marrow (BM), which can involve T (T-ALL) or B (B-ALL) lineages. ALL accounts for more than 75% of acute leukemias in children, making it the most prevalent pediatric malignancy.[1-3] The age distribution of ALL occurrences peaks in childhood and around the age of 50.[4] ALL is diagnosed by the confirmed presence of at least 20% lymphoblasts in patients' BM or peripheral blood (PB). Symptoms may include anemia, thrombocytopenia, leukopenia, fever, weight loss, night sweats, easy bleeding or bruising, weariness, dyspnea, and infection.[5,6]

The standard induction chemotherapy for ALL involves vincristine, corticosteroids, and anthracyclines.[7,8] Cyclophosphamide, an alkylating agent introduced in 1958 as an anti-cancer drug, is used to treat various malignancies. Cyclophosphamide irreversibly binds to DNA and results in strand breaks, and therefore, cell cycle arrest in the S phase.[9] Growing evidence illustrates the efficacy of cyclophosphamide treatment in ALL. Despite the access to different chemotherapeutics, a relapse rate of 15%-20% exists in ALL pediatric patients.[10] In addition, in the elderly, only 30%-40% of patients attain sustained recovery, which indicates the need for novel treatments to improve the prognosis of patients.[11]

The mammalian target of rapamycin (mTOR), a protein kinase, is responsible for various procedures in cells, including cell proliferation, survival, metabolism, etc.[12] The *MTOR* gene, coding for mTOR, is located on chromosome 1p36.2.[13] mTOR acts as a core subunit of two different complexes including mTOR complex 1

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(mTORC1), and mTOR complex 2 (mTORC2). Each of these complexes plays various roles in the biology of cells.[14,15] mTORC1 contributes to mRNA translation and autophagy inhibition, and functions as an integrator for different signals.[16] Whereas, mTORC2 helps with the organization of actin cytoskeletal, cell polarization and is also involved in generating pro-survival signals.[17]

The altered expression and function of mTOR is observed in various diseases, including diabetes, obesity, and cancer. The aberrant activation of mTOR results in tumor growth and metastasis, in ALL in particular, thereby, making it an ideal target for cancer treatment.[12,18,19] It is reported that PI3K/AKT/mTOR pathway is over-activated in high-risk B-ALL and is significantly associated with chemoresistance and poor survival.[20,21] Moreover, it is reported that in imatinib (a tyrosine kinase inhibitor)-resistant Philadelphia chromosome + (Ph +) ALL cells, BCR/ABL oncoprotein induces the activation of this pathway, and mTOR inhibition by rapamycin (an mTOR inhibitor) helps overcome imatinib-resistance.[22] In addition, it has been proved that rapalogs (rapamycin analogs that can inhibit mTORC1) have therapeutic properties in acute myeloid leukemia (AML).[23-27] According to another study, cyclophosphamide in combination with mTOR inhibition demonstrated a synergistic anti-tumor effect in sarcoma models *in vivo*.[28] Moreover, it has been shown that the combinational treatment of rapalogs with several T-ALL conventional therapies, such as doxorubicin, idarubicin, cyclophosphamide, and methotrexate induces further apoptosis in T-ALL cells.[29]

We hypothesized that mTOR inhibition and cyclophosphamide treatment could induce further cell cycle arrest and help eliminate the disease in ALL patients. In the following study, mTOR was inhibited using siRNA in ALL cells isolated from the PB and BM of patients. The effect of mTOR silencing combined with cyclophosphamide treatment on cell viability was also studied.

Methods

Materials

Cyclophosphamide was purchased from Cayman Chemical Company (IRC No: 7069750324959135). Human mTOR gene targeting siRNA (catalogue number: sc-35409), and negative control siRNA (NC-siRNA) were bought from Santa Cruz Biotechnology, Inc. The MTT Cell Proliferation Assay Kit was supplied by Sigma-Aldrich and used following the directions provided by the company.

Primary cells

After the acquisition of the ethical code (code number: IR.TBZMED.REC.1399.202) from the ethics review board of Tabriz University of Medical Sciences, heparinized PB and BM specimens were taken from eleven patients with diagnosed ALL, accompanied by informed consent. Using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden), primary cells were isolated. Table 1 displays the patient demographic information.[30] Patients-derived peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were cultured in RPMI-1640 supplemented with 20% FBS and 2% L-glutamine. Viable cells were enumerated before any downstream test.

Cell transfection method

Following the seeding of 1×10^4 cells/well in a 96-well plate and incubation for 24 hours at 37 ℃, according to the company's directions, cells were transfected with siRNA via Lipofectamine 2000 (Invitrogen).

cDNA synthesis and qRT-PCR for gene expression studies

In short, after treating cells with different mixtures for 48 hours, the total RNA of cells was extracted via a TRIzol reagent (Invitrogen). Subsequently, 2 µg of RNA was transcribed into cDNA. Using a Light-Cycler 480 real-time PCR system (Roche) and a SYBR Green PCR Master Mix, the qRT-PCR test was performed. Melting curves were used to quantify mTOR mRNA levels by normalizing the expression of mTOR with β-actin (a constantly expressed gene) via the $2^{-\Delta\Delta CT}$ method. The sequences of the used primers in the current study are provided in Table 2.

Analysis of cytotoxicity and cell death

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was carried out to ascertain the combined effect of mTOR inhibition and cyclophosphamide. 2×10^4 cells were seeded in each well and incubated for 24 hours. Subsequently, the plate was centrifuged and 100 µL of the upper medium was removed. The cells were then subjected to the following treatments: untreated, lipofectamine, scramble siRNA (60 pM), mTOR siRNA (60 pM), cyclophosphamide (optimized concentration), lipofectamine-mTOR siRNA (60 pM), and lipofectamine-mTOR siRNA + cyclophosphamide. Cells were then incubated with the aforementioned treatments for 24 or 48 hours. After that, 100 µL of fresh RPMI containing 10 µL of MTT mixture was added to each well. Followed by a 3 hours incubation, 100 µL of DMSO replaced the supernatant of each well. Eventually, after 30 minutes, the absorbance of wells was assessed using a spectrophotometer (Synergy 4, BioTek, USA). The test was performed in triplicate form. The viability index was calculated by the following equation[33]:

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

Statistical analysis

GraphPad Prism V9 software and a two-way ANOVA test was used for all the statistical analysis. Statistical significance was set at $P < 0.05$.

Table 1. the demographic data of patients

Patients	Age (y)	Gender	WBC ($x10^3$/mL)	Plt ($x10^3$/mL)	Hb (g/dL)	LDH level (Nl : Up to 480 U/L)	Hepatomegaly	Splenomegaly	Lymphadenopathy	C- ALL Ag (CD10)(%)	ALL 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

Abbreviations: WBC, white blood cells; Plt, Platelet; LDH, lactate dehydrogenase; C-ALL Ag, Common ALL antigen; Hb, hemoglobin.

Table 2. Primer sequences

Gene	Primer type	Sequence	Reference
mTOR	Forward	5'-AAAACCTCAGCATCCAGAGATACGC-3'	[31]
	Reverse	5'-CATCAGAGTCAAGTGGTCATAGTCCG-3'	
B-actin	Forward	5'-GAGACCTTCAACACCCCAGC-3'	[32]
	Reverse	5'- ATGTCACGCACGATTTCCC -3'	

Results

Cells were efficiently transfected with siRNA using lipofectamine

After treating cells with different pharmacological groups, to evaluate the efficiency of gene silencing, mTOR mRNA levels were assessed using qRT-PCR. The results, including the changes in mRNA levels of mTOR in different treatment categories, are provided in Figure 1. Based on the data acquired from qRT-PCR, no notable difference was observed in cells under treatment with scramble siRNA or lipofectamine alone. In addition, cells treated with free mTOR siRNA alone did not demonstrate any significant change in mRNA level, indicating that efficient siRNA transfection into cells requires a carrier. However, treating cells with cyclophosphamide decreased the expression level of mTOR slightly. Whereas, treating cells with lipofectamine-mTOR siRNA efficiently reduced the expression of mTOR mRNA when results were compared to other treatment groups. Also, cyclophosphamide synergistically decreased the expression level of mRNA in the cells treated with both the drug and lipofectamine-mTOR siRNA.

mTOR inhibition sensitizes ALL cells to cyclophosphamide and improves apoptosis

An MTT test was run to evaluate the effect of different treatments on the viability of patient-derived ALL cell groups following 24 or 48 hours of incubation. Cyclophosphamide was used in its optimal suggested concentration. The results are provided in Figure 2.

Controls, including untreated, cells treated with lipofectamine, and mTOR siRNA alone, did not show any significant viability change. On the other hand, cells treated with cyclophosphamide or lipofectamine-mTOR siRNA showed a considerable reduction in viability. Also, according to the results, the maximum apoptosis rate was observed in the group treated with lipofectamine-mTOR siRNA + cyclophosphamide compared to the controls.

Moreover, the apoptosis was further increased after 48 h of incubation, indicating the time-dependency of the treatment on the viability of ALL cells.

Discussion

ALL, a blood-born neoplasm, is characterized by the excessive proliferation of lymphoid progenitors in the PB, BM, and extra-nodal sites. ALL mostly (80%) occurs in children, while it is associated with more severe diseases in adults (20%).[1,34] Despite all the advances in chemotherapy strategy for ALL treatment, the adult response rate is still unfavorable. Thus, ALL patients need novel therapies to help overcome possible chemoresistance.

Several studies indicated the correlation between mTOR activation and chemoresistance, cancer progression, and poor prognosis in ALL.[14,15] mTOR is a protein kinase responsible for several processes happening in the cells.[12] mTOR mediates most of its function by incorporating into complexes called mTORC1 and mTORC2.[14,15] Increased activity of mTORC1 and mTORC2 is alleged to have been a part of the development, metastasis, and relapse of leukemias.[35] Based on another study, PI3K/AKT/mTOR pathway is over-activated in patient-derived B-ALL[36] and T-ALL cells.[37] mTOR hyperactivity is also reported to be involved in the inhibition of apoptosis through an indirect control over specific molecules, including p53, BAD, Bcl-2, p21, and c-myc.[38] Thus, making mTOR an ideal target for cancer therapy.

Therefore, in the following study, the result of mTOR inhibition in combination with cyclophosphamide was studied. Lipofectamine was used to transfect cells with

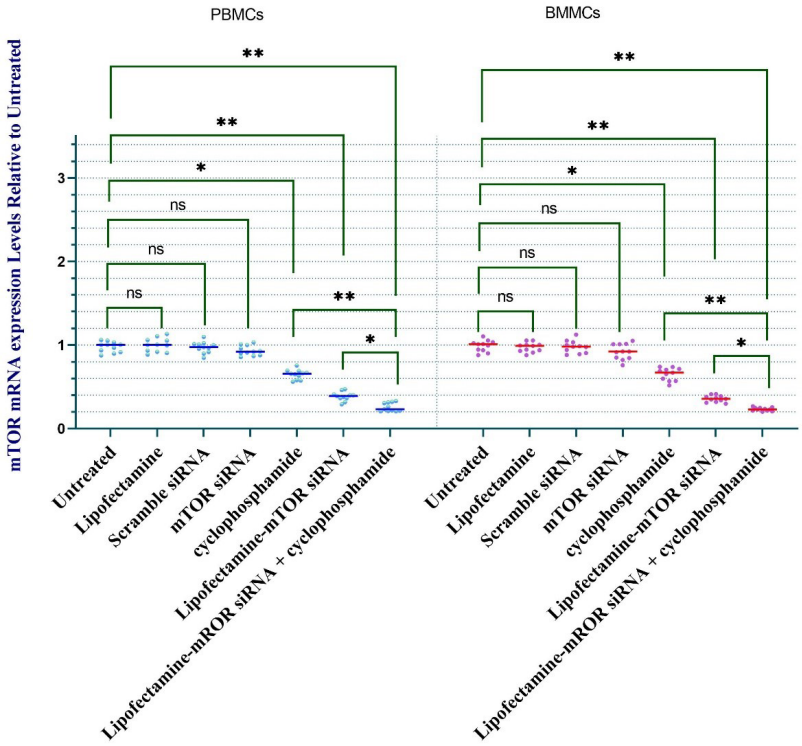

Figure 1. Transfection of leukemic cells with lipofectamine-mTOR siRNA suppressed the expression of mTOR. Treatment of ALL cells purified from the peripheral blood and bone marrow of 11 patients with anti-mTOR siRNA using Lipofectamine led to suppressing the mTOR expression as investigated by qRT-PCR. * represents $P<0.05$ and ** indicates $P<0.01$. Abbreviations: PBMC, peripheral marrow mononuclear cell; BMMC, bone marrow mononuclear cell; ns: non-significant.

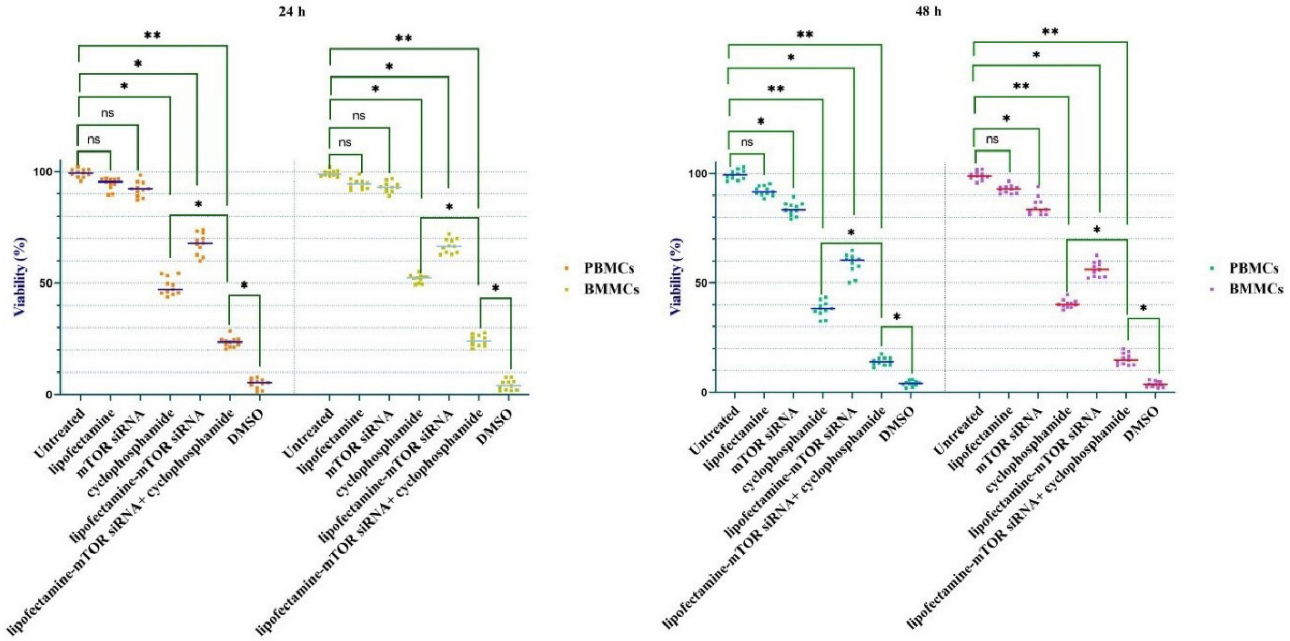

Figure 2. Suppressing mTOR increased the sensitivity of ALL primary cells to cyclophosphamide. ALL primary cells isolated from peripheral blood and bone marrow of ALL patients (n=11) were treated with the combination of anti-mTOR siRNA and cyclophosphamide. Next, the toxic effect was evaluated using an MTT assay after 24 and 48 hours of incubation. * 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.

mTOR siRNA. qRT-PCR results demonstrated that cells were efficiently transfected with mTOR siRNA, as the mTOR mRNA level was decreased significantly (Figure 1). Moreover, controls (untreated, treated with scramble siRNA and mTOR siRNA alone) did not demonstrate any significant difference in the expression level of mTOR. Also, it was found that cyclophosphamide reduces the expression of mTOR in cells. This data is in support of a study conducted by a group of researchers, reporting that in mice treated with cyclophosphamide,

after scarification, the expression of mTOR in mice kidneys was found to be decreased, which was found to be mediated by cyclophosphamide.[39]

Following the treatment of patient-derived ALL cells with different treatment groups for 24 hours or 48 hours (illustrated in Figure 2), an MTT test was run. According to the results, cells treated with cyclophosphamide or Lipofectamine-mTOR siRNA demonstrated a moderate apoptosis rate. The cell group that received the lipofectamine-mTOR siRNA and cyclophosphamide showed the highest amount of apoptosis compared to the controls and cells treated only with cyclophosphamide, indicating the synergism between cyclophosphamide and mTOR inhibition. Also, cell viability in response to the mentioned treatment demonstrated a time-dependent trait, as the apoptosis was higher in 48 hours treatment compared to 24 hours treatment. Additionally, we did not note any significant difference between T-ALL and B-ALL cells in response to the treatment. This could be due to the scarcity of T-ALL samples (three samples only) compared to B-ALL cells (eight samples). Therefore, future studies with a greater number of samples could help evaluate the difference between these two subgroups of disease.

Our results support other studies conducted about the role of mTOR inhibition alone or in combination with other chemotherapeutics in ALL,[22,40,41] indicating that mTOR inhibition is an effective therapeutic option along with chemotherapy. A recent study reports that mTORC1 inhibition by rapamycin in combination with cyclophosphamide improved the survival of T-ALL-bearing mice versus treatment with just one agent,[42] which is in line with the results of our study. Therefore, it appears that silencing mTOR synergizes with cyclophosphamide treatment that could help with ALL treatment.

Conclusion

To sum up, based on the results of the current study, it seems that mTOR inhibition could be used as a complementary treatment along with cyclophosphamide to help overcome its resistance and effectively eliminate ALL cells, which clinical trials can further prove.

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Authors' Contribution

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Competing Interests

Nothing to state.

Ethical Approval

This study was approved by the ethics review board of Tabriz University of Medical Sciences (the ethical code: IR.TBZMED.REC.1399.202).

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