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Original Article

Selective ERK inhibitor ulixertinib inhibits proliferation and induces apoptosis in lymphoma cell lines

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Abstract: Aberrant MAPK pathway signaling and resultant ERK kinase activity is evident in many oncogene-dependent cancers and the intrinsic and acquired drug resistance is often associated with activated ERK signaling. Here, we reported ulixertinib, an ERK1/2 kinase inhibitor, potently and selectively inhibits ERK1 and ERK2 kinases in a reversible, ATP-competitive fashion. Consistent with its mechanism of action, ulixertinib inhibited ERK1/2 activation and cell proliferation, and induced cell cycle arrest and apoptosis in human lymphoma cell lines SUDHL-10 and Raji cells. Furthermore, the expression of VEGFA, VEGFR2 and Bcl-2 was significantly decreased in SUDHL-10 and Raji cells treated with ulixertinib, but the expression of Bax and caspase-3 was notably increased. In total, *in vitro* studies will help elucidate how ulixertinib may be used as a novel agent in therapeutic strategies for human lymphoma.

Keywords: Lymphoma, ulixertinib, ERK1/2, proliferation, apoptosis

Introduction

Lymphomas are solid tumors of the immune system. Non-Hodgkin lymphoma accounts for about 90% of all lymphomas and has a wide range of histological appearances and clinical features at presentation, which can make diagnosis difficult [1]. Diffuse large B-cell lymphoma is the most common lymphoma in adults, accounting for 30%-40% of cases of non-Hodgkin lymphoma [2]. Burkitt lymphoma is another type of lymphoma characterized by highly aggressive and rapidly proliferating B-cell neoplasm, accounting for 30%-50% of cases of lymphomas in children and 1%-2% in adults [3]. The cause of most diffuse large B-cell and Burkitt lymphomas remain unknown, however, dysregulation of apoptosis or defective repair plays a role in lymphogenesis [4].

Several advances in recent years have focused increasing attention on the role of the RAF-MEK-ERK1/2 pathway in cancer progression. ERK1/2 is activated in several cancers, including non-small-cell lung [5], breast [6] and ovarian cancer [7], and associated with advanced tumors. At the same time, studies defined the importance of the RAF-MEK-ERK1/2 pathway in G1/S cell cycle progression, leading to a general perception that the ERK1/2 pathway controlled cell cycle reentry [8]. In some cases, studies showed that miR-28 and ERK1/2 inhibitor AZD8330 induced cell apoptosis through down-regulating the activation of ERK1/2 pathway in Burkitt's lymphoma cell lines [9, 10]. However, the molecular mechanism of ERK1/2 involved in lymphogenesis is still unknown.

To explore the role of ERK1/2 in the proliferation, cell cycle and apoptosis of diffuse large B-cell and Burkitt lymphoma cell lines, we used SUDHL-10 and Raji cells to examine the involvement of ERK1/2 inhibitor ulixertinib. We next examined whether inhibition of ERK1/2 by treatment of SUDHL-10 and Raji cells with ulixertinib plays any role in the regulation of proliferation and apoptosis-related members in materializing the induction of apoptosis and cell proliferation inhibition.

Materials and methods

Reagents

ERK1/2 kinase inhibitor ulixertinib was purchase from Selleck (Shanghai, China). Antibodies

Table 1. Primes sequences used in this study

Gene	Sequences
VEGF-forward	5'-ATGAACTTTCTGCTGTCTTGG-3'
VEGF-reverse	5'-TCACCGCCTCGGCTTGTCACA-3'
VEGFR2-forward	5'-CATCACATCCACTGGTATTGG-3'
VEGFR2-reverse	5'-GCCAAGCTTGTACCATGTGAG-3'
Bcl-2-forward	5'-CCACCTGTGGTCCATCTGAC-3'
Bcl-2-reverse	5'-CAATCCTCCCCCAGTTCACC-3'
Bax-forward	5'-GTCATCTCGCTCTGGTACGG-3'
Bax-reverse	5'-CACACACACAAAGCTGCTCC-3'
Caspase-3-forward	5'-CTGACTGGAAAGCCGAAAC-3'
Caspase-3-reverse	5'-GCAAAGGGACTGGATGAAC-3'
GAPDH-forward	5'-GGAATCCACTGGCGTCTTCA-3'
GAPDH-reverse	5'-GGTTCACGCCCATCACAAAC-3'

for p-ERK1/2 (1:1000), ERK1/2 (1:1000) and GAPDH (1:1500) were purchased from Cell Signaling Technology, Inc. (Beverley, MA, USA). Antibodies for VEGFA (1:200), VERFR2 (1:500) and caspase-3 (1:1500) were purchased from Abcam (Cambridge, MA, USA). Bcl-2 (1:150) and Bax (1:100) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibody IgG was purchased from Beyotime Institute of Biotechnology (Haimen, China). DMEM, fetal calf serum, penicillin, streptomycin and TRIzol were purchase from Invitrogen Life Technologies (Carlsbad, CA, USA). Annexin-V fluorescein isothiocyanate and propidium iodide were purchase from Biovision Technologies, (Mountain View, CA, USA). cDNA synthesis kit was purchase from Thermo Fisher Scientific Inc. (Rockford, IL, USA). SYBR Green was purchase from Takara Biotechnology Co., Ltd. (Dalian, China).

Cell culture

The human lymphoma cell lines SUDHL-10 and Raji were obtained from the Academia Sinica Cell Bank (Shanghai, China), grown in DMEM supplemented with 10% fetal calf serum and 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in 5% CO_2.

Cell viability assay

The Cell Count Kit-8 (CCK-8, Dojindo, Rockville, MD, USA) was used to assess the effects of ulixertinib, on cell viability. In brief, SUDHL-10 and Raji cells were collected, and 5×10^3 cells/well were dispensed into 96-well culture plates with 100 μL culture medium. After 24 h culture different concentrations of ulixertinib (0.1, 0.4 and 1.0 nM) were put in different wells. Each of the concentrations above was regarded as one treated group while there was no ulixertinib in the control group. Culture plates were then incubated for 0, 24, 48, 72 and 96 h. Subsequently the cell viability was evaluated by CCK-8 following the manufacturer's instructions. The absorbance at wavelength 450 nm was measured for the supernatant of each well using the plate reader Multiskan EX (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Flow cytometry (FCM) detection

Cell cycles were examined using propidium iodide (PI) and flow cytometry. SUDHL-10 and Raji cells were seeded in 12-well plates at the density of 3×10^3 cells/well and then treated with 0.1, 0.4 and 1.0 nM of ulixertinib for 48 h. After treatments, the percentages of cells in the different phases of cell cycle were evaluated by determining the DNA content after PI staining. Briefly, cells were washed with PBS, trypsinized and centrifuged at 400 ×*g* at 4°C for 5 min. Pellets were fixed overnight in 70% cold ethanol. After fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/mL) for 10 min at room temperature. Finally, samples were stained with PI (1 mg/mL) for 30 min at 4°C. Data acquisition was done by flow cytometry (Epics XLM.CL, Beckman Coulter, Fullerton, CA, USA) using Cell Quest software (BD, Franklin Lakes, NJ, USA).

Apoptosis was determined by flow cytometry analysis. SUDHL-10 and Raji cells were collected after treatment with ulixertinib (0.1, 0.4 and 1.0 nM) for 48 h. Annexin-V fluorescein isothiocyanate (FITC)/PI double stain assays were performed following the manufacturer's protocol. Both floating and trypsinized adherent cells were collected, resuspended in 500 μL of binding buffer containing 2.5 μL of annexin-V FITC and 5 μL of PI, and then incubated for 10 min in the dark at room temperature before flow cytometry analysis.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions. The complementary DNA was synthesized using a cDNA synthesis kit. RT-qPCR anal-

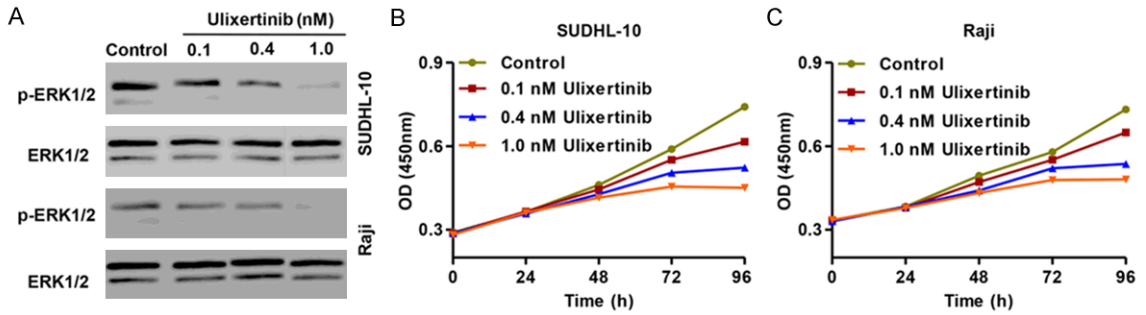

Figure 1. Ulixertinib inhibited the proliferation of SUDHL-10 and Raji cells. (A) The activation of ERK1/2 was analyzed by Western blotting. Ulixertinib (0.1, 0.4 and 1.0 nM) significantly inhibited ERK1/2 activation in a dose-dependent manner compared with control cells. Cell proliferation was measured by the CCK-8 assay. Ulixertinib (0.1, 0.4 and 1.0 nM) significantly inhibited proliferation of SUDHL-10 (B) and Raji cells (C) in a time- and dose-dependent manner compared with control cells.

yses were performed using SYBR Green, and data collection was conducted using an ABI 7500 (Applied Biosystems Life Technologies, Foster City, CA, USA). RT-qPCR was performed to detect the mRNA expression levels of the genes, as indicated in **Table 1**. GAPDH was used an internal control for normalization. The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [11].

Western blotting

Following treatment with ulixertinib at the desired concentrations and time, SUDHL-10 and Raji cells were harvested. The expression of related proteins was detected by Western blot according to the manufacturer's instructions and previous reports [12]. GAPDH antibody was used as an internal control for whole cell lysates. The experiment was repeated three times independently.

Statistical analysis

Values were expressed as means ± SD (standard deviation). Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The unpaired, two-tail Student's t-test was used to analyze the significance of the difference between groups. Probability (*p*) values less than 0.05 were considered significant.

Results

Ulixertinib treatment in lymphoma cell lines impairs proliferation

To investigate the possible role of ERK1/2 pathway in lymphomagenesis, we studied the effects of ulixertinib, an ERK1/2 kinase inhibitor, on proliferation. We engineered two lymphoma cell lines (SUDHL-10 and Raji) to treatment of ulixertinib. Expression of ERK1/2 phosphorylation in SUDHL-10 and Raji cells was confirmed by Western blot analysis 6 h upon ulixertinib treatment. After 6 h, the expression of ERK1/2 phosphorylation was significantly reduced in a dose-dependent manner, and was about 80% lower in 1.0 nM of ulixertinib treated SUDHL-10 and Raji cells compared with cells without ulixertinib treatment (**Figure 1A**). We then monitored proliferation for 96 h and observed that ulixertinib-treated cells displayed reduced or impaired proliferation in both cell lines (**Figure 1B** and **1C**).

Ulixertinib treatment in lymphoma cell lines arrests cell cycle

SUDHL-10 and Raji cells were treated with different concentrations of ulixertinib for 48 h, and then stained with PI and analyzed by flow cytometry. As shown in **Figure 2A** and **2B**, the percentage of G0-G1 phase cells in the SUDHL-10 was increased and that of S phase cells was decreased in cells treated with 1.0 nM ulixertinib. However, the percentage of G2-M phase cells in the SUDHL-10 decreased after treatment with 0.4 nM ulixertinib but not 1.0 nM ulixertinib. While, the percentage of G0-G1 phase cells in the Raji was increased and that of S phase cells was decreased in cells treated with ulixertinib at both 0.4 and 1.0 nM concentrations, compared with control cells without ulixertinib treatment (**Figure 2C** and **2D**). However, the percentage of G2-M phase cells in

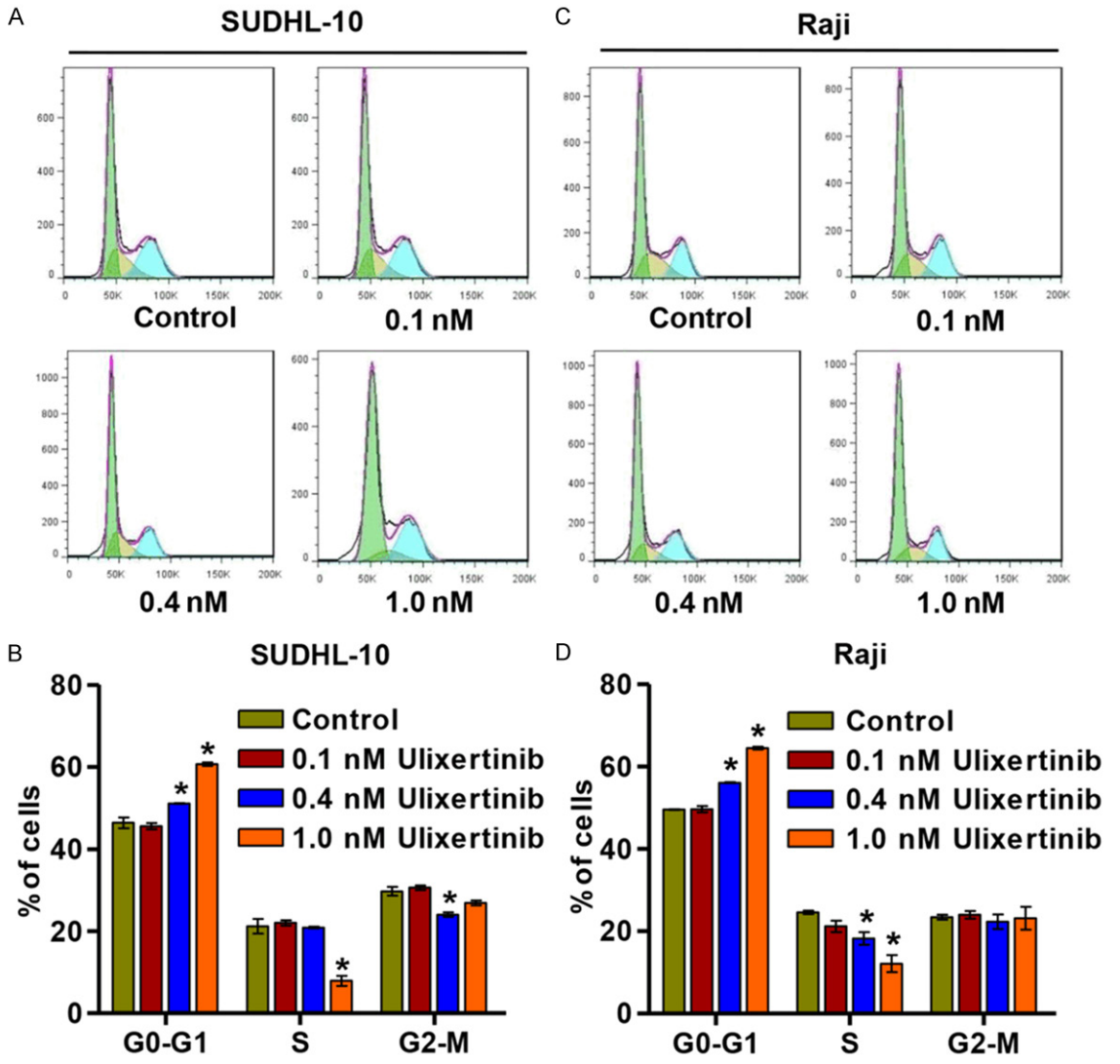

Figure 2. Ulixertinib arrested cell cycle of SUDHL-10 and Raji cells. PI stain assay and flow cytometry were carried out to analyze the cell cycle after SUDHL-10 (A) and Raji cells (C) were treated with ulixertinib at the dose of 0.1, 0.4 and 1.0 nM for 48 h. The percentages of G0-G1, S and G2-M phase cells in SUDHL-10 (B) and Raji cells (D) after treatment with different doses of ulixertinib were shown. $^{*}P<0.01$ compared with control.

the Raji was not statistically significant between cells with and without ulixertinib treatment.

Ulixertinib treatment in lymphoma cell lines induces apoptosis

An annexin-V fluorescein isothiocyanate (FITC)/propidium iodide (PI) double stain assay and flow cytometry analysis were carried out to substantiate cell apoptosis induced by ulixertinib treatment under various concentrations. The number of apoptotic cells was counted as late apoptotic cells shown in the upper right quadrant and early apoptotic cells as shown in lower right quadrant of the histograms. As shown in **Figure 3**, treatment of ulixertinib at the dose of 0.1, 0.4 and 1.0 nM for 48 h significantly increased the number of early apoptotic SUDHL-10 and Raji cells, respectively, in a dose-dependent manner compared with corresponding control cells. The significant induction of apoptosis indicated the anticancer effect of ulixertinib against SUDHL-10 and Raji cells.

Ulixertinib directly targets gene involved in proliferation and apoptosis

To identify the effect of ulixertinib on key genes involved in proliferation and apoptosis, we performed RT-qPCR and Western blotting to exam-

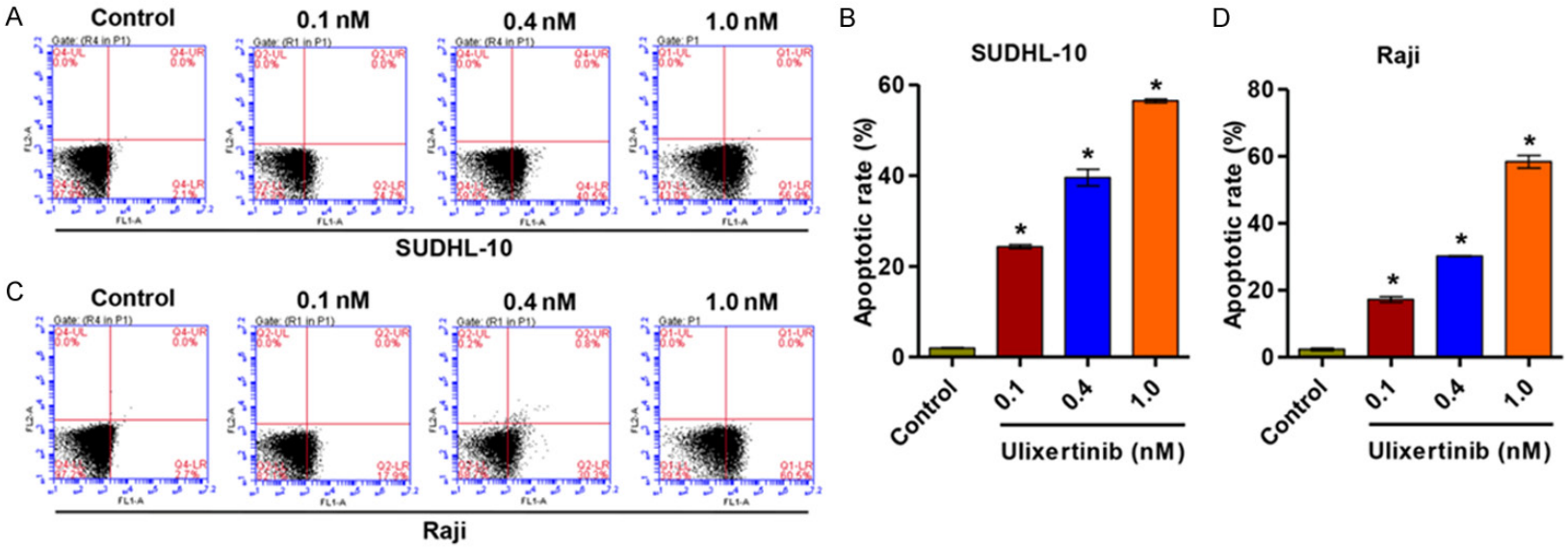

Figure 3. Ulixertinib induced apoptosis of SUDHL-10 and Raji cells. Annexin-V/PI double stain assay and flow cytometry analysis were carried out to substantiate apoptosis of SUDHL-10 (A) and Raji cells (C). Treatment of ulixertinib at doses of 0.1, 0.4 and 1.0 nM for 48 h dose-dependently increased the apoptotic population of SUDHL-10 (B) and Raji cells (D). $^{*}P<0.01$ compared with control.

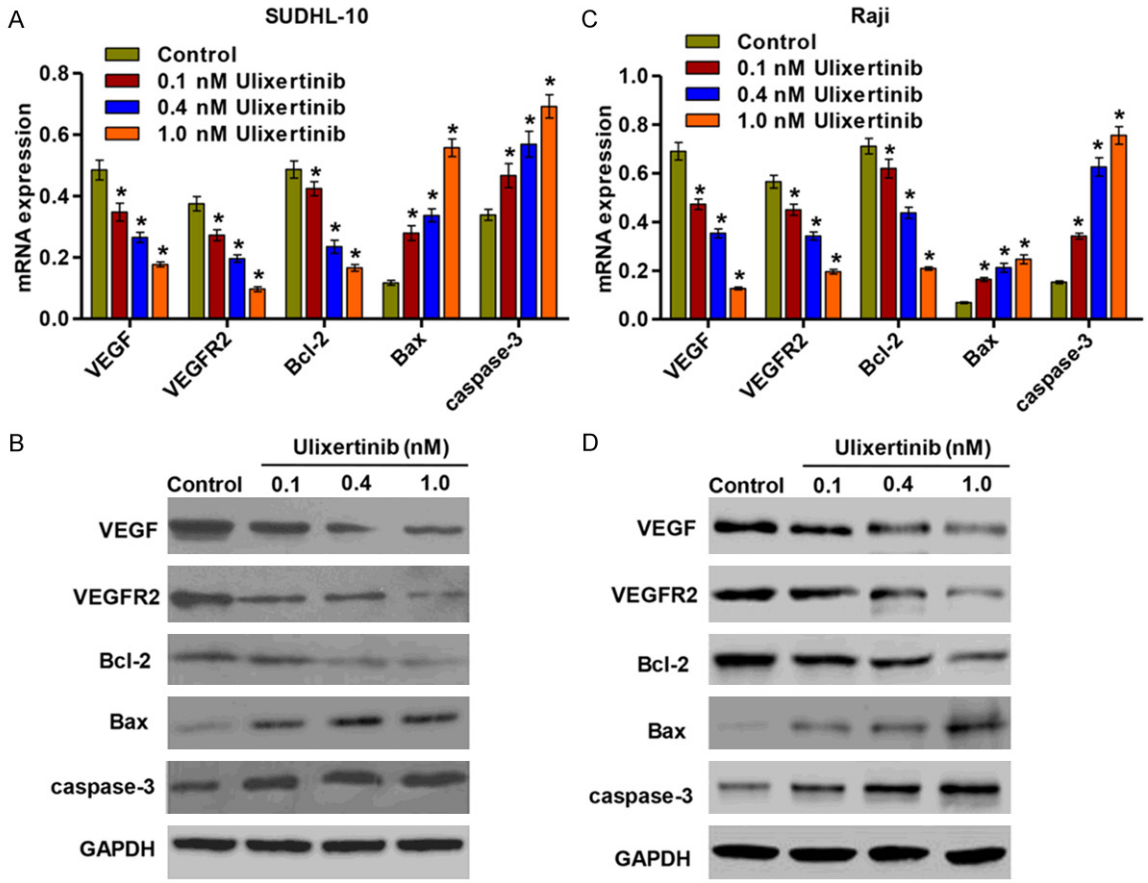

Figure 4. Effects of ulixertinib on the expression of key genes involved in cell proliferation and apoptosis. The mRNA expression of related genes in SUDHL-10 (A) and Raji cells (C) was detected by RT-qPCR. The protein expression of related genes in SUDHL-10 (B) and Raji cells (D) was detected by Western blotting. Ulixertinib significantly decreased the mRNA and protein expression of VEGF, VEGFR2 and Bcl-2, and increased the mRNA and protein expression of Bax and caspase-3 in SUDHL-10 and Raji cells compared with control cells without ulixertinib treatment. $^{*}P<0.01$ compared with control.

ine the genes expression at both mRNA and protein levels. As shown in **Figure 4**, the VEGFA, VEGFR2 and Bcl-2 genes showed a reduction in both mRNA and protein expression upon treatment of SUDHL-10 and Raji cells with ulixertinib. While, the Bax and caspase-3 genes showed an increase in both mRNA and protein expression upon treatment of SUDHL-10 and Raji cells with ulixertinib.

Discussion

In the present study, we provide evidence that ERK1/2 signaling pathway plays a critical role in regulating the proliferation, cell cycle and apoptosis of lymphoma cell lines. Our data showed that ulixertinib, a selective inhibitor of ERK1/2 kinase at 0.1 to 1.0 nM, caused apoptosis as determined by the increase of both G1 phase of cells and annexin-V positive cells. We also identified several genes that showed response to ulixertinib-induced apoptosis. The oncogenic role of the deregulated ERK1/2 pathway is probably related to its simultaneous actins on proliferation and apoptosis. Different mechanisms have been reported that facilitate nuclear translocation of phospho-ERK1/2 [13, 14]. Upon stimulation, ERK1/2 becomes phosphorylated at threonine and tyrosine residues and the latter results in the dissociation of ERK1/2 from MEK1/2 [15]. The rapid and persistent nuclear transfer of ERK1/2 during the entire G0-G1 period is crucial for the function of these kinases in mediating the growth response [16].

Many studies defining the role of the ERK1/2 pathway have used the MEK inhibitors, PD-

98059 or U0126. However, PD98059 is not very potent and has poor solubility [17]. Here, we used a novel small molecule ERK1/2 kinase inhibitor, ulixertinib, effective in several models that show intrinsic or acquired resistance to other MAPK pathway inhibitors [18] and as only one ERK1/2 inhibitor used in clinical phase 1/2 for advanced malignancies [19]. In this study, we found that inhibition of ERK1/2 by treatment of lymphoma cell lines with ulixertinib significantly decreased the expression of VEGFA and VEGFR2 at both mRNA and protein levels. VEGFA is expressed by tumor cells and exhibits proangiogenic functions by binding to the VEGFR2 on endothelial cells. VEGFA stimulation caused upregulation of phosphorylated ERK1/2 indicating an intracellular pathway via ERK1/2 [20]. Usnic acid inhibited breast tumor growth by suppressing VEGFR2-mediated ERK1/2 signaling pathway, suggesting that VEGFR2 is associated with activation of ERK1/2 [21].

Although ERK1/2 activation has generally been associated with cell proliferation and apoptosis, a number of studies show that depending on the stimuli and cell types involved, activation of ERK1/2 can also mediate cell apoptosis [22]. However, the molecular mechanisms that define the conditions for ERK1/2-mediated cell apoptosis remain poorly understood. Apoptosis is a multistep process and an increasing number of genes have been identified that are involved in the control or execution of apoptosis [23]. The BCL-2 family consists of proapoptotic proteins (such as Bcl-2 and Bcl-x_L) and antiapoptotic proteins (such as Bax and Bak). In this study, we showed that inhibition of ERK1/2 downregulation of Bcl-2 and activated Bax in ulixertinib-sensitive lymphoma cell lines. This implies that Bcl-2 and Bax are involved in ulixertinib-induced apoptosis. There is abundant evidence that apoptosis factors can use the ERK1/2 pathway to increase the expression of several proapoptotic BCL-2 proteins, notably Bcl-2, Bcl-x_L and MCL-1, by promoting gene expression in a variety of cell types [24]. ERK1/2 inhibition caused a decrease in Bcl-2 and increase in Bax and apoptosis in human pancreatic [25] and prostate cancer cells [26]. Moreover, caspases play a crucial role in apoptosis and caspase-3 has been shown to be a key component of the apoptotic machinery [27]. Our results indicated that inhibition of ERK1/2 significantly activated caspase-3 in ulixertinib-treated lymphoma cell lines. In agreement with our findings, previous studies showed that fisetin, an inhibitor of cyclin-dependent kinase 6, induced apoptosis through ERK1/2-mediated activation of caspase-3-dependent pathway in human cervical cancer HeLa cells [28].

In conclusion, our results show that inhibition of ERK1/2 leads to proliferation inhibition, cell cycle arrest and apoptosis in lymphoma cell lines. These studies may have important implications for future animal, preclinical and clinical studies in lymphoma.

Disclosure of conflict of interest

None.

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