Apoptotic Effect of Phosphatidylinositol 3-Kinase Inhibition on Acute Lymphoblastic Leukemia Cells Using Buparlisib

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ABSTRACT

BACKGROUND AND OBJECTIVE: Resistance to chemotherapy is one of the most important problems in treatment of patients diagnosed with acute lymphoblastic leukemia (ALL). Pathway interruption of the phosphatidylinositol -3 kinase (PI3K) and its relation to resistance phenomena cause the inhibitors of this pathway, particularly buparlisib are introduced as one of the most promising cancer drugs. The aim of this study was to evaluate the effect of PI3K pathway inhibition on reducing the survival and induction of apoptosis in Nalm-6 cells using buparlisib.

METHODS: In this experimental study, the phosphorylation level of Akt was evaluated using western blot to measure the effect of buparlisib on PI3K/Akt pathway in Nalm-6 cells. Nalm-6 cells were treated with different concentrations of buparlisib (0.5-4 μM) for 24, 36 and 48 hours to study the cytotoxic effect of this inhibitor and then, the metabolic activity, induction of apoptosis and changes in expression of genes involved in apoptosis were evaluated using MTT assay, Annexin/PI staining and Rq-PCR, respectively.

FINDINGS: Results showed that PI3K pathway inhibition using buparlisib causes the cytotoxic effect on Nalm-6 cells in a dose- and time-dependent manner through reducing p-Akt. These findings suggested that probably, the anti-leukemic effect of buparlisib is mediated through almost 17-fold increase in apoptotic cells (p≤0.001) and rising the mRNA expression level of pro-apoptotic genes (p≤0.01).

CONCLUSION: The results indicated that buparlisib has anti-tumor activity against Nalm-6 cells so this inhibitor can be used as a promising agent for the treatment of ALL.

KEY WORDS: *Apoptosis, phosphatidylinositol 3-kinase, Acute lymphoblastic leukemia, Buparlisib, Nalm-6.*

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Introduction

Despite dramatic advances in the therapeutic approaches of acute lymphoblastic leukemia (ALL), this malignancy is still remain as one of the leading causes of person-years of life lost worldwide (1, 2). By identifying the contribution of several signaling pathways in the pathogenesis of ALL, it is not surprising that tremendous attentions have recently been attracted to the treatment strategies targeting the critical components of such pathways (3, 4).

An overwhelming number of clinic and genetic studies indicated that the PI3K/Akt signaling pathways, aberrantly activated in 80% of ALL cases (5), possess a pivotal impact not only on unlimited proliferative potential of leukemic cells, but also on acquisition of chemo-resistant phenotype (4). Taking advantage of these facts, the therapeutic application of PI3K inhibitors in this malignancy seems to be promising for ALL patients.

Successful drug design has yielded three classes of PI3K inhibitors with different pharmacologic and pharmacokinetics characteristics, which among them, Buparlisib, a potent pan-PI3K inhibitor, has incorporated into the management of different cancers (6, 7).

Buparlisib, a synthetic selective PI3K inhibitor, exerts a cytotoxic effect in broad spectrum of human malignancies from solid tumors to hematological malignancies (8, 9).

Previous study conducted on a panel of acute myeloid leukemia cell lines indicated that although Buaprlisib is able to reduce the survival and proliferative potential of AML cell lines, this agent had no cytotoxic effect on normal cells (10). In another study, it was also demonstrated that Buparlisib induced the apoptotic cell death in chronic lymphocytic leukemia (CLL) primary cells (8).

Based on the favorable cellular potency and preclinical safety profile, the anti-tumoral activity of this pan-PI3K inhibitor, either alone or in combination of chemotherapeutic drugs have been examined in several solid tumors (11).

In this study, we aimed to investigate the anti-cancer effects of Buparlisib on pre-B ALL-derived Nalm-6 cells. Moreover it was of great interest to underscore the underlying mechanism of action of the inhibitor in this cell line.

Methods

Cell culture and drug treatment: Nalm-6 cells were grown in RPMI 1640 medium supplemented with antibiotics, 10% fetal bovine serum (Invitrogen) and 2 mM l-glutamine (Invitrogen) in the presence of 5% CO_2 at 37°C. For the drug treatment, we used Buparlisib, a derivative of 2,6-dimorpholino pyrimidine (Selleckchem, USA) (Fig. 1).

A stock solution of the inhibitor at a concentration of 50 μM was prepared by dissolving the compound in 0.1% sterile dimethyl sulfoxide (DMSO), divided into aliquots, and stored at −20°C until use. For determining the cytotoxic effect of the inhibitor on Nalm-6, cells were treated with increasing concentrations of the agent (0.5- 4 μM) at different time intervals (24, 36 and 48 h). In addition to the negative control (no inhibitor), cells were treated with the corresponding concentration of DMSO as an alternative negative control.
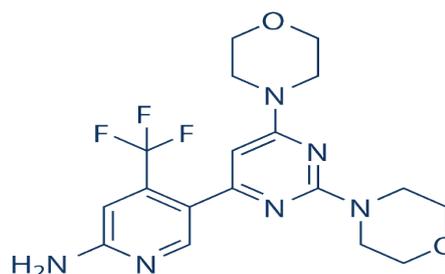
Fig. 1. Chemical structure of Buparlisib.

Evaluating the metabolic activity using MTT assay: To explore the cytotoxic effect of Buparlisib on Nalm-6 cells and to calculate the IC_{50} value, microculture tetrazolium assay (MTT) was applied. The cells (5000/well) were plated in 96-well plates and incubated with the indicated concentrations of the agents (0.5-4 μM) up to 48 h. After removing the media, cells were further incubated with MTT solution (5 mg/ml in PBS) at 37°C for 3 h and untreated cells were defined as the control group. The resulting formazan was solubilized with DMSO and the absorption was measured at 570 nm in an enzyme-linked immunosorbent assay (ELISA) reader.

Assessment of apoptosis using flow cytometry: To investigate the effect of pan-PI3K inhibitor on the induction of apoptosis, Nalm-6 cells were seeded into 24-well cell culture plates in the presence of Buparlisib for 36 h and then the externalization of phosphatidylserine

were analyzed using Annexin-V/PI double staining kit. Following centrifugation at 600g for 5min, cells were washed with PBS and were resuspended in a total volume of 100 μl of the incubation buffer containing 1 μl Annexin-V at the concentration of 0.5 mg/ml and 0.1 mg/ml of PI for 15 min in the dark at room temperature. Fluorescence was then measured using flowcytometery and the data were evaluated using the Flowmax Software (Partec GmbH, Munster, Germany).

RNA extraction and cDNA synthesis: Total RNA from Nalm-6 cells was extracted at 48 h after treatment using a High Pure RNA Isolation Kit according to the manufacturer's recommendation (Roche). The quantity of RNA samples was assessed spectrophotometrically using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE).

The reverse transcription (RT) reaction was performed using a RevertAid First Strand cDNA Synthesis kit (Takara BIO). A 20 μl reaction was carried out containing 4 μl 5X PCR buffer, 2 μl dNTP (10 mM), 1 μl random hexamers, 1 μl DEPC-treated water, 1 μl RNase inhibitor (20 U/μl), 1 μL M-MuLV reverse transcriptase (200U/μl) and 1 μg total RNA per reaction. Incubation was for 5 min at 65°C, 5 min at 25°C followed by 60 min at 42°C. The reaction was terminated by heating for 5 min at 70°C.

Quantitative real-time PCR: Changes in mRNA expression level of the desired genes were assessed by real-time PCR that was performed with a light cycler instrument (Roche Diagnostics, Germany) using SYBR Premix Ex Taq technology (Takara Bio, Inc). For this purpose, PCR assay was performed in a ultimate volume of 20 μl of reaction mixture containing 10 μl of SYBR Green master mix, 2 μl of cDNA product, 0.5 μl of each forward and reverse primers (10 pmol) and 7 μl of nuclease-free water (Qiagen, Hilden, Germany). Thermal cycling conditions included an initial activation step for 30 s at 95°C followed by 40 cycles including a denaturation step for 5 s at 95°C and a combined annealing/extension step for 20 s at 60°C. Melting curves were analyzed to verify single PCR product of each primer. Hypoxanthine phosphoribosyl transferase (HPRT) was amplified as an internal control and fold change in expression of each target mRNA relative to HPRT was calculated on the basis of comparative on $2^{-\Delta\Delta Ct}$ relative expression formula. The sequences of the primers used for Real-Time RT-PCR were listed in table 1.

Table 1. Nucleotide sequences of primers used for real-time RT-PCR.

Gene	Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Size (bp)
HPRT	NM_000194	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTTA	111
Bax	NM_138761	CGAGAGGTCTTTTTCCGAGTG	GTGGGCGTCCCAAAGTAGG	242
Bad	NM_004322	CCCAGAGTTTGAGCCGAGTG	CCCATCCCTTCGTCGTCCT	249
PUMA	NM_014417	GACCTCAACGCACAGTACGAG	AGGAGTCCCATGATGAGATTGT	98
Noxa	NM_021127	CAAGAACGCTCAACCGAG	GGAAGTTCAGTTTGTCTCC	95

Western blot analysis: Cells were centrifuged at 12 h after treatments, and cellular pellets were washed with cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (Sigma). After determination of protein concentrations according to Brad-ford method, equivalent amounts of total cellular protein were separated by 10% SDS-PAGE, and subsequently transferred to nitro-cellulose membrane using a semidry transfer cell (Bio-Rad).

The proteins were detected using specific primary antibodies and the enhanced chemiluminescence detection system according to the manufacturer's protocol. Band intensity was calculated using ImageJ software and the ratio of proteins to actin expression was normalized.

Statistical analysis: Data are expressed as the mean±standard deviation (SD) of three independent experiments.

All tests were performed in triplicate. The significance of differences between experimental variables was determined by the use of two-tailed student's test and by one-way variance analysis. In order to compare between the control group and the experimental ones, the Dunnett's multiple comparison

test was used. A probability level of p<0.05 was considered statistically significant.

Results

Evaluating the amount of Akt phosphorylation in response to Buparlisib in Nalm-6 cells: As a primary step, to evaluate whether PI3K signaling pathway is activated in Nalm-6 leukemic cells, the baseline expression and phosphorylation level of Akt, an important member of PI3K axis, were evaluated either in the presence or absence of Buparlisib (2 and 4 μM) using western blot analysis. Our results showed that Nalm-6 cells express p-Akt (Ser473), which is indicative of the sustained activation of PI3K axis in this cell line (Fig. 2). Moreover, we found that unlike the total protein level of Akt, the amount of p-Akt decreased in a concentration-dependent manner upon 12 h treatment of cells with indicated concentrations of the inhibitor, substantiating the effectiveness of the drug in inhibiting the PI3K network in pre-B-ALL-derived Nalm-6 cells (Fig. 2).
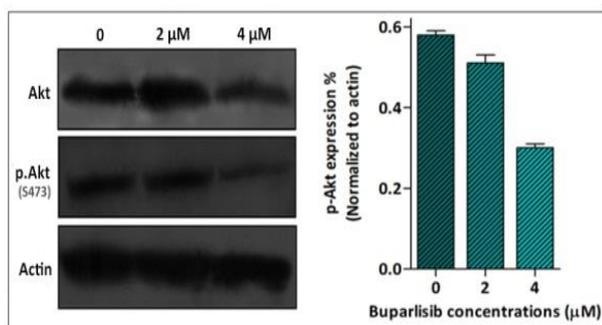
Figure 2. Evaluating the amount of Akt phosphorylation in Nalm-6 cells. As presented, PI3K/Akt signaling pathway is aberrantly activated in this cell line. Moreover, the results showed that treatment of NAlm-6 cells with Buparlisib reduced the phosphorylation of Akt in a dose-dependent manner.

Evaluating the effect of Buparlisib on the metabolic activity of Nalm-6 cells: To investigate whether the abrogation of PI3K/Akt signaling pathway in pre-B ALL is associated with the reduction of cell survival rate, Nalm-6 cells were treated with increasing concentrations of Bupalisib up to 48 h and then the metabolic activity was assessed using MTT assay. The results showed that Buraplisib decreased the metabolic activity of Nalm-6 cells in both dose- and time-dependent manner. As presented in Fig. 3, 24 h

treatment of cells with 0.5, 1, 2, 3, and 4 μM concentration of the inhibitor reduced the metabolic activity of Nalm-6 cells by 5, 10, 22, 34 and 44%, respectively. This inhibitory effect become even more evident after 48 h, as incubating cells with Buparlisib at the concentration of 3 μM reduced the metabolic activity of cells nearly by 50% (p≤0,001).

Buparlisib induced apoptotic cell death in Nalm-6 cells: To determine whether the cytotoxic effect induced in inhibitor-treated cells were likely due to the induction of apoptosis, the externalization of phosphatidylserine was analyzed by flowcytometry. The results obtained from Annexin-V/PI staining demonstrated that Buparlisib significantly induced apoptosis in Nalm-6 cells. As presented in Fig 4, Buparlisib not only increased the proportion of Annexin-V positive cells, but also elevated the percentage of Annexin-V/PI positive cells as compared with the control group (p≤0.001). The resulting data suggested that Buparlisib decreased the survival rate of Nalm-6 cells through induction of apoptotic cell death.
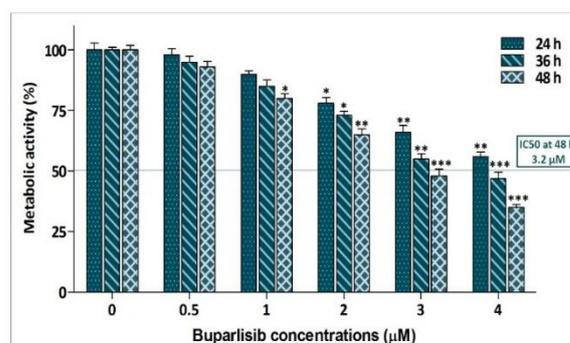
Figure 3. Evaluating the effect of Buparlisib on the metabolic activity of Nalm-6 cells. After treatment of cells with indicated concentrations of Buparlisib, the metabolic activity reduced in both dose- and time-dependent manner. Calculating the IC_{50} value also demonstrated that Buparlisib at the concentration of 3.2 μM is capable to reduced 50% of Nalm-6-viable cells. Values are given as mean ± SD of three independent experiments. *, P<0.05; **, P<0.01; and ***, p<0.001 represent significant changes from untreated control.

Buparlisib increased the transcriptional activity of pro-apoptotic related genes in Nalm-6 cells: Since the PI3K/Akt signaling pathway play crucial role in the regulation of the expression level of pro-apoptotic-related genes, such as Bax, Bad, PUMA and Noxa, it was of particular of interest to investigate the effect of

inhibition of this pathway on the transcriptional activity of aforementioned genes. Results of RQ-PCR analysis showed that treating cells with Buparlisib at the concentrations of 2 and 4 µM upon 36 h elevated in the transcriptional activity of pro-apoptotic members of Bcl-2 family. Buparlisib at the concentration of 2 µM is able to elevate the mRNA expression level of Bad, Bax, PUMA and Noxa nearly by 5.7-, 5.99-, 4.2- and 4.1-fold (p≤0.05), respectively. This inductionary effect become even more strengthen when cells were treated with 4 µM of Buparlisib, which increased the mRNA expression level of Bad, Bax, PUMA and Noxa by 7.78-, 6.91-, 6- and 6.3-fold (p≤0.001), respectively (Fig 5).
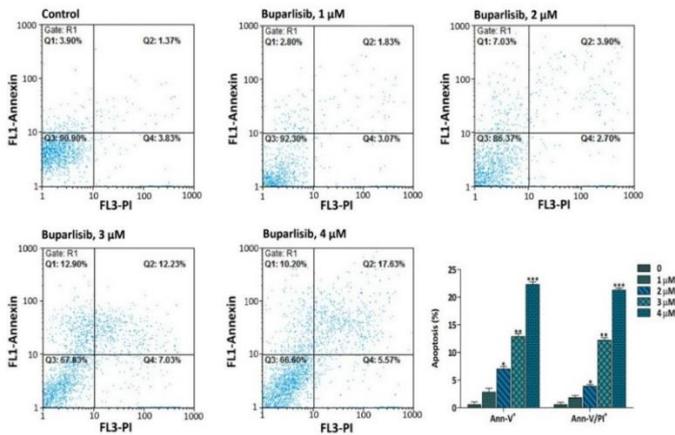
Figure 4. The apoptotic effect of Buparlisib on Nalm-6 cells. The percentages of both annexin-V and annexin-V/PI double-positive inhibitor-treated-6 cells were increased in response to the drug treatment. Values are given as mean ± SD of three independent experiments. *, p< 0.05; **, p< 0.01; and ***, p< 0.001 represent significant changes from untreated control.
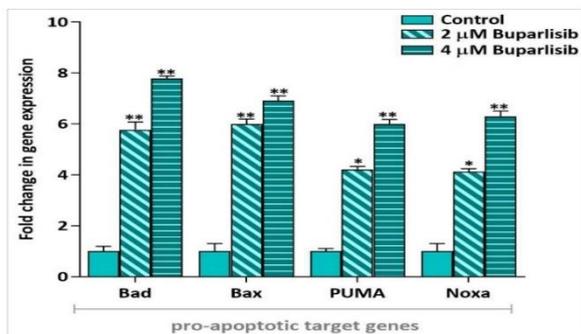
Figure 5. The effects of Buparlisib on the transcriptional activity of pro-apoptotic target genes in Nalm-6 cells. The results obtained from RQ-PCR analysis demonstrated that Buparlisib significantly increased the mRNA expression level of pro-apoptotic target genes. Values are represented as mean±SD of three independent experiments. *, p<0.05 and **, p<0.01 represent significant changes from untreated control.

Discussion

The results of the present study showed that Buparlisib through abrogation of the PI3K signaling pathway, as revealed by the reduction of p-Akt, exerts its cytotoxic effect against Nalm-6 cells both in a dose- and time-dependent manner. Moreover, our results indicated that upon 48 h treatment of cells with 3 µM concentration of the inhibitor, the survival rate of Nalm-6 cells reduced by 50%, highlighting the potent anti-leukemic activity of Buparlisib in Nalm-6 cells.

Of particular of interest, previous phase I clinical study reported that the maximum dose of Buparlisib, which could be used in pre-clinical investigations is around 4 µM (12).

In corroboration with the results of this study, Allegretti et al., also showed that Buparlisib promoted cell growth arrest and significant apoptosis in a dose- and time-dependent manner in AML cells through blockade of Akt phosphorylation (10).

Evasion of apoptosis is a hallmark of cancer cells and suppression of programmed cell death is thought to contribute to carcinogenesis by several mechanisms. Moreover, multiple lines of evidence indicated that the imbalanced expression of pro-apoptotic and anti-apoptotic genes endow cancer cells a survival advantage (13, 14).

Over the last decades, it was well-established that the aberrant activation of PI3K/Akt signaling pathway hampered the induction of apoptotic cell death in cancer cells through inhibition of p53, a well-known tumor suppressor protein, and subsequently down-regulation of the Bcl-2 pro-apoptotic-related genes (15).

In this study, we found that Buparlisib not only induced apoptosis in pre-B ALL-derived Nalm-6 cells, but also elevated the transcriptional activity of pro-apoptotic target genes of p53, such as Bad, Bax, PUMA and Noxa. Our finding was in harmony with the results of Koul et al, who demonstrated that inhibition of PI3K/Akt signaling pathway using a pan-PI3K inhibitor induced p53-dependent apoptosis in glioblastoma cells (16).

Overall, this study highlighted the potent efficacy of Buparlisib in pre-B-ALL cells. Given the pharmacologic safety of this inhibitor and its broad clinical effectiveness in variety of human malignancies, our study suggests that Buparlisib could be considered a promising agent for the treatment of ALL. However,

further investigation, including clinical trials and a detailed understanding of the underlying mechanism of action of Buparlisib, will provide valuable clues to add this PI3K inhibitor for the treatment of pre-B-ALL patients.

Acknowledgements

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