Dual PI3K/mTOR inhibitor NVP-BEZ235 decreases the proliferation of doxorubicin-resistant K562 cells

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Abstract. Acute myelogenous leukemia (AML) is frequently accompanied by a poor prognosis. The majority of patients with AML will experience recurrence due to multiple drug resistance. Our previous study reported that targeting the mTOR pathway may increase cell sensitivity to doxorubicin (Doxo) and provide an improved therapeutic approach to leukemia. However, the effect and mechanism of action of NVP-BEZ235 (BEZ235), a dual inhibitor of PI3K/mTOR, on Doxo-resistant K562 cells (K562/A) is yet to be elucidated. Therefore, the aim of the present study was to investigate the effects of BEZ235 on K562/A cell proliferation. K562/A cells was investigated using CCK-8, flow cytometry and western blotting, following BEZ235 treatment. It was observed that BEZ235 significantly decreased the viability of K562/A cells. In addition, BEZ235 arrested K562/A cells at the G_0/G_1 phase, and reduced the protein expression levels of CDK4, CDK6 and cyclin D1. Apoptotic cells were more frequently detected in K562/A cells treated with BEZ235 compared with the control group (12.97±0.91% vs. 7.37±0.42%, respectively; $P<0.05$). Cells treated with BEZ235 exhibited downregulation of Bcl-2 and upregulation of Bax. Furthermore, BEZ235 treatment markedly decreased the activation of the PI3K/AKT/mTOR pathway and its downstream effectors. Thus, these results demonstrated that BEZ235 inhibited cell viability, induced G_0/G_1 arrest and increased apoptosis in K562/A cells, suggesting that BEZ235 may reverse Doxo resistance in leukemia cells. Therefore, targeting the PI3K/mTOR pathway may be of value as a novel therapeutic approach to leukemia.

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Introduction

Leukemias, presenting with increased numbers of leucocytes, are a group of malignant disorders. Globally, the number of newly diagnosed leukemia patients increased from 3,545,000 in 1990 to 5,185,000 in 2017 (1). It is estimated there were a total of 4,370,000 new cases of and 3,090,000 deaths from leukemia worldwide in 2018 (2). Myeloid leukemia, including acute myelogenous leukemia (AML) and chronic myelogenous leukemia (CML), is the most common hematological malignancy. CML, which is characterized by the Philadelphia chromosome (a fusion of chromosomes 9 and 22), gives rise to the Bcr/Abl oncogene (3,4). Although Bcr/Abl kinase inhibitors have achieved a promising clinical response in CML, certain patients progressing to the blast phase may develop resistance to Bcr/Abl kinase inhibitors (5). Furthermore, AML is frequently accompanied by a poor prognosis. The DA regimen, mainly comprising cytarabine and doxorubicin (Doxo), has been the standard treatment for AML (6). However, the majority of the patients with AML will experience recurrence due to the development of multiple drug resistance, with decreasing cancer cell capability for apoptosis (7). Therefore, novel therapeutic targets must be investigated to enhance the therapeutic efficacy against leukemia.

It was previously demonstrated that the mTOR pathway is upregulated in CML, and rapamycin can arrest cells at the G_0/G_1 phase and promote apoptosis of K562 cells (8). In addition, rapamycin enhances the antitumor effects of Doxo by downregulating the mTOR/ribosomal protein S6 kinase (p70S6K) pathway (9). Therefore, targeting the mTOR pathway may increase cell sensitivity to Doxo and serve as an effective therapeutic approach to leukemia. NVP-BEZ235 (BEZ235), a dual inhibitor of the PI3K/mTOR pathway, was reported to induce cancer cell apoptosis in multiple studies (10,11). It has been established that the PI3K/AKT/mTOR signaling pathway regulates cell survival, proliferation and metabolism under physiological and pathological conditions (12,13). At present, previous studies have revealed that BEZ235 exerts an inhibitory effect on cancer cell proliferation by downregulating the PI3K/AKT/mTOR pathway (14-19). Moreover, BEZ235 was observed to reverse chemoresistance in several

human cancer types, such as ovarian, pancreatic, prostate and gastric cancer (14-17). BEZ235 can also induce apoptosis and enhance autophagic activity, as well as inhibit the proliferation of leukemia cells (18,19). Furthermore, BEZ235 was identified to enhance the accumulation of Doxo in drug-resistant ovarian and pancreatic cancer cells (9). However, the effect of BEZ235 on Doxo-resistant K562 cells (K562/A) has not been extensively reported.

In the present study, the proliferation and apoptosis of K562/A cells were investigated following BEZ235 treatment. In addition, the effects of BEZ235 on the PI3K/AKT/mTOR signaling pathway in K562/A cells were examined, with the aim of determining the underlying mechanism and identifying a novel therapeutic target for clinical application in hematological malignancies.

Materials and methods

Chemicals and reagents. The K562 human erythromyeloblastoid leukemia cell line was purchased from the Beijing Institute for Cancer Research, and K562/A cells were obtained from the Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences.

The primary antibodies used in the present study included rabbit anti-human AKT (1:1,000; cat. no. 4685), phosphorylated (p)-AKT (1:2,000; cat. no. 4060), mTOR (1:1,000; cat. no. 2983), p-mTOR (1:1,000; cat. no. 5536), eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) (1:1,000; cat. no. 9644), p-4E-BP1 (1:1,000; cat. no. 2855), p70S6K (1:800, cat. no. 2708) and p-p70S6K (1:500; cat. no. 9234), which were purchased from Cell Signaling Technology, Inc. Rabbit anti-human β-actin (1:10,000; cat. no. AC026) monoclonal antibody was purchased from ABclonal Biotech Co., Ltd., while rabbit anti-human Bax (1:500; cat. no. sc-23959) and mouse anti-human Bcl-2 (1:500; cat. no. sc-509) antibodies were obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-human CDK4 (1:5,000; cat. no. ab108357), CDK6 (1:20,000; cat. no. ab124821), cyclin B1 (1:10,000; cat. no. ab32053) and cyclin D1 (1:200; cat. no. ab16663) were purchased from Abcam. Goat anti-mouse IgG (H&L), horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; cat. no. S0002) and goat anti-rabbit IgG (H&L), HRP-conjugated secondary antibody (1:5,000; cat. no. S0001) were obtained from Affinity Biosciences.

Cell culture and treatment. K562 and K562/A cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with penicillin (100 U/ml), streptomycin (100 U/ml) and 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified atmosphere containing 5% CO_2. Doxo (cat. no. 22386; Cayman Chemical Company) and BEZ235 (cat. no. 21185; Cayman Chemical Company) were diluted in DMSO (Merck KGaA) at a concentration of 1 mM as the primary stock solution and stored at 4˚C, respectively.

Cell Counting Kit-8 (CCK-8) assay. A CCK-8 assay (Dojindo Molecular Technologies, Inc.) was used to examine the viability of K562 and K562/A cells, according to the manufacturer's instructions. BEZ235 was dissolved in DMSO at a concentration of 1 mM as the primary stock solution and stored at 4˚C. K562 and K562/A cells were cultured at 37˚C and treated with various concentrations of BEZ235 (25, 50, 100, 200, 400, 800 and 1,600 nM) for 24 h or with 200 nM BEZ235 for 0.5, 1, 2 and 3 days. Meanwile, the cells were treated with various concentrations of Doxo (31.25, 62.5, 125, 250, 500, 1,000 and 2,000 nM) according to our previously published paper (9). After the cells were incubated with 10 μl CCK-8 reagent at 37˚C for 4 h, the cytotoxic effect of BEZ235 on K562/A cells was measured on a spectrophotometer microplate reader (BioTek Instruments, Inc.) at a wavelength of 450 nm. Cell viability was assayed and compared with the control group. Based on the results of CCK-8 and our previous study, 200 nM BEZ235 and 250 nM Doxo were chosen for the following experiments.

Flow cytometry. K562/A cells were cultured as described above and treated with BEZ235 (200 nM) for 24 h, then were harvested in the buffer (PBS-0.05% trypsin). K562/A cells were incubated with 5 μl Annexin V and 10 μl PI in the dark for 5 min at room temperature, and then resuspended in 1X binding buffer. The fluorescence of the cells was analyzed on a FACSCalibur (BD Biosciences). Cell apoptosis was analyzed using an Annexin V/PI apoptosis detection kit [Hangzhou Multi Sciences (Lianke) Biotech Co., Ltd.], according to the manufacturer's protocol. The frequency of intact cells (annexin V^-/PI^-), early apoptotic cells (annexin V^+/PI^-) and late apoptotic/necrotic cells (annexin V^+/PI^+) were analyzed using FlowJo 10 software (FlowJo LLC).

For cell cycle analysis, flow cytometry analysis with propidium iodide staining was performed on a FACSCalibur (BD Biosciences). After 24-h treatment with 200 nM BEZ235, cells were fixed with 70% ethanol and incubated with PI/RNase Staining Buffer (BD Biosciences; cat. no. 550825) for 15 min at room temperature, according to the manufacturer's instructions. Cells in the G_2/M phases of the cell cycle have twice the DNA content of those in G_0/G_1 phases. Cells in the S phase have a DNA content lying between these extremes. The data were analyzed using FlowJo 10 software (FlowJo LLC).

Western blot analysis. K562/A were cultured as aforementioned and treated with 200 nM BEZ235 for 24 h. Total protein was extracted from K562/A cells using lysis buffer (1% Triton X-100; 150 mM NaCl; 2 mM EDTA; 50 mM Tris-HCl) supplemented with phosphatase inhibitor cocktail. Total protein concentration was measured using Gen5 1.0 software (BioTek Instruments, Inc.) on a spectrophotometer microplate reader (BioTek Instruments, Inc.). A total of 20-50 μg protein was separated by SDS-PAGE using 10-15% gels and transferred onto a PVDF membrane. Following blocking non-specific binding sites with 5% non-fat dry milk in Tris-buffered saline with 0.05% Tween-20 at room temperature for 60 min, the membranes were incubated with primary antibodies at 4˚C overnight. Next, the membranes were probed with HRP-conjugated secondary antibody (1:5,000) for 60 min at 37˚C. Subsequently, the immunoreactive membranes were developed using Pierce™ Electrochemiluminescent Western Bloting Substrate (cat. no. UC280185, Thermo Fisher Scientific, Inc.) on ImageQuant™ LAS 4000 (GE Healthcare Life Sciences) and the density was quantified and normalized to β-actin.

Table I. Primers for reverse transcription-quantitative PCR amplification.

Target	Sequence	Product length, bp
CDK4	F: 5'-AGTTCGTGAGGTGGCTTTAC-3'	162
	R: 5'-GCCTTGTCCAGATATGTCCTTAG-3'	
CDK6	F: 5'-CTGCCTTGTTGGCAAAGTATC-3'	229
	R: 5'-CCAGGTAGAAGGACTGCATTAG-3'	
cyclin D1	F: 5'-CCACTCCTACGATACGCTACTA-3'	219
	R: 5'-GGACTGAAAGTGCTTGGAAATG-3'	
cyclin B1	F: 5'-GGTGTCACTGCCATGTTTATTG-3'	179
	R: 5'-CGAAGGAAGTGCAAAGGTAGA-3'	
Bcl-2	F: 5'-GCCAGGGTCAGAGTTAAATAGAG-3'	143
	R: 5'- GCCTCTCTTGCGGAGTATTT-3'	
Bax	F: 5'-GTCACTGAAGCGACTGATGT-3'	128
	R: 5'-CTTCTTCCAGATGGTGAGTGAG-3'	
β-actin	F: 5'-AGCGAGCATCCCCCAAAGTT-3'	285
	R: 5'-GGGCACGAAGGCTCATCATT-3'	

F, forward; R, reverse.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted using TRIzol® reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After cDNA was obtained by GoScript™ Reverse Transcription System (Promega Corporation), PCR amplification was performed with forward and reverse primers using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc.) in an Mx3005p instrument (Agilent Technologies GmbH). RT was performed under the following conditions: 25˚C for 5 min, 42˚C for 60 min and 70˚C for 15 min. The thermocycling conditions of PCR amplification consisted of an initial denaturation at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec, annealing at 60˚C for 30 sec and elongation at 60˚C for 30 sec. The Cq values of the test genes were normalized to the mean Cq values of β-actin. Fold change was calculated using the $2^{-\Delta\Delta Cq}$ method (20). The primers used in the present study are listed in Table I.

Statistical analysis. All experiments were performed ≥3 times. Data are presented as the mean ± standard deviation. Statistically significant differences were evaluated using an unpaired Student's t-test or the comparative data among groups was analyzed using one-way ANOVA followed by Tukey's test, using SPSS 21.0 for Windows (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

BEZ235 inhibits the viability of K562 and K562/A cells. The cytotoxic effect of BEZ235 and Doxo on K562/A cells was measured using a CCK-8 assay after treating cells with increasing concentrations of each reagent for 24 h. The viability of K562 cells was significantly inhibited in the Doxo-treated group from 31.25-2,000 nM (P<0.05); however, that of K562/A cells was decreased in only the 1,000 and 2,000 nM treatment groups (P<0.05; Fig. 1C and D). In addition, cell viability was significantly decreased in a dose-dependent manner in K562 and K562/A cells treated with BEZ235 from 25-1,600 nM (P<0.05; Fig. 1A and B).

Based on the aforementioned results and our previous study (9), 200 nM BEZ235 and 250 nM Doxo were used to treat K562 and K562/A cells for 0.5, 1, 2 or 3 days. At these concentrations, the effect of BEZ235 was observed to be time-dependent (P<0.05; Fig. 1E-G). However, Doxo exerted little effect on the viability of K562/A cells over time (P>0.05, Fig. 1H). These findings suggested that K562/A cells were resistant to Doxo, while BEZ235 significantly inhibited the viability of K562/A cells.

BEZ235 arrests K562/A cells at the G_0/G_1 phase. To investigate the inhibitory effects of BEZ235 on the proliferation of Doxo-resistant cells, cell cycle progression was evaluated following BEZ235 treatment. The proportion of K562/A cells at the G_0/G_1 phase was 52.97±0.47% in the BEZ235 treatment group, which was significantly higher compared with that in the control group (36.17±2.31%; P<0.05; Fig. 2A and B). Consistent with the increasing number of G_0/G_1 phase cells, the proportion of S phase cells was significantly decreased in the BEZ235 treatment group (P<0.05; Fig. 2A and B).

The protein and mRNA expression levels of several cell cycle-related molecules were further assessed following BEZ235 treatment (Fig. 2C-E). The protein expression levels of CDK4, CDK6 and cyclin D1 were significantly decreased in the BEZ235 group compared with the control group, as indicated via western blotting (P<0.05; Fig. 2D and E). However, cyclin B1 expression displayed no change in K562/A cells after incubation with BEZ235 (P>0.05). The RT-qPCR results demonstrated the same trend (Fig. 2C). Thus, these observations suggested that BEZ235 induced G_0/G_1 arrest in K562 cells.

BEZ235 induces apoptosis of K562/A cells. To assess whether apoptosis contributed to cell proliferation inhibition in the BEZ235 treatment group, the effects of BEZ235 on

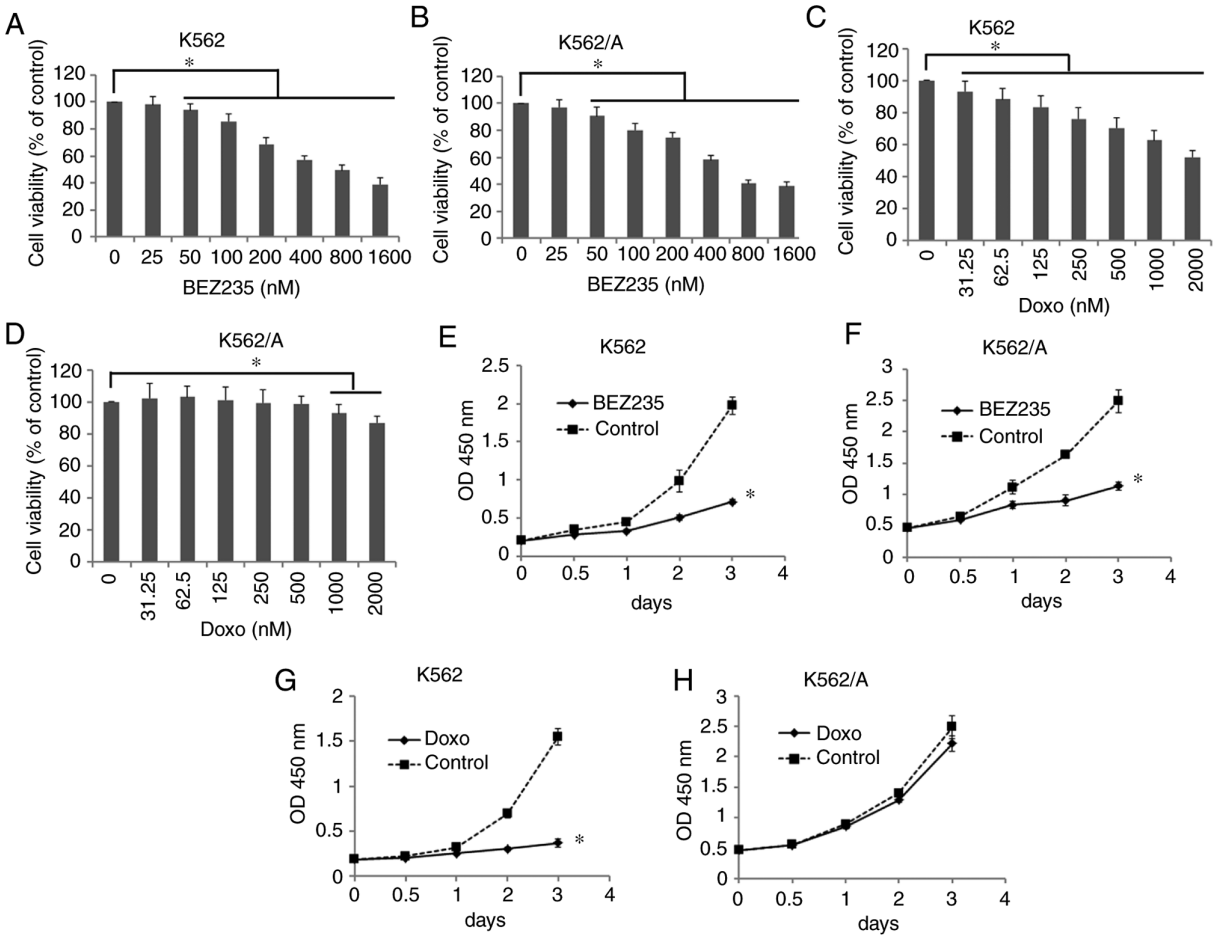

Figure 1. BEZ235 decreases the proliferation of K562 and K562/A cells. (A) K562 and (B) K562/A cells were treated with various concentrations of BEZ235 (0-1,600 nM) for 24 h, and cell viability was determined using a Cell Counting Kit-8 assay. The percentage of viable (A) K562 and (B) K562/A cells treated with BEZ235 decreased in a dose-dependent manner. (C) Doxo (0-2,000 nM for 24 h) decreased cellular viability in treated K562 cells (D) Viability of K562/A cells decreased significantly following Doxo treatment (1,000 and 2,000 nM) for 24 h. The control value was measured as 100%. Viability of (E) K562 and (F) K562/A cells decreased in the BEZ235 (200 nM) treatment group in a time-dependent manner. (G) Doxo (250 nM) treatment inhibited the viability of K562 cells in a time-dependent manner. (H) Doxo treatment exerted no effect on the viability of K562/A cells. Data are presented as mean ± standard deviation of triplicate experiments. *P<0.05 vs. control group. Doxo, doxorubicin; BEZ235, NVP-BEZ235; OD, optical density.

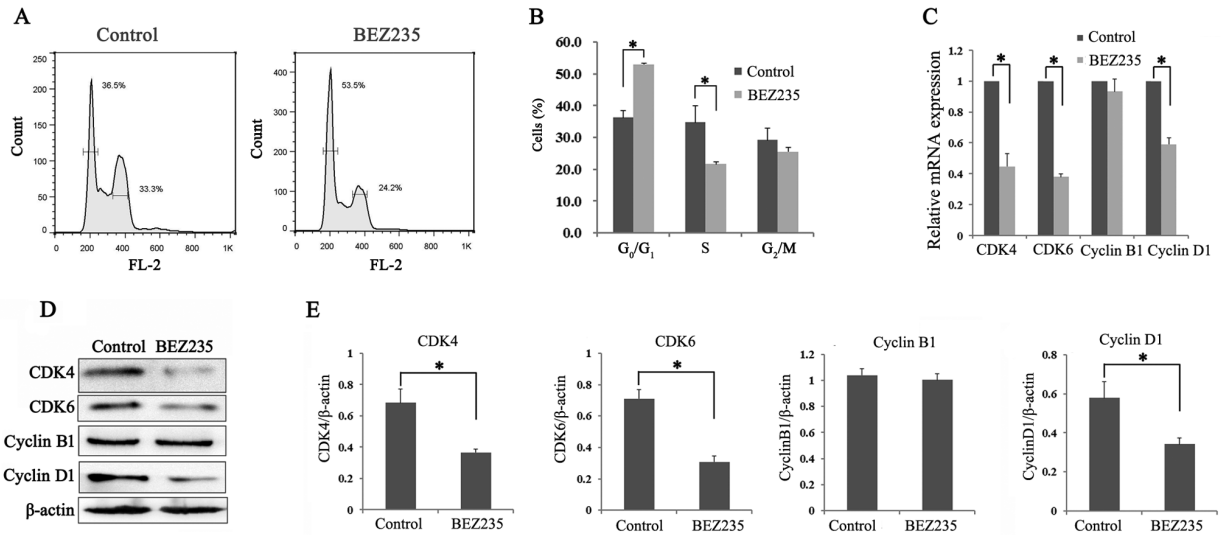

Figure 2. BEZ235 arrests the cell cycle at the G_0/G_1 phase. K562/A cells were incubated with BEZ235 (200 nM) for 24 h. (A) Cell cycle at each phase of K562/A cells treated with BEZ235 and measured using flow cytometry. (B) Results of cell cycle analysis demonstrated a higher proportion of G_0/G_1 phase cells and a lower proportion of S phase cells in the BEZ235 treatment group compared with the control group. (C) Reverse transcription-quantitative PCR analysis identified lower mRNA expression levels of CDK4, CDK6 and cyclin D1 in BEZ235-treated cells. (D) Cell cycle-related proteins were detected using western blot analysis. (E) Protein expression levels of CDK4, CDK6 and cyclin D1 were significantly decreased in K562/A cells after incubation with BEZ235. *P<0.05. BEZ235, NVP-BEZ235.

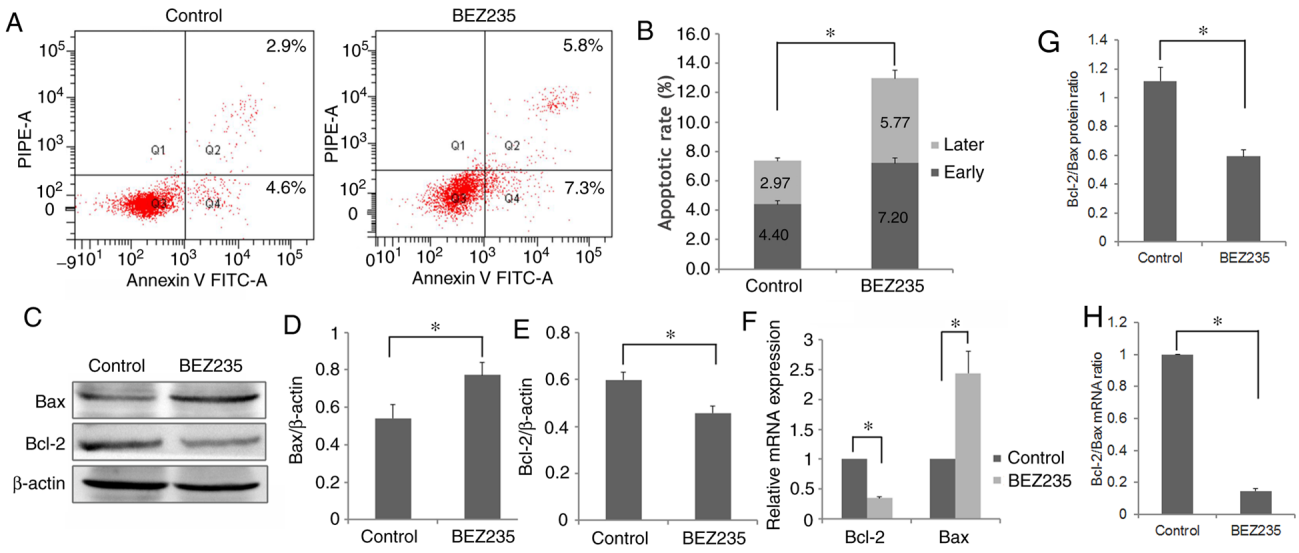

Figure 3. BEZ235 induces apoptosis of K562/A cells. (A) Apoptosis of K562/A cells was determined using an Annexin V/PI apoptosis detection kit. (B) BEZ235 (200 nM) treatment significantly increased the number of early and late apoptotic cells compared with the control group. (C) Protein expression levels of Bax and Bcl-2 in K562/A cells. BEZ235 significantly (D) increased the expression of Bax and (E) decreased the expression of Bcl-2. (F) mRNA expression levels of Bcl-2 and Bax in K562/A cells after BEZ235 treatment. (G and H) A significantly lower Bcl-2/Bax ratio was observed in the BEZ235-treated cells. Data are presented as mean ± standard deviation, and experiments were performed in triplicate. *P<0.05. BEZ235, NVP-BEZ235.

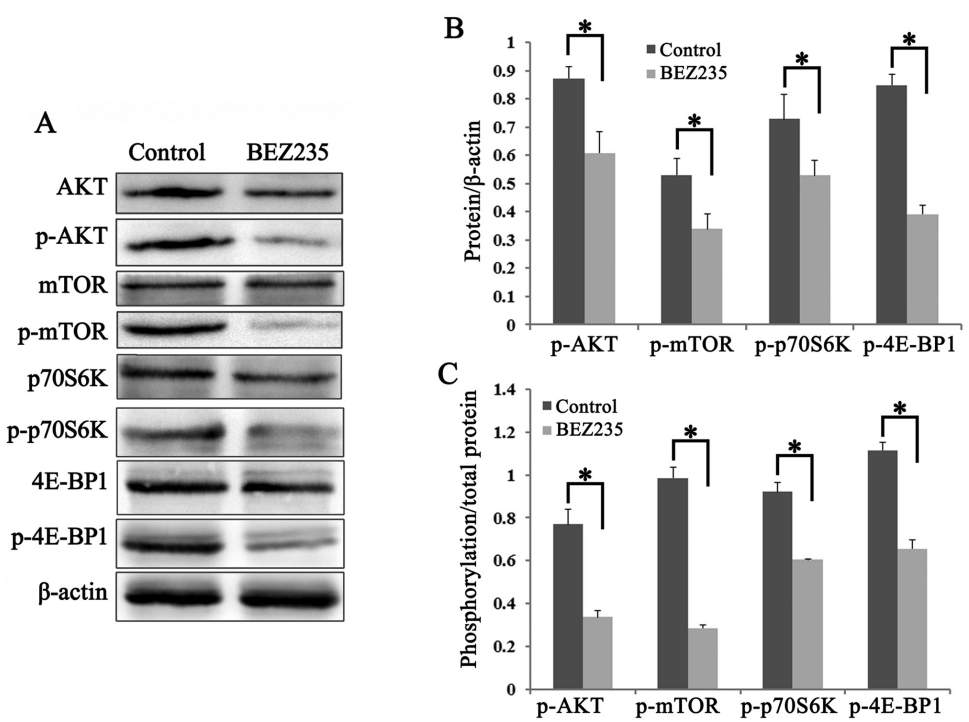

Figure 4. BEZ235 downregulates the PI3K/AKT/mTOR signaling pathway in K562/A cells. Cells were cultured with BEZ235 (200 nM) for 24 h. Total protein was extracted, followed by immunoblotting with the antibodies against the PI3K/AKT/mTOR pathway. Experiments were repeated three times and data are presented as mean ± standard deviation. (A) Protein expression levels of AKT, mTOR, 4E-BP1 and p70S6K, and their phosphorylation levels were detected in K562/A cells using western blot analysis. (B) Semi-quantification of the results demonstrated lower expression levels of p-AKT (Ser-473), p-mTOR (Ser-2448), p-p70S6K (Thr-389) and p-4E-BP1 (Thr37/46) in K562/A cells with BEZ235 treatment. (C) Ratio of p-/total protein were significantly downregulated in BEZ235 treated cells, as compared with the control group. *P<0.05. BEZ235, NVP-BEZ235; 4E-BP1, eukaryotic translation initiation factor 4E binding protein 1; p-, phosphorylated; p70S6K, ribosomal protein S6 kinase.

K562/A cell apoptosis were examined. The total apoptotic rate following treatment with BEZ235 was 12.97±0.91%, which was significantly higher compared with that in the control group (7.37±0.42%; P<0.05; Fig. 3A and B). In addition, the both rates of early and late apoptosis were significantly increased in BEZ235-treated cells (P<0.05; Fig. 3B).

To investigate the apoptotic mechanism induced by BEZ235, the expression levels of Bcl-2 and Bax, two pivotal apoptosis-related proteins, were detected using western

blotting and RT-qPCR analysis. Downregulation of Bcl-2 and upregulation of Bax were observed in the BEZ235 treatment group (P<0.05; Fig. 3C-F). Thus, compared with the control group, a significantly lower Bcl-2/Bax ratio was observed in the BEZ235-treated cells (P<0.05, Fig. 3G and H), indicating that BEZ235 may induce cell apoptosis by downregulating the Bcl-2/Bax ratio in K562/A cells. Therefore, these findings demonstrated that BEZ235 could inhibit K562/A cell proliferation via inducing apoptosis.

BEZ235 downregulates the PI3K/AKT/mTOR pathway and its downstream proteins in K562/A cells. The effects of BEZ235 on the PI3K/AKT/mTOR pathway in K562/A cells were also investigated using western blot analysis. The phosphorylation levels of AKT (Ser473) and mTOR (Ser-2448) were significantly lower in the BEZ235 group compared with those in the control group (P<0.05; Fig. 4A and B). The two major downstream targets of mTOR, p70S6K and 4E-BP1, were examined, and the expression levels of p-p70S6K (Thr-389) and p-4E-BP1 (Thr37/46) were significantly decreased in K562/A cells treated with BEZ235 (P<0.05; Fig. 4A and B). Furthermore, the ratio of p-/total protein for these factors were significantly downregulated in BEZ235-treated cells, compared with the control group (P<0.05; Fig. 4C). Collectively, the results demonstrated that BEZ235 inhibits cell proliferation via downregulating the PI3K/AKT/mTOR pathway in K562/A cells.

Discussion

Despite Doxo-induced rapid and durable responses in patients with myelogenous leukemia, a proportion of patients will develop drug resistance and therefore treatment failure (21). Thus, it is important to identify an alternative novel target that can achieve a satisfactory clinical response in patients who experience relapse and have a poor prognosis. In the present study, a Doxo-resistant cell line (K562/A) was selected and the viability of K562/A cells after Doxo treatment was detected. The results demonstrated that K562/A cells exhibited lower sensitivity to Doxo, which provided an opportunity to search for new drugs that are potentially effective against Doxo-resistant leukemia cells.

PI3K/AKT/mTOR is a central signaling pathway involved in the regulation of cell proliferation, differentiation and survival (12,13). Moreover, the BCR/ABL protein encoded by the Bcr/Abl oncogene can activate PI3K/AKT/mTOR via its regulatory subunit (22). mTOR activation further phosphorylates its two downstream targets, p70S6K and 4E-BP1. Phosphorylated p70S6K affects cell proliferation by inducing protein synthesis and cell survival, while 4E-BP1 phosphorylation results in the release of the eukaryotic initiation factor 4E (23,24). Our previous study reported that the mTOR pathway was upregulated in CML, and rapamycin arrested cells at the G_0/G_1 phase and increased apoptosis in K562 cells (8). In addition, a mTOR inhibitor, everolimus is currently being investigated as an anticancer agent due to its potential action by inhibiting tumor cell proliferation, and has been used in clinical trials (25-27). However, it has been reported that everolimus plus only or Imatinib demonstrate limited activity in progressing advanced cancer types (28,29), and the clinical application of everolimus does not exert the same effect as that in *in vitro* experiments. Thus, this may explain why inhibition of mTOR complex 1 (mTORC1), which is sensitive to rapamycin, may paradoxically enhance the PI3K/AKT axis (30,31). Therefore, a dual PI3K/mTOR inhibitor may have improved anticancer activity compared with a mTORC1 inhibitor alone (32,33). It was reported that BEZ235 inhibits the activity of PI3K, mTOR and Rad3-related protein, as well as possesses an anticancer potential (34). Therefore, BEZ235 has been used to reverse temozolomide resistance in glioma and to suppress paclitaxel-resistant gastric cancer (35,36). In the present study, BEZ235 was found to significantly inhibit the viability of K562/A cells, which suggested that BEZ235 may reverse Doxo resistance of leukemia cells.

To identify the mechanism via which BEZ235 regulates cell proliferation, the present study further detected cell cycle progression following BEZ235 treatment of K562/A cells. The present results demonstrated that BEZ235 arrested K562/A cells at the G_0/G_1 phase. In addition, the expression levels of several cell cycle-related proteins, including CDK4, CDK6 and cyclin D1, were significantly decreased in BEZ235-treated cells, indicating that BEZ235, as an inhibitor of PI3K/mTOR, blocked cell cycle progression from the G_1 to the S phase, ultimately arresting cell proliferation. To assess whether apoptosis contributes to cell proliferation inhibition following BEZ235 treatment, cell apoptosis was further investigated. The present study identified an increase in apoptotic K562/A cells following treatment with BEZ235. Moreover, significantly lower Bcl-2 and higher Bax expression levels were observed in BEZ235-treated cells. Bcl-2 family members are key regulators of cell apoptosis, and an increasing Bax expression and/or decreasing Bcl-2 expression have been reported to induce apoptosis (37). Therefore, in the current study, it was suggested that BEZ235 may induce cell apoptosis by downregulating the Bcl-2/Bax ratio in K562/A cells. These findings indicated that BEZ235 may reverse Doxo resistance via inducing cell apoptosis and G_0/G_1 arrest.

The present study demonstrated that p-AKT and p-mTOR expression levels were significantly decreased in BEZ235-treated K562/A cells, as well as those of p-4E-BP1 and p-p70S6K compared with the control group. These findings suggested that BEZ235 inhibited the PI3K/mTOR pathway and decreased the phosphorylation levels of its downstream proteins. Previous studies have revealed that suppression of AKT/mTOR pathway activation, acting additively or synergistically with trastuzumab, carboplatin and Doxo, may suppress cancer cell proliferation *in vitro* (38-40). In addition, rapamycin may enhance the antitumor effects of Doxo by inhibiting mTOR/p70S6K (9). Collectively, the present results suggested that BEZ235 can inhibit the proliferation of Doxo-resistant leukemia cells by downregulating the PI3K/mTOR pathway, and thus targeting PI3K/mTOR may be a novel approach to leukemia treatment.

In conclusion, the present findings indicated that BEZ235 inhibited cell proliferation, induced apoptosis and G_0/G_1 arrest and decreased PI3K/mTOR signaling in K562/A cells, suggesting that BEZ235 may reverse resistance of K562/A cells to Doxo by inhibiting the PI3K/mTOR pathway. One limitation of the study is that the results were derived from *in vitro* studies rather than an appropriate animal model of

leukemia. Moreover, the mechanism related to PI3K/mTOR in leukemia remains unclear. Nevertheless, the present findings may provide insight into the underlying mechanism and identification of a novel therapeutic target for clinical application in myelogenous leukemia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JL and LX designed the study, analyzed the data and wrote the manuscript. XW, CM, SX and MX performed the experiments and prepared the figures. RW and JY prepared the figures and analyzed the data. JL and LX performed critical revision of the manuscript and supervised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hebei General Hospital (Shijiazhuang, China; approval no. 202041).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Dong Y, Shi O, Zeng Q, Lu X, Wang W, Li Y and Wang Q: Leukemia incidence trends at the global, regional, and national level between 1990 and 2017. Exp Hematol Oncol 9: 14, 2020.
2. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
3. Rowley JD: Ph1-positive leukaemia, including chronic myelogenous leukaemia. Clin Haematol 9: 55-86, 1980.
4. Groffen J, Stephenson JR, Heisterkamp N, de Klein A, Bartram CR and Grosveld G: Philadelphia chromosomal breakpoints are clustered within a limited region, bcr, on chromosome 22. Cell 36: 93-98, 1984.
5. Gorre ME, Mohammed M, Ellwood K, Hsu N, Paquette R, Rao PN and Sawyers CL: Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 293: 876-880, 2001.
6. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, Dombret H, Ebert BL, Fenaux P, Larson RA, *et al*: Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood 129: 424-447, 2017.
7. Amico D, Barbui AM, Erba E, Rambaldi A, Introna M and Golay J: Differential response of human acute myeloid leukemia cells to gemtuzumab ozogamicin in vitro: Role of Chk1 and Chk2 phosphorylation and caspase 3. Blood 101: 4589-4597, 2003.
8. Li J, Xue L, Hao H, Han Y, Yang J and Luo J: Rapamycin provides a therapeutic option through inhibition of mTOR signaling in chronic myelogenous leukemia. Oncol Rep 27: 461-466, 2012.
9. Li J, Liu W, Hao H, Wang Q and Xue L: Rapamycin enhanced the antitumor effects of doxorubicin in myelogenous leukemia K562 cells by downregulating the mTOR/p70S6K pathway. Oncol Lett 18: 2694-2270, 2019.
10. Seo BR, Min KJ, Cho IJ, Kim SC and Kwon TK: Curcumin significantly enhances dual PI3K/Akt and mTOR inhibitor NVP-BEZ235-induced apoptosis in human renal carcinoma Caki cells through down-regulation of p53-dependent Bcl-2 expression and inhibition of Mcl-1 protein stability. PLoS One 9: e95588, 2014.
11. Xie G, Wang Z, Chen Y, Zhang S, Feng L, Meng F and Yu Z: Dual blocking of PI3K and mTOR signaling by NVP-BEZ235 inhibits proliferation in cervical carcinoma cells and enhances therapeutic response. Cancer Lett 388: 12-20, 2017.
12. Shi X, Wang J, Lei Y, Cong C, Tan D and Zhou X: Research progress on the PI3K/AKT signaling pathway in gynecological cancer (Review). Mol Med Rep 19: 4529-4535, 2019.
13. Vivanco I and Sawyers CL: The phosphatidylinositol 3-kinase AKT pathway in human cancer. Nat Rev Cancer 2: 489-501, 2002.
14. Durrant DE, Das A, Dyer S and Kukreja RC: A dual PI3 kinase/mTOR inhibitor BEZ235 reverses doxorubicin resistance in ABCB1 overexpressing ovarian and pancreatic cancer cell lines. Biochim Biophys Acta Gen Subj 1864: 129556, 2020.
15. Park H, Kim Y, Sul JW, Jeong IG, Yi HJ, Ahn JB, Kang JS, Yun J, Hwang JJ and Kim CS: Synergistic anticancer efficacy of MEK inhibition and dual PI3K/mTOR inhibition in castration-resistant prostate cancer. Prostate 75: 1747-1759, 2015.
16. Li L, Zhang S, Xie D, Chen H, Zheng X and Pan D: Dual inhibitor of PI3K and mTOR (NVP-BEZ235) augments the efficacy of fluorouracil on gastric cancer chemotherapy. Onco Targets Ther 11: 6111-6118, 2018.
17. Xia A, Li H, Li R, Lu L and Wu X: Co-treatment with BEZ235 enhances chemosensitivity of A549/DDP cells to cisplatin via inhibition of PI3K/Akt/mTOR signaling and downregulation of ERCC1 expression. Oncol Rep 40: 2353-2362, 2018.
18. Deng L, Jiang L, Lin XH, Tseng KF, Liu Y, Zhang X, Dong RH, Lu ZG and Wang XJ: The PI3K/mTOR dual inhibitor BEZ235 suppresses proliferation and migration and reverses multidrug resistance in acute myeloid leukemia. Acta Pharmacol Sin 38: 382-391, 2017.
19. Xin P, Xu W, Zhu X, Li C, Zheng Y, Zheng T, Cheng W and Peng Q: Protective autophagy or autophagic death: Effects of BEZ235 on chronic myelogenous leukemia. Cancer Manag Res 11: 7933-7951, 2019.
20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Estey EH: Acute myeloid leukemia: 2019 update on risk-stratification and management. Am J Hematol 93: 1267-1291, 2018.
22. Ly C, Arechiga AF, Melo JV, Walsh CM and Ong ST: Bcr-Abl kinase modulates the translation regulators ribosomal protein S6 and 4E-BP1 in chronic myelogenous leukemia cells via the mammalian target of rapamycin. Cancer Res 63: 5716-5722, 2003.
23. Harada H, Andersen JS, Mann M, Terada N and Korsmeyer SJ: p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci USA 98: 9666-9670, 2001.
24. Hua H, Kong Q, Zhang H, Wang J, Luo T and Jiang Y: Targeting mTOR for cancer therapy. J Hematol Oncol 12: 71, 2019.
25. Miklja Z, Yadav VN, Cartaxo RT, Siada R, Thomas CC, Cummings JR, Mullan B, Stallard S, Paul A, Bruzek AK, *et al*: Everolimus improves the efficacy of dasatinib in PDGFRα-driven glioma. J Clin Invest 130: 5313-5325, 2020.
26. Lee JS, Yost SE, Blanchard S, Schmolze D, Yin HH, Pillai R, Robinson K, Tang A, Martinez N, Portnow J, *et al*: Phase I clinical trial of the combination of eribulin and everolimus in patients with metastatic triple-negative breast cancer. Breast Cancer Res 21: 119, 2019.

27. Grignani G, Palmerini E, Ferraresi V, D'Ambrosio L, Bertulli R, Asaftei SD, Tamburini A, Pignochino Y, Sangiolo D, Marchesi E, *et al*: Sorafenib and everolimus for patients with unresectable high-grade osteosarcoma progressing after standard treatment: A non-randomised phase 2 clinical trial. Lancet Oncol 16: 98-107, 2015.
28. Stacchiotti S, Morosi C, Lo Vullo S, Casale A, Palassini E, Frezza AM, Dinoi G, Messina A, Gronchi A, Cavalleri A, *et al*: Imatinib and everolimus in patients with progressing advanced chordoma: A phase 2 clinical study. Cancer 124: 4056-4063, 2018.
29. Kim ST, Lee J, Park SH, Park JO, Park YS, Kang WK and Lim HY: Prospective phase II trial of everolimus in PIK3CA amplification/mutation and/or PTEN loss patients with advanced solid tumors refractory to standard therapy. BMC Cancer 17: 211, 2017.
30. Fruman DA and Rommel C: PI3K and cancer: Lessons, challenges and opportunities. Nat Rev Drug Discov 13: 140-156, 2014.
31. Aylett CH, Sauer E, Imseng S, Boehringer D, Hall MN, Ban N and Maier T: Architecture of human mTOR complex 1. Science 351: 48-52, 2016.
32. Hall CP, Reynolds CP and Kang MH: Modulation of glucocorticoid resistance in pediatric T-cell acute lymphoblastic leukemia by increasing BIM expression with the PI3K/mTOR inhibitor BEZ235. Clin Cancer Res 22: 621-632, 2016.
33. Gazi M, Moharram SA, Marhäll A and Kazi JU: The dual specificity PI3K/mTOR inhibitor PKI-587 displays efficacy against T-cell acute lymphoblastic leukemia (T-ALL). Cancer Lett 392: 9-16, 2017.
34. Chiarini F, Evangelisti C, McCubrey JA and Martelli AM: Current treatment strategies for inhibiting mTOR in cancer. Trends Pharmacol Sci 36: 124-135, 2015.
35. Yu Z, Xie G, Zhou G, Cheng Y, Zhang G, Yao G, Chen Y, Li Y and Zhao G: NVP-BEZ235, a novel dual PI3K-mTOR inhibitor displays anti-glioma activity and reduces chemoresistance to temozolomide in human glioma cells. Cancer Lett 367: 58-68, 2015.
36. Chen D, Lin X, Zhang C, Liu Z, Chen Z, Li Z, Wang J, Li B, Hu Y, Dong B, *et al*: Dual PI3K/mTOR inhibitor BEZ235 as a promising therapeutic strategy against paclitaxel-resistant gastric cancer via targeting PI3K/Akt/mTOR pathway. Cell Death Dis 9: 123, 2018.
37. Zheng JH, Viacava Follis A, Kriwacki RW and Moldoveanu T: Discoveries and controversies in BCL-2 protein-mediated apoptosis. FEBS J 283: 2690-2700, 2016.
38. Mondesire WH, Jian W, Zhang H, Ensor J, Hung MC, Mills GB and Meric-Bernstam F: Targeting mammalian target of rapamycin synergistically enhances chemotherapy-induced cytotoxicity in breast cancer cells. Clin Cancer Res 10: 7031-7042, 2004.
39. Shen YQ, Guerra-Librero A, Fernandez-Gil BI, Florido J, García-López S, Martinez-Ruiz L, Mendivil-Perez M, Soto-Mercado V, Acuña-Castroviejo D, Ortega-Arellano H, *et al*: Combination of melatonin and rapamycin for head and neck cancer therapy: Suppression of AKT/mTOR pathway activation, and activation of mitophagy and apoptosis via mitochondrial function regulation. J Pineal Res 64: Jun 9, 2018 (Epub ahead of print).
40. Hsu PY, Wu VS, Kanaya N, Petrossian K, Hsu HK, Nguyen D, Schmolze D, Kai M, Liu CY, Lu H, *et al*: Dual mTOR Kinases MLN0128 inhibitor sensitizes $HR^+/HER2^+$ breast cancer patient-derived xenografts to trastuzumab or fulvestrant. Clin Cancer Res 24: 395-406, 2018.