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The second-generation ALK inhibitor alectinib effectively induces apoptosis in human neuroblastoma cells and inhibits tumor growth in a TH-*MYCN* transgenic neuroblastoma mouse model

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Abstract

Activating germline mutations of anaplastic lymphoma kinase (ALK) occur in most cases of hereditary neuroblastoma (NB) and the constitutively active kinase activity of ALK promotes cell proliferation and survival in NB. Therefore, ALK kinase is a potential therapeutic target for NB. In this study, we show that the novel ALK inhibitor alectinib effectively suppressed cell proliferation and induces apoptosis in NB cell lines with either wild-type *ALK* or mutated *ALK* (*F1174L* and *D1091N*) by blocking ALK-mediated PI3K/Akt/mTOR signaling. In addition, alectinib enhanced doxorubicin-induced cytotoxicity and apoptosis in NB cells. Furthermore, alectinib induced apoptosis in an orthotopic xenograft NB mouse model. Also, in the TH-*MYCN* transgenic mouse model, alectinib resulted in decreased tumor growth and prolonged survival time. These results indicate that alectinib may be a promising therapeutic agent for the treatment of NB.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

Keywords

Neuroblastoma; ALK inhibitor; alectinib; PI3K/Akt/mTOR; apoptosis

1. Introduction

Neuroblastoma (NB) is the most common childhood extracranial malignant tumor [2, 31]. With current treatments, the outcomes for low- and intermediate-risk NB patients have improved. However, the prognosis for patients with high-risk NB remains dismal. Due to the lack of effective therapies, identification of novel therapeutic targets for high-risk NB is urgently needed [20, 36].

Anaplastic lymphoma kinase (ALK), known as ALK tyrosine kinase receptor or cluster of differentiation 246 (CD246), has been identified as one of the major oncogenes in tumor pathogenesis [8, 11, 12, 16, 21, 26, 51]. In NB, high expression levels of ALK closely correlates with poor outcomes, especially in high-risk NB [22, 42, 49]. It is reported that *ALK* with activating mutations in its tyrosine kinase domain occur in most cases of hereditary NB [17, 33]. In addition, *ALK*-activating point mutations have been identified in approximately 8% of investigated NB tumors [1]. In prior studies, inhibition of ALK led to a significant decrease in cell proliferation in *ALK*-positive cancers, including non-small cell lung cancer (NSCLC) [26, 27, 29], anaplastic large cell lymphoma (ALCL) [6, 34] and NB [15, 44, 50, 52]. ALK-targeted chemotherapies have been shown to downregulate PI3K/Akt signaling, leading to cell apoptosis and tumor regression [4, 15, 51, 52].

Alectinib (CH5424802) is an orally available, highly selective, potent second-generation inhibitor of ALK [13, 43, 46, 53]. It exhibits ten-fold greater potency in kinase assays than the first generation of ALK inhibitor crizotinib [23, 39]. Alectinib is derived from a carbonitrile in physical structure and shows potent inhibitory effects against tumors addicted to ALK activity, such as NSCLC which expresses the EML4-ALK fusion protein [30, 32, 38, 39, 54]. Unlike other ALK inhibitors, the crystal structural analysis of alectinib exhibits only one hinge hydrogen bond with kinase, indicating that this compound may achieve higher selectivity for ALK [39]. In prior studies, alectinib has shown substantial inhibitory effects against tumors with *ALK* mutations, including ALK^{L1196M}, ALK^{L1152R}, ALK^{F1174L}, and ALK^{R1275Q} [28, 35, 40, 48, 55]. Furthermore, chemoresistant cells harboring crizotinib-mediated *ALK* mutations ALK^{F1174L} [10, 47] are sensitive to alectinib. Alectinib demonstrated a favorable safety profile and clinically meaningful response in patients with *ALK*-positive metastatic NSCLC who progressed on crizotinib and, therefore, was granted accelerated approval by the United States Food and Drug Administration (FDA) on December 11, 2015 [25]. Because of the established oncogenic role of ALK in NB [3], and the potent inhibitory efficacy of alectinib against advanced/recurrent *ALK*-mutated tumors, we hypothesize that alectinib is a promising treatment for NB.

In this study, we evaluate the inhibitory effects of alectinib *in vitro* in NB cell lines and *in vivo* in two mouse models of the disease. Our results demonstrated that ALK inhibitor alectinib suppresses ALK-induced PI3K/Akt/mTOR signaling and induces apoptosis in both *ALK* wild-type (WT) and *ALK* mutant NB cells. These results suggest that alectinib is a

potential therapeutic compound for NB patients and should be investigated further in this devastating pediatric malignancy.

2. MATERIALS AND METHODS

2.1 Cell lines

IMR-32, SH-SY5Y and LA-N-6 cell lines were cultured in RPMI 1640 medium (Lonza, Walkersville, MD, USA), 20% (v/v) heat-inactivated Fetal Bovine Serum (FBS) (SAFC Biosciences, Lenexa, KS, USA), and 100 units/mL penicillin/streptomycin. Kelly, NB-19 and SK-N-AS were grown in RPMI 1640 medium (Lonza, Walkersville, MD, USA), 10% (v/v) heat-inactivated FBS (SAFC Biosciences, Lenexa, KS, USA), and 100 units/mL penicillin/streptomycin. All cells were maintained in a humidified incubator at a constant temperature of 37 °C and 5% CO2.

2.2 Antibodies and Reagents

Alectinib (HY-13011) was purchased from MedChem Express (NJ 08852, USA). Doxorubicin (Dox, D1515) and anti-β-Actin antibody (A2228) were purchased from Sigma (Sigma-Aldrich Corp, St. Louis, MO, USA). Anti-PARP (9532 S), anti-Caspase-3 (9662S), anti-phospho-Akt (4060S), anti-Akt (9272), anti-phospho-S6 (4858S), anti-S6 (2217S), anti-Mouse (7076S) and anti-Rabbit (7074S) antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Danvers, MA, USA).

2.3 Cell viability assay

Cell viability experiments were performed with the Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Rockville, MA, USA) according to the manufacturer's instructions. All assays were conducted in replicates of six in 96-well plates. The absorbance at 450 nm of each well was measured by the Synergy™ 4 Hybrid Microplate Reader (Biotek). The IC50 value based on the cell viability data was calculated with Prism 5 (Graphpad Software Inc., La Jolla, CA).

2.4 Flow Cytometry and Propidium Iodide (PI) Staining Assay

Cells were seeded into 6-cm dishes with 1×10^6 cells/dish. Twenty-four hours later, cells were treated with 10 μM alectinib for 0 or 24 hours. Cells were then trypsinized and resuspended in RPMI-1640 medium followed by centrifugation at 450 g for 5 min (4 °C). Cells were then washed with cold 1× PBS with 1% BSA twice, and resuspended at a density of 1×10^6 cells/ml in 1× binding buffer (51-66121E; BD Biosciences, San Jose, CA, USA). Afterwards, 100 μl of cell suspension was transferred into a new tube and stained with 10 μl of 10 μg/mL Propidium Iodide solution (40017; Biotium, Inc.). The cells in the tubes were gently vortexed and incubated for 15 min at RT (25 °C) in the dark. Unstained cells were used as a negative control, and untreated cells were used as a control for treated cells. Then flow cytometry analyses were performed on a LSR-II flow cytometer (BD Biosciences) using BD FACDiva software v.6.0.

2.5 Colony Formation Assay

A 5% (w/v) base agar layer was prepared by adding agar (214220, Difco Laboratories, Detroit, MI, USA) into distilled water and then autoclaving the mixture for 50 min before cooling in a 56 °C water bath. The 2 mL bottom agar layer was made of 0.5% agar and media solution, and added to each well until semi-solid. The 1.5 ml top agar layer was made of 0.3% agar and media solution, and each NB cell line was counted and added to the mixture at 1×10^4 cells/well along with the indicated concentrations of alectinib. Cells were grown at 37 °C for 2 to 3 weeks, and subsequently stained with 500 μL of 5 mg/mL Thiazolyl Blue Tetrazolium Bromide (MTT, M5655, Sigma). After 4 hours, images were captured by the microscope and colonies were counted. Each assay was performed in triplicate.

2.6 Western blot analysis

2×10^6 cells were plated in 60 × 15 mm tissue culture dishes and cultivated in a humidified incubator for 24 h. Cells were then treated and harvested. Pellets were washed twice with ice cold PBS and then lysed on a rotator at 4 °C for 30 min in cooled RIPA buffer (150 mM NaCl, 50 mM Tris-HCl at pH 7.4, 50 mM sodium fluoride, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.1 mM sodium orthovanadate, 10 μg/mL leupeptin, 1% NP-40, 0.25% sodium deoxycholate, and phosphatase inhibitor cocktail 2 and 3 (p5726 and p0044, Sigma)). Lysates were then centrifuged at 13,000 rpm for 15 min, and supernatants were collected. Protein concentration of each sample was quantified by the Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Lysates (100 μg protein) were separated by the SDS-PAGE, and then transferred to PVDF (polyvinylidence fluoride) membranes (BioRad). Membranes were blocked in 5% milk for 1 h at room temperature, and then incubated with the indicated primary antibodies overnight at 4°C. The membranes were then incubated with anti-mouse or anti-rabbit IgG conjugated with horseradish peroxidase at room temperature for 1 h. Chemiluminescent visualization was detected by The ECL-Plus Western detection system (GE Health Care, Buckinghamshire, UK).

2.7 Orthotopic mouse model of NB

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine. Female athymic NCR nude mice were purchased from Taconic (Taconic, Hudson, NY, USA) and maintained under pathogen-free conditions. The preclinical mouse model of NB was established using orthotopic (intrarenal) implantation of the NB cells as described previously [9]. 1.5×10^6 human *luciferase*-transduced NGP cells in 0.1 ml of PBS were surgically injected into the left renal capsule and toward the superior pole of the left kidney of the animal. Several weeks later, mice bearing similar-sized tumors were randomly divided into two groups and treated with either DMSO or alectinib (25 mg/kg, intraperitoneal injection once daily). Three days later, the mice were sacrificed and the tumors were harvested and lysed for immunoblotting.

2.8 TH-*MYCN* transgenic Mouse Model of NB

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Baylor College of Medicine. Homozygous TH-*MYCN* transgenic mice were identified via PCR genotyping. At four weeks of age, these mice were randomly divided into groups and treated with 25 mg/kg alectinib or an equal volume of DMSO every other day by i.p. injection for three weeks. Mice were then sacrificed when they were 7 weeks old or kept until death. The survival time of each group was recorded, and tumor and corresponding kidneys were photographed and weighed. The inhibitory effects of alectinib on the tumor of TH-*MYCN* transgenic mice was conducted as followings: seven-week-old homozygous TH-*MYCN* transgenic mice were treated with 25 mg/kg alectinib or an equal volume of DMSO i.p. once daily for two days. At the end of treatment, the mice were sacrificed and the tumors were harvested. The tumor tissues were then lysed for protein immunoblotting with the indicated antibodies.

2.9 Statistical Analysis

The results were presented as mean ± standard deviation (SD). A two-tailed Student's t-test was used to determine the statistical significance among control and drug treatment groups. $P<0.05$ was considered as statistically significant.

3. Results

3.1 ALK inhibitor alectinib suppresses cell proliferation in both *ALK*-WT and *ALK*-mutant NB cells

First, we hypothesized that treatment of NB cells *in vitro* with the ALK inhibitor alectinib would result in decreases in cell viability. To test this hypothesis, we examined relative cell viability in NB cells treated with alectinib with CCK-8 assays. We selected six NB cell lines, including three *ALK*-WT cell lines (IMR-32, NB-19, and SK-N-AS) and three *ALK*-mutant cell lines (Kelly, SH-SY5Y, and LA-N-6). The results show that cell viability was greatly reduced by alectinib treatment in all six cell lines tested (Figure 1A), and there were morphological changes in the NB cells (Figure 1C). The IC50s of the cell lines tested ranges from 3.181 μM to 9.6 μM. *ALK* mutated cells Kelly and SH-SY5Y were relatively more sensitive to the treatment of alectinib and showed greater inhibition of cell growth. In addition, alectinib showed potent inhibitory effects on *ALK*-WT cells (Figure 1B) with IC50s ranging from 4 to 9.4 μM. These results indicated that alectinib significantly inhibits cell growth in both *ALK*-WT and *ALK*-mutant NB cells.

3.2 ALK inhibitor alectinib inhibits colony formation abilities of NB cells

The ability to form colonies in an anchorage-independent manner in soft agar is regarded as one of the characteristics of cancer cells [7]. To evaluate if alectinib inhibits anchorage-independent colony formation of NB cells, we conducted soft agar assays with the same six NB cell lines, including *ALK*-WT and *ALK*-mutant cells. Our results show that the number of colonies were significantly reduced in all alectinib-treated groups, indicating that alectinib inhibited colony formation in all NB cell lines tested (Figure 2A, 2B).

3.3 Alectinib inhibits ALK-mediated PI3K/Akt/mTOR signaling and induces apoptosis in NB cells

In prior studies of NB, gain-of-function mutations in *ALK* primarily signal through the activation of the PI3K/Akt/mTOR pathways [1]. We aimed to determine if alectinib inhibits the activation of PI3K/Akt/mTOR signaling by blocking ALK in NB. We measured the phosphorylation levels of Akt in six NB cell lines including three N-*myc* amplified cells, NB-19, IMR-32 and Kelly, and three N-*myc* non-amplified cells, SH-SY5Y, LA-N-6 and SK-N-AS. Our results show that alectinib effectively suppresses the phosphorylation levels of Akt at S473 and S6 at S235/236 in all cell lines tested (Figure 3). We observed that the phosphorylation of Akt and S6 was inhibited in Kelly and SH-SY5Y cells (Figure 3), indicating that *ALK*-mutant cell lines are relatively more sensitive to alectinib compared to *ALK*-WT cell lines. In addition, treatment with alectinib greatly induced apoptosis in all NB cells tested, as shown by caspase 3 and PARP cleavages (Figure 3). Consistent with these results, the flow cytometry analysis showed that alectinib could induce cell death in Kelly, NB-19, SH-SY5Y and SK-N-AS cell lines (Supplement 1A). These findings demonstrate that alectinib induced apoptosis by blocking PI3K/Akt/mTOR pathways in both N-*myc* amplified and N-*myc* non-amplified NB cells.

3.4 ALK inhibitor alectinib enhances dox-induced apoptosis in NB

Because of the genomic instability and heterogeneity, cancer cells can rapidly recover from oncogene addiction, resulting in failure to achieve long-lasting efficacy with monotherapies. Therefore, combined cancer therapies were designed and demonstrated better efficacy compared to single agent therapies [18]. Thus, we evaluated the combined effect of alectinib with the traditional chemotherapeutic drug doxorubicin (dox) on cell viability in a panel of six NB cell lines, including *ALK*-WT cell lines, *ALK*-mutant cell lines, and the chemoresistant LA-N-6 cell line. Our results showed that alectinib enhanced dox-induced cytotoxicity in all cell lines tested, including in LA-N-6 cells, which are resistant to dox alone (Figure 4A). In addition, we observed that alectinib greatly increased dox-induced PARP and Caspase 3 cleavages in all cells tested including LA-N-6 (Figure 4B). These results show that alectinib enhances dox-induced apoptosis and even overcomes acquired chemoresistance to dox in NB cells.

3.5 ALK inhibitor alectinib induces apoptosis in xenograft NB mouse model

Having shown the inhibitory effects of alectinib on NB cells *in vitro*, we evaluated the *in vivo* effects of the drug on apoptosis in orthotopic xenograft mouse models of NB. Mice bearing xenograft tumors generated with NGP cells were randomly divided into two groups and treated with either alectinib or dimethylsulfoxide (DMSO) (carrier control) for 3 days at a dose of 25 mg/kg intraperitoneally daily, tumors were harvested and analyzed for PI3K/Akt/mTOR signaling and apoptotic effectors. Our results show that, in contrast to tumors treated with DMSO, tumors treated with alectinib exhibited significantly decreased levels of p-Akt and p-S6. Furthermore, we observed increased PARP and Caspase 3 cleavages in tumors treated with alectinib compared to controls (Figure 5A). These results suggest that alectinb inhibites the PI3K/Akt/mTOR pathways and induces apoptosis in a xenograft mouse model of NB.

3.6 ALK inhibitor alectinib suppresses of tumor growth in the TH-*MYCN* transgenic mouse model

To determine if alectinib could successfully inhibit NB tumor development, we assessed the efficacy of alectinib in the TH-*MYCN* transgenic mouse model, which is the most widely used murine NB model [37]. We observed that treatment with alectinib resulted in decreased tumor growth in TH-*MYCN* transgenic mice when compared with the control animals treated with placebo (Figure 5C, 5D). In addition, alectinib induced PARP and Caspase 3 cleavages in TH-*MYCN* tumor tissue and blocked PI3K/Akt/mTOR signaling as shown by the loss of phosphorylation of Akt at S473 and S6 at S235/236 (Figure 5B). Furthermore, compared with DMSO treatment, alectinib exposure prolonged the survival time of TH-*MYCN* transgenic mice (Figure 5E, Supplement 1B). These data suggest that alectinib inhibits NB tumor growth, as well as induces ALK inhibition-mediated apoptosis and blocks PI3K/Akt/mTOR in the TH-*MYCN* transgenic mouse model.

4. Discussion

Constitutively activated ALK plays an oncogenic role in a variety of tumors, including NSCLC, ALCL, and NB [5, 45]. In NB, ALK-related aberrations are closely linked with poor clinical outcome, especially in high-risk NB [24]. Herein, we evaluated the effects of the ALK inhibitor alectinib in NB. Our results indicate that alectinib shows significant inhibitory activity against NB cells by suppressing cell proliferation and inducing apoptosis through the inhibition of PI3K/Akt/mTOR signaling. In addition, alectinib enhances dox-induced apoptosis and increases sensitivity to dox treatment *in vitro*. Furthermore, treatment of alectinib blocks PI3K/Akt/mTOR pathways and, in turn induces apoptosis in two murine models of NB, a xenograft mouse model and the TH-*MYCN* transgenic model. Most notably, alectinib successfully inhibited tumor development in the TH-*MYCN* transgenic NB mouse model, resulting in slowed or regressed tumor growth as well as prolonged lifespan of the animals. Since we observed potent efficacy of alectinib combined with dox *in vitro*, we hypothesize that combining alectinib with current therapeutic agents would enhance efficacy and improve outcomes.

Inhibition of ALK signaling is an effective therapeutic strategy for cancer. The clinical efficacy of crizotinib, a first generation of ALK inhibitor, has been demonstrated in several cancer types, including *ALK*-mutated NB, *ALK*-rearranged NSCLC and *ALK*-positive ALCL [39]. On August 26, 2011, crizotinib was approved by the FDA for treating *ALK*-rearranged NSCLC, which is regarded as one of the milestones for clinical development of small molecular inhibitors in cancer therapy [14]. However, with prolonged exposure, crizotinib treatment might lead to the development of mutations within the ALK tyrosine kinase domain [41]. In prior studies, crizotinib-induced *ALK* activations have been detected in approximately 10% of cases of NB, most commonly as ALK^{R1275Q} and ALK^{F1174L} [41]. Therefore, to overcome acquired drug resistance generated by treatment with crizotinib, and to effectively treat tumors with crizotinib-induced *ALK* mutations, the second and third generation of ALK inhibitors have been developed.

Based on our experiments, the ALK inhibitor alectinib shows significant inhibitory effects on both *ALK*-WT and *ALK*-mutant NB cells. Importantly, *ALK*-mutant cells Kelly (N-*myc*

amplified, ALK^{F1174L} mutation) and SH-SY5Y (N-*myc* non-amplified, ALK^{F1174L} mutation) were more sensitive to alectinib. These results are encouraging since ALK^{R1275}, ALK^{F1174} and ALK^{F1245} account for more than 85% of *ALK* mutations in NB [19, 52]. In addition, *MYCN* amplification is reported as a crucial factor driving NB tumor development [31]. However, our results show that there were no obvious differences in the effects of alectinib between N-*myc* amplified and N-*myc* non-amplified NB cells. These results suggest that the cytotoxicity and sensitivity of alectinib are dependent more on *ALK* activation status, rather than on the N-*myc* amplification status in NB.

Our findings may have important clinical implications for NB patients who develop acquired resistance for crizotinib. The crizotinib-resistant ALK^{F1174L} and ALK^{R1275Q} mutations that arise *de novo* during crizotinib treatment in NB could result in acquired chemoresistance [10]. Thus alectinib may be very effective treatment option since it induces apoptosis in Kelly and SH-SY5Y, both of which harbor the ALK^{F1174L} mutation. This result indicates that alectinib is able to overcome crizotinib-induced chemoresistance. Our study supports further work evaluating the efficacy of alectinib in NB patients, especially in patients whose tumors have become resistant to crizotinib. The efficacy of alectinib as a single agent therapy or in combination with conventional agents should be further investigated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NB	neuroblastoma
ALK	anaplastic lymphoma kinase
WT	wild type
dox	doxorubicin
DMSO	dimethyl sulfoxide
FDA	Food and Drug Administration
ALCL	anaplastic large cell lymphoma
NSCLC	non-small cell lung cancer

FBS Fetal Bovine Serum

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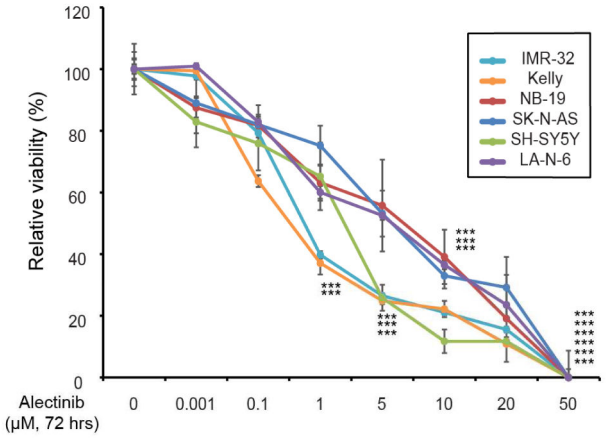
Highlights

- Alectinib treatment strongly induces apoptosis in human neuroblastoma cells.
- Alectinib inhibits neuroblastoma tumorigenesis.
- Alectinib is able to overcome crizotinib-induced chemoresistance.

A



B

IC50 of Alectinib and ALK status in NB cell lines

Cell lines	IC50 (μM)	ALK status
IMR-32	3.995	WT
Kelly	3.181	F1174L
NB-19	9.43	WT
SK-N-AS	7.619	WT
SH-SY5Y	3.841	F1174L
LA-N-6	9.62	D1091N

C

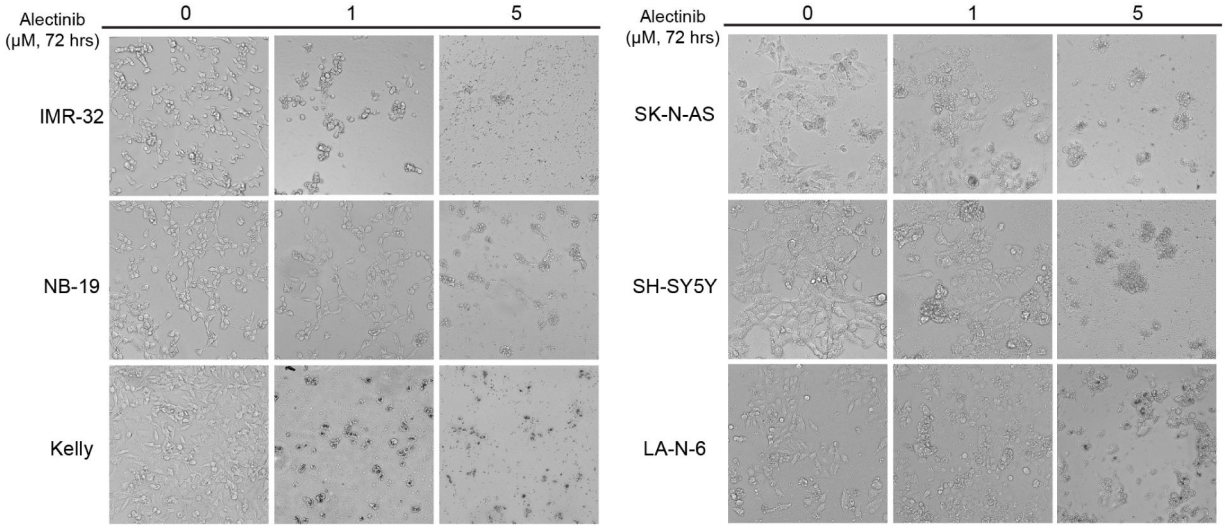


Figure 1.

Alectinib shows anti-proliferation effects on NB cell lines. (**A**) Six NB cell lines were treated with increasing concentrations of alectinib for 72 hours. Cell viability was assessed by CCK-8 assay. Data was represented as % vehicle ± S.D. with $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.001$ (***) indicated. (**B**) The IC50 values of alectinib in each cell line listed were calculated based on the data collected in the cell viability assays in (A). *ALK* status in NB cell lines was also shown. (**C**) Morphological changes of the six different NB cell lines treated with concentrations (0, 1, 5 μM) of alectinib were shown.

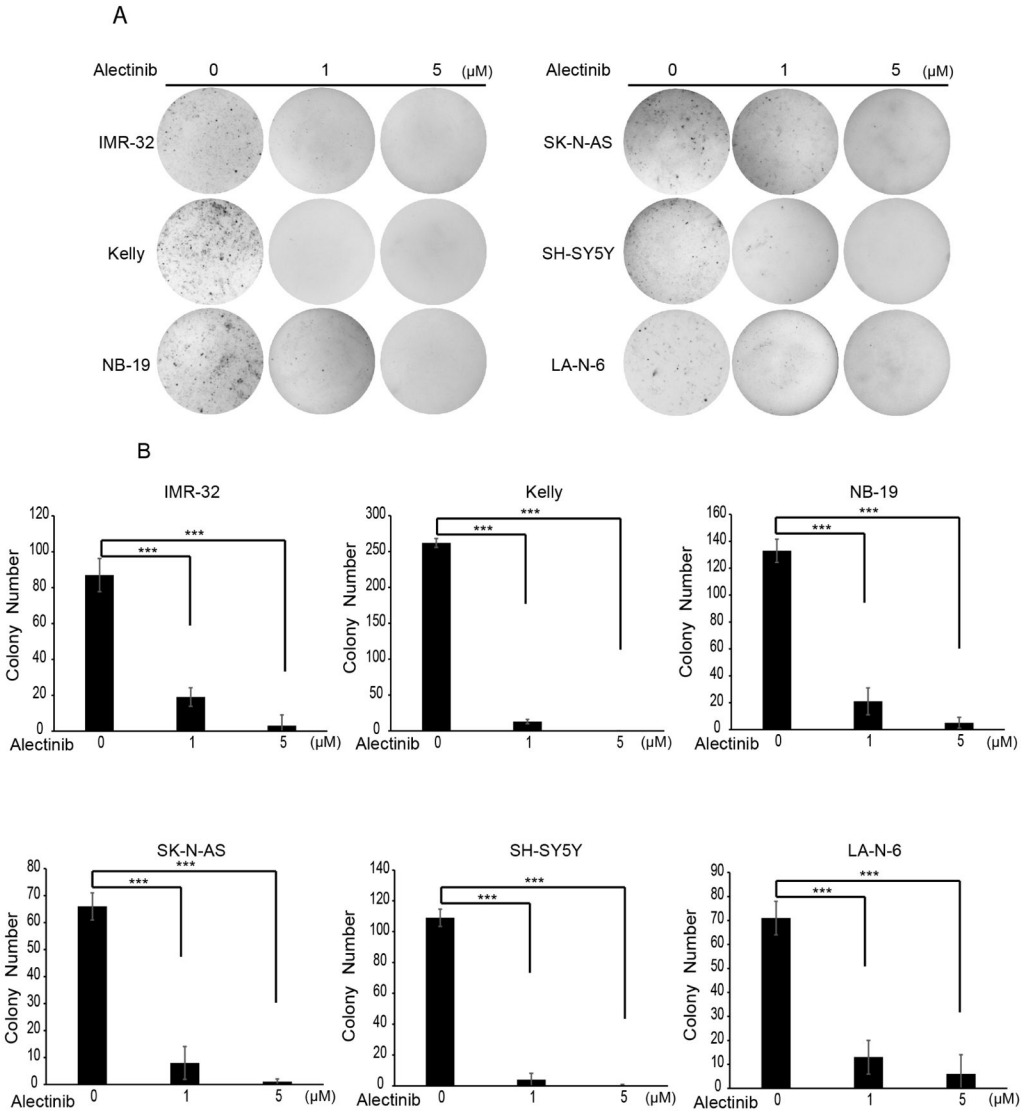


Figure 2.

Alectinib suppresses anchorage-independent growth of NB cells. (**A**) A panel of six NB cell lines were grown on soft agar with 0, 1, or 5 μM of alectinib for 2 to 3 weeks. Cells were stained with crystal violet to visualize colonies. (**B**) Colonies were counted and colony numbers were represented as % vehicle ± S.D. with $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) indicated.

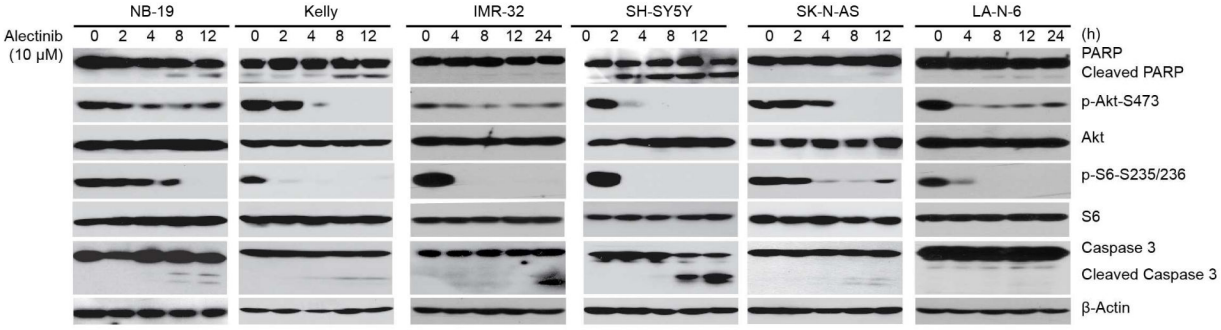


Figure 3.

Alectinib inhibits PI3K/Akt/mTOR signaling and induces apoptosis in NB cells. NB-19, Kelly, IMR-32, SH-SY5Y, SK-N-AS and LA-N-6 cells were treated with 10 μM alectinib for various time points as indicated. The anti-β-Actin antibody was used as a loading control for whole cell extracts.

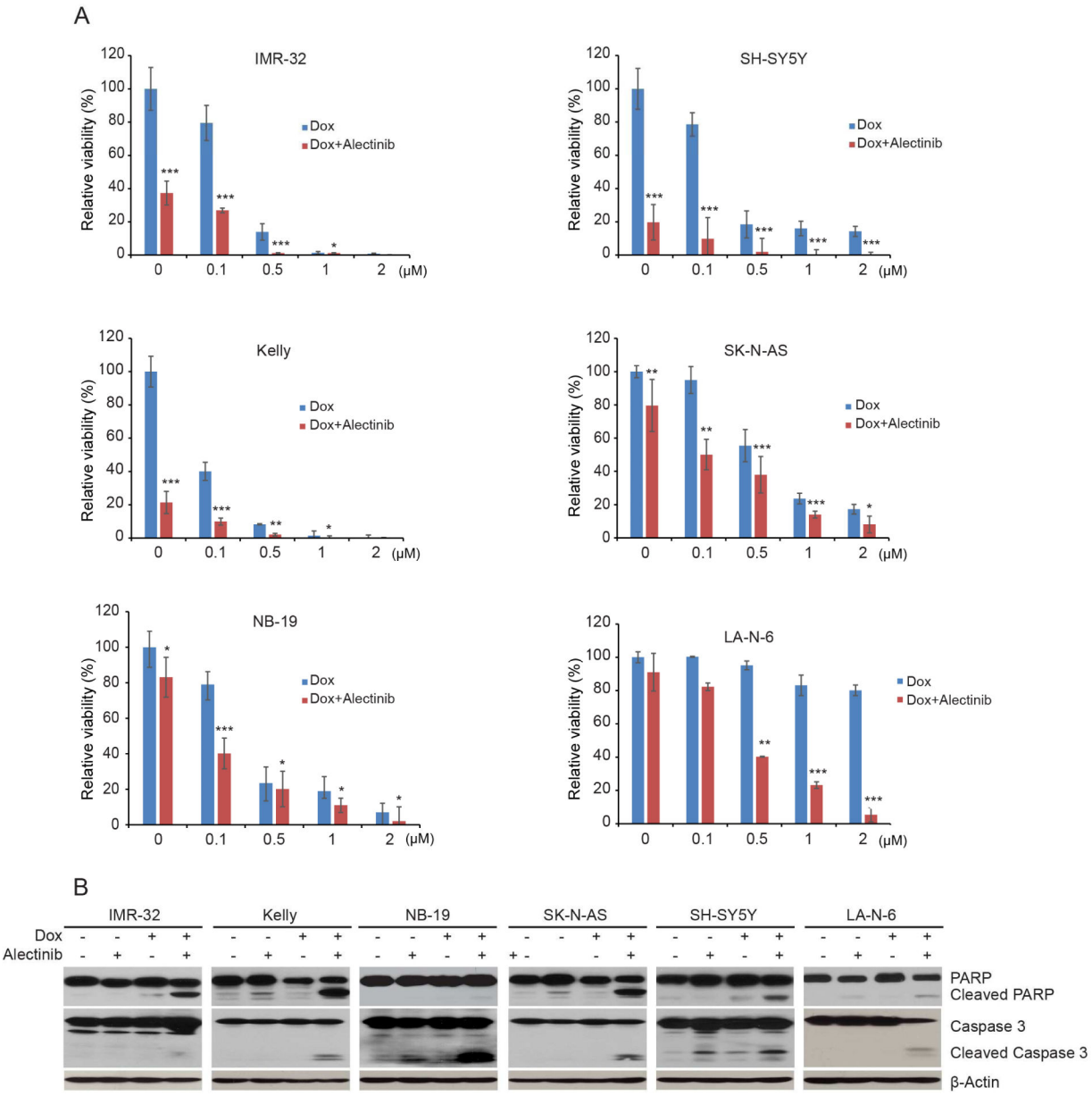


Figure 4.

Alectinib enhances the cytotoxic effect of dox on NB cell lines. (**A**) Six NB cells were seeded and incubated with the indicated concentrations (0, 0.1, 0.5, 1, 2 μM) of dox or 5 μM alectinib for 24 hrs. Cell viability was then measured by CCK-8 assay. Cell viabilities were represented as % vehicle ± S.D. with $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***) as indicated. (**B**) NB-19 and SH-SY5Y cells were treated with either Dox (0.2 μM), alectinib (5 μM), or a combination of both for 12 hrs; IMR-32, Kelly and SK-N-AS cells were treated with either dox (0.5 μM), alectinib (5 μM), or a combination of both for 12 hrs; LA-N-6 cells were treated with either dox (2 μM), alectinib (5 μM), or a combination of both for 12 hrs. PARP and Caspase 3 cleavages were examined.

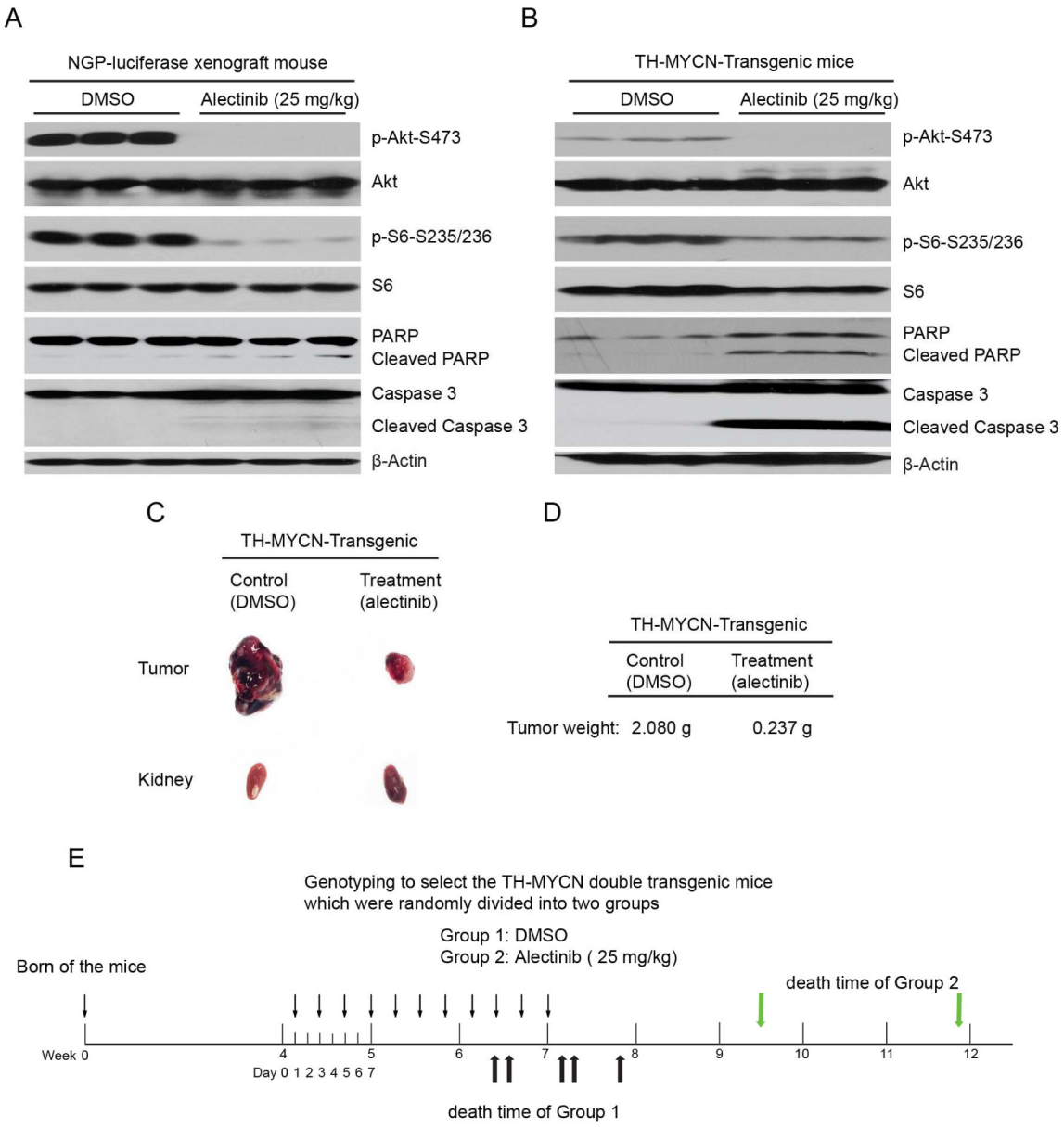


Figure 5.

Alectinib induces apoptosis in an orthotopic mouse model of NB and the TH-*MYCN* transgenic mouse model. (**A–B**) NGP-*luciferase* xenograft mouse bearing tumors (A) and the TH-*MYCN* transgenic mice bearing tumors (B) were treated with DMSO or 25 mg/kg of alectinib daily for 3 days. Then tumors were harvested and analyzed for apoptotic marker and for Akt and S6 phosphorylation. (**C–D**) Four-week-old TH-*MYCN* transgenic mouse were treated with DMSO or 25 mg/kg of alectinib every other day until the end of seven-week-old (11 times). Then mice were sacrificed, tumors and corresponding kidneys in each group were photographed (C) and weighted (D). (**E**) Four-week old TH-*MYCN* transgenic mouse were treated with DMSO or 25 mg/kg of alectinib every other day for another three

weeks (11 times). Mice were kept until natural death, the survival time points of control and treatment groups were recorded.