Alisertib induces apoptosis and autophagy through targeting the AKT/mTOR/AMPK/p38 pathway in leukemic cells

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Abstract. Alisertib, a potent and selective Aurora kinase A inhibitor, has been demonstrated to exert potent anti-cancer effects in pre-clinical and clinical studies. However, mechanisms of action of alisertib, including the molecular pathways involved in alisertib-induced apoptosis and autophagy of leukemic cells, have remained elusive. The aim of the present study was to investigate the effects of alisertib on cell growth, apoptosis and autophagy and to delineate the possible molecular mechanisms in leukemic cells. Acid phosphatase, MTT and Annexin V/propidium iodide staining assays as well as immunostaining for light chain 3B showed that treatment of the REH leukemia cell line with alisertib exerted potent growth inhibitory effects, and induced apoptosis and autophagy in a dose-dependent manner. Western blot analysis indicated that these effects may be attributed to the suppression of the activity of the Akt/mammalian target of rapamycin/5'-AMP-dependent kinase/p38 mitogen-activated protein kinase signaling pathways in REH cells. The present study confirmed that alisertib may represent a promising autophagy-inducing drug for the treatment of leukemia and shed light on its molecular mechanism of action.

Introduction

Leukemia is a hematopoietic malignancy caused by acquired somatic mutation and ranks among the ten types of malignant neoplasm with the highest incidence rate. Chronic myeloid leukemia is a common hematopoietic malignancy, accounting for 20% of all types of leukemia (1). Cancer fusion protein p210bcr/abl generated by Ph chromosome has an important role in these malignant phenotypes, as it has been demonstrated to induce leukemia-like syndrome in cancerous mice, to promote tumor-cell proliferation and survival *in vitro* as well as to be implicated in the evasion of apoptosis of tumor cells (2).

Autophagy is a conserved self-degradation system in eukaryotic cells involved in numerous physiological and pathological processes. Autophagosomes are a typical feature of autophagy, and the control of their formation and degradation is the main regulatory factor in autophagy (3). Autophagy has a dual nature of promoting cell survival and death, and is also closely associated with the development, metastasis and drug resistance of cancer (4). Targeting autophagy may be a novel strategy to treat cancer and address drug resistance. In-depth study of autophagy in leukemia will lead to the elucidation of the induction and regulatory mechanisms of leukemia-cell autophagy and provide novel targets and strategies for leukemia treatment and possible cures (5).

The mechanisms of action of the promising anti-cancer drug candidate alisertib are complex and have remained to be fully elucidated. As alisertib has been demonstrated to induce apoptosis (6), the present study assessed the underlying molecular mechanisms. A recent study showed that alisertib exerts pro-autophagic effects on human osteosarcoma cell lines through the activation of a mitochondria-mediated pathway and inhibition of the p38 mitogen-activated protein kinase (MAPK)/phosphoinositide-3 kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway (7). To assess whether alisertib exerts its effects against leukemia cells via similar mechanisms, its ability to inhibit proliferation, induce autophagy and affect MAPK/PI3K/Akt/mTOR signaling were assessed in the REH leukemia cell line.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Mediatech Inc. (Manassas, VA, USA). Alisertib (chemical structure shown in Fig. 1) was purchased from Selleckchem Inc. (Houston, TX, USA). Acid phosphatase assay (AP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) were obtained from BD Biosciences (San Diego, CA,

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USA). All antibodies used for western blot analysis were from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA).

Cell lines and culture. The REH human acute lymphocytic leukemia cell line was purchased from Central South University (Hunan, China). REH cells were cultured in DMEM medium supplemented with 2 mM L-glutamine and 1% of antibiotic/antimycotic mixture (Sigma-Aldrich, St. Louis, MO, USA). Cells were maintained in a humidified atmosphere of 5% CO_2/95% air at 37˚C.

Cell viability assay. Cells were seeded into 96-well plates at a density 1.0-2.0x10^4 cells/well and treated with alisertib (0, 0.1, 1 or 5 μM) for 24 h. For the PA assay, the cells were subsequently incubated for 2 h at 37˚C with 100 ml p-nitrophenyl phosphate solution (5 mM) and 0.1% Triton X-100. The reaction was terminated by addition of 10 ml NaOH (1 M) and the number of viable cells was measured at 405 nm using a Multiskan Spectrum microplate reader (Thermo Fisher Scientific, Inc.). For the MTT assay, 20 μl MTT was added to the cells, which were then cultured for an additional 4 h. Following aspiration of the MTT solution, dimethylsulfoxide (Nanjing Chemical Reagent Co., Ltd., Nanjing, China) was added to each well and plates were agitated for 20 min. The absorbance was then measured with Multiskan Spectrum microplate reader at 490 nm.

Apoptosis assay. Cells were seeded into six-well plates at a density 1.0-2.0x10^6 cells/well and treated with alisertib (0, 0.1, 1 and 5 μM) for 24 h. Following harvesting, cells were washed twice with ice-cold phosphate-buffered saline (PBS), re-suspended in binding buffer and then incubated with 10 μl Annexin V-FITC and 5 μl PI in the dark for 30 min. The samples were then analyzed by flow cytometry (FACSAria III; BD Biosciences).

Immunofluorescence microscopy. Cells were seeded into six-well plates at a density 1.0-2.0x10^6 cells/well and treated with alisertib (0, 0.1, 1 and 5 μM) for 24 h. Cells were then fixed with 4% paraformaldehyde (Solarbio Science and Technology, Ltd., Beijing, China) for 30 min at room temperature and permeabilized using pre-cooled methanol on ice for 10 min. Binding sites were then blocked with 5% normal goat serum (Thermo Fisher Scientific, Inc.) and 0.3% Triton X-100 (Thermo Fisher Scientific, Inc.) for 1 h. Cells were then incubated with mouse monoclonal anti-light chain (LC)3B (cat. no. sc-376404; 1:300; Santa Cruz Biotechnology, Inc.) followed by 1 h of incubation with Alexa Fluor 488 goat anti-mouse immunoglobulin G (cat. no. 8878; 1:1,000 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) as a secondary antibody. After incubation, cells were washed with ice-cold PBS, mounted onto microscopic slides with Flouromount-G (Southern Biotech, Birmingham, AL, USA) and observed using a confocal laser scanning microscope (TCS SP5; Leica Microsystems GmbH, Wetzlar, Germany).

Western blot analysis. Cells was seeded into six-well plates at a density 1.0-2.0x10^6 cells/well and treated with alisertib (0, 0.1, 1 and 5 μM) for 24 h. Cells were then lysed with ice-cold lysis buffer (1 mM phenylmethylsulfonylfluoride, 30 mM Tris-HCl pH 8.0, 150 mM NaCl, protease/phosphatase
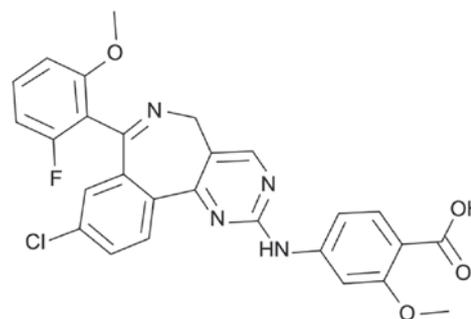
Figure 1. Chemical structure of alisertib.
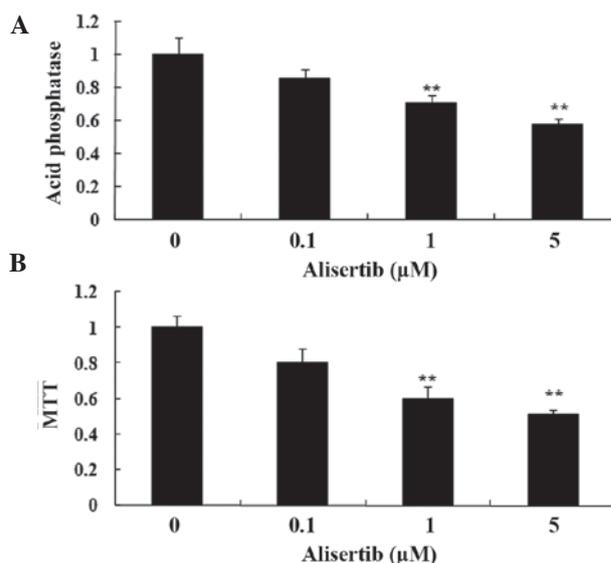
Figure 2. Alisertib suppresses the viability of REH cells. (A) Acid phosphatase and (B) MTT assays were used to assess the suppressive effects of alisertib on the viability of REH cells. Values are expressed as mean ± standard error of the mean. **P<0.01 compared with 0 μM alisertib-treatment group.
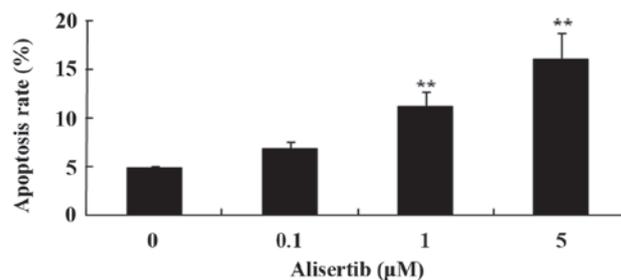
Figure 3. Alisertib induces autophagy in REH cells. The apoptotic rate was determined by Annexin V/propidium iodide double staining and flow-cytometric analysis. Values are expressed as mean ± standard error of the mean. **P<0.01 compared with 0 μM alisertib-treatment group.

inhibitor cocktail and 1% nonidet P-40) for 30 min. The lysate was then centrifuged at 12,000 x g for 10 min at 4˚C. Equal amounts of protein (40-50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel (Sangon Biotech Co., Ltd., Shanghai, China) electrophoresis and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Following incubation with mouse monoclonal anti-phosphorylated (p)-mTOR (cat. no. sc-293132;
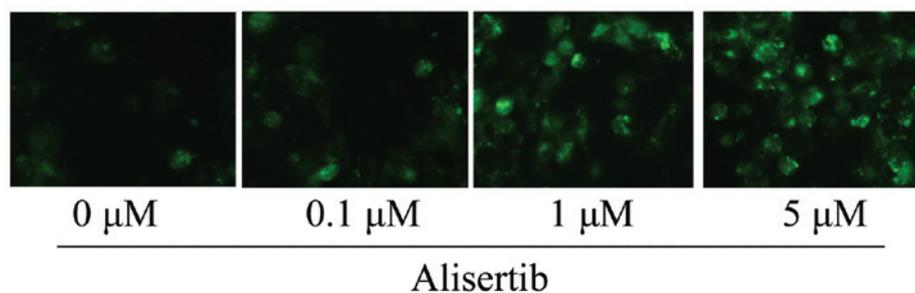
Figure 4. Alisertib induces autophagy of REH cells. Cells were immunostained with light chain 3B antibody (green). Magnification, x100.

1:1,000 dilution; Santa Cruz Biotechnology, Inc.), mouse monoclonal p-Akt (cat. no. sc-293125; 1:1,000 dilution; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-p-adenosine monophosphate-activated kinase (AMPK) (cat. no. 4186; 1:2,000 dilution; Cell Signaling Technology, Inc.) and rabbit polyclonal anti-β-actin (cat. no. R1207-1; 1:800 dilution; HangZhou HuaAn Biotechnology Co., Ltd., Hangzhou, China). Subsequently, membranes were incubated with goat anti-rabbit (cat. no. A0208) and goat anti-mouse (cat. no. A0216) horseradish peroxidase-conjugated secondary antibodies from Beyotime Institute of Biotechnology (Haimen, China) at 1:5,000 dilution for 2 h at room temperature. Proteins were visualized using an enhanced chemiluminescence western blot detection system (EMD Millipore) and visualization was performed using Image J (version 1.31; National Institutes of Health, Bethesda, MA, USA).

Statistical analysis. Values are expressed as the mean ± standard error. Statistical significance of the differences was analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. All statistical analysis was performed using SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed three times. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Alisertib reduces the viability of REH cells. The present study assessed the effects of alisertib on viability of REH cells using AP and MTT assays. Incubation of REH cells with alisertib at 1 and 5 μM for 24 h significantly decreased the cell viability (Fig. 2). These results showed that alisertib potently decreases the viability of REH cells.

Alisertib induces apoptotic death of REH cells. Next, the present study explored the effects of alisertib on apoptosis of REH cells using flow cytometry following Annexin V-FITC and PI staining. As shown in Fig. 3, treatment with alisertib at 1 μM for 24 h caused a two-fold increase of the apoptotic rate of REH cells, while 5 μM alisertib increased the apoptotic rate by three-fold. These results demonstrated that alisertib exerts its anti-cancer effects on REH cells by inducing apoptosis.

Alisertib induces autophagy in REH cells. Since a previous study reported on the ability of alisertib to induce authophagy in osteosarcoma cells (7), the present study assessed whether alisertib induces autophagy in REH leukemia cells. As

demonstrated by immunofluorescence microscopy, incubation of REH cells with alisertib at 1 and 5 μM for 24 h resulted in marked expression of the autophagic marker LC3B (Fig. 4). These results indicated that alisertib may exert its anti-cancer effects via inducing autophagy of REH cells.

The mechanism of action of alisertib on leukemia cells may be mediated via deactivation of the MAPK/PI3K/Akt/mTOR pathway and activation of AMPK. To elucidate the possible molecular mechanisms via which alisertib induces apoptosis and autophagy in leukemic cells, its effects on the levels of p-mTOR, p-Akt, p-AMPK and p-p38 were assessed by western blot analysis. As shown in Fig. 5, treatment with 1 and 5 μM alisertib significantly reduced the levels of p-mTOR protein in REH cells; furthermore, the levels of p-Akt were reduced (Fig. 6), the levels of p-AMPK were increased (Fig. 7) and the levels of p-p38 (Fig. 8) were decreased ($P<0.01$ for all). These results suggested that deactivation of MAPK/PI3K/Akt/mTOR signaling and activation of AMPK may be involved in the mechanism of action of alisertib in leukemia cells.

Discussion

Studies have indicated a significant link between autophagy and neoplastic transformations of blood cells. Autophagy facilitates the removal of the mitochondria in red blood cells, which has an important regulatory role in early differentiation and maturation of red blood cells as well as in the development of myelodysplastic syndrome (8). In addition, autophagy may be the main cause of megakaryocyte dysfunction in myelodysplastic syndrome and idiopathic thrombocytopenic purpura (9). A variety of bioactive compounds and irradiation are known to induce autophagic death of leukemia cells. The results of the present study indicated that treatment with alisertib significantly reduced the viability of REH cells and promoted apoptotic cell death in a dose-dependent manner. Qi *et al* (10) indicated that alisertib inhibits the proliferation and induces apoptosis of T-Hodgkin lymphoma cells. Melichar *et al* (11) revealed that alisertib suppressed the grow of small-cell lung cancer, non-small-cell lung cancer, gastro-oesophageal adenocarcinoma, breast cancer and head and neck squamous-cell carcinoma. Furthermore, the results of the present study indicated that alisertib possesses activity against leukemia cells.

Autophagy is an important intracellular self-degradation mechanism, during which macromolecules and organelles within the cell are transported to the lysosomes for digestion and degradation by double-membrane vesicles, releasing free
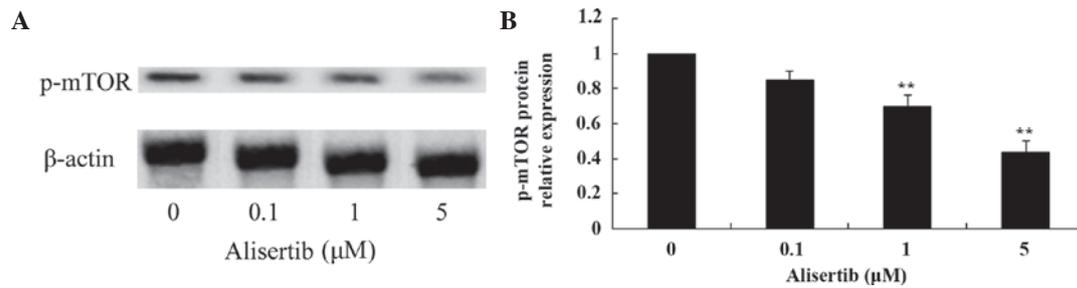
Figure 5. Alisertib suppresses autophagy through mTOR activation. (A) Representative western blot of p-mTOR protein levels in REH cells following Alisertib treatment and (B) quantified p-mTOR protein levels as determined by densitometric analysis normalized to β-actin. Values are expressed as mean ± standard error of the mean. **P<0.01 compared with 0 μM alisertib group. p-mTOR, phosphorylated mammalian target of rapamycin.
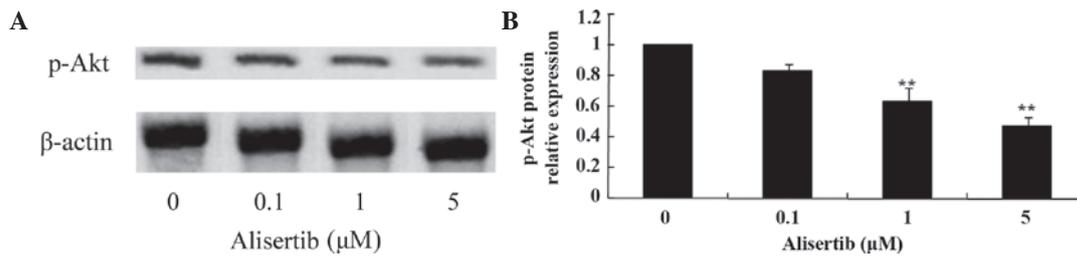
Figure 6. Alisertib suppresses autophagy through Akt activation. (A) Representative western blot of p-Akt protein levels in REH cells following Alisertib treatment and (B) quantified p-Akt protein levels as determined by densitometric analysis normalized to β-actin. Values are expressed as mean ± standard error of the mean. **P<0.01 compared with 0 μM alisertib group. p-Akt, phosphorylated Akt.
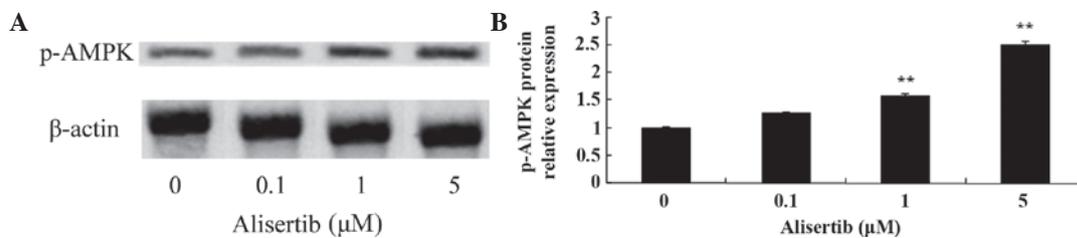
Figure 7. Alisertib induces autophagy through AMPK activation. (A) Representative western blot of p-AMPK protein levels in REH cells following Alisertib treatment and (B) quantified p-AMPK protein0 levels as determined by densitometric analysis normalized to β-actin. Values are expressed as mean ± standard error of the mean. **P<0.01 compared with 0 μM alisertib-treatment group. p-AMPK, phosphorylated 5'-adenosine monophosphate-dependent kinase.
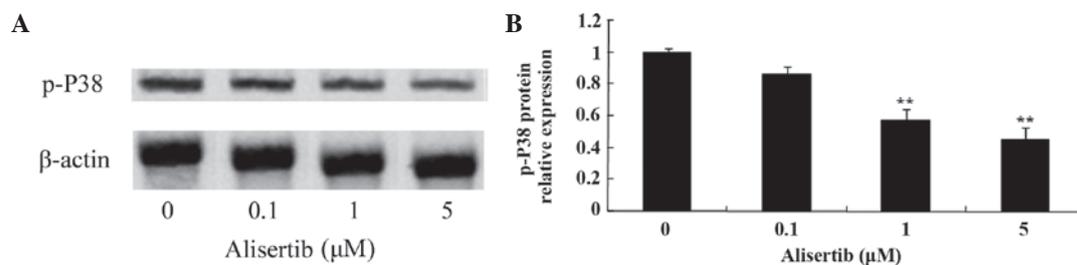
Figure 8. Alisertib induces autophagy through p38 activation. (A) Representative western blot of p-P38 protein levels in REH cells following Alisertib treatment and (B) quantified p-P38 protein levels as determined by densitometric analysis normalized to β-actin. Values are expressed as mean ± standard error of the mean. **P<0.01 compared with 0 μM alisertib-treatment group. p-P38, phosphorylated P38.

small molecules for recycling in the cell (12). Almost all types of eukaryotic cell have the capacity to undergo autophagy (13). It is known that this mechanism has an important role in cell physiological processes, including cell development, differentiation, senescence and death, while the specific mechanisms have remained to be fully elucidated. Autophagy can be divided into macro-autophagy, micro-autophagy and chaperone-mediated autophagy (14). In contrast to the ubiquitin protein degradation system, autophagy can avert pathogenic attacks as well as cell damage caused by physical and chemical factors, apart from acting as a energy retrieval by self digestion (15). Autophagy also acts in a power switch-like manner to change the threshold level of the regulation of cell survival in response to apoptotic stimuli. In the embryonic development period as

well as in adult organisms, excessive levels of autophagy lead to autophagic death (16). The findings of the present study showed that alisertib dose-dependently induced autophagy in REH cells. Wang et al (17) reported that alisertib induces cell cycle arrest and autophagy of human pancreatic cancer cells through PI3K/Akt/mTOR. Furthermore, Ding et al (18) also reported that alisertib induced apoptosis and autophagy of human epithelial ovarian cancer cells. These results suggested that induction of autophagy represents a major mechanism of action of alisertib, which is a desired mechanism of cancer treatments.

mTOR is an important factor regulating cell growth and proliferation, and influences processes including nutrition-associated molecules in signal transduction, regulation of protein translation and cell cycle progression; furthermore, activated mTOR is an inhibitor of autophagy (19). The initiation of autophagy is mainly inhibited by molecules targeting mTOR, including TOR complex 1 (TORC1) and type I PI3K (20). Signaling of growth factors within the cell and information regarding the nutrient status and energy levels are conveyed to TORC1 through Type I PI3K and Akt/protein kinase B pathways, resulting in the activation of TORC1 at an appropriate level, thereby activating mTOR to inhibit autophagy (21). Therefore, the activity of mTOR is influenced by the activity of TORC1 activity to regulate the level of autophagy (22). The results of the present study revealed that alisertib decreased the phosphorylation/activation of mTOR as well as that of Akt and p38, thereby inducing autophagy in REH cells, which was in line with the findings of a previous study (17). Furthermore, alisertib significantly activated AMPK in REH cells. Yuan et al (23) indicated that alisertib induces apoptosis and autophagy via activation of AMPK and suppression of p38 MAPK in human gastric cancer. These results indicated that alisertib induces autophagy in REH cells and other cancer cell types via activation of AMPK and suppression of the PI3K/Akt/mTOR pathway.

In conclusion, the present study reported that alisertib exerted marked anti-cancer effects against the REH leukemia cell line by suppressing cell grow, promoting apoptosis and inducing autophagy. The underlying molecular mechanism of the autophagy-inducing effects of alisertib in REH cells were indicated to include the deactivation of the PI3K/Akt/mTOR pathway and activation of AMPK.

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