Abstract. The aberrant activation of PI3K/Akt/mTOR signaling pathway plays an important role in the oncogenesis, prognosis and chemotherapy resistance of neuroblastoma. However, NVP-BEZ235, a potent dual PI3K and mTOR inhibitor have not shown beneficial effects on neuroblastoma especially in terms of apoptosis induction as a single agent. We therefore attempted to explore an effective combination regimen to enhance the anticancer activity of NVP-BEZ235. Interestingly, we found that oridonin, a natural biologically active compound extracted from the Chinese medicinal herb *Rabdiosa rubescens*, combined with NVP-BEZ235 markedly induced apoptosis of neuroblastoma cells. Notably, the synergistic activation of the apoptotic pathway was accompanied with enhanced autophagy as evidenced by significant decreased p62 expression as well as upregulated conversion of LC3-II. Suppression of the Beclin-1, a core component of the autophagy machinery, by means of shRNA resulted in diminished synergistic antitumor effect. Furthermore, the co-treatment with oridonin and NVP-BEZ235 was also much more effective than either agent alone in inhibiting the growth of neuroblastoma xenografts and in inducing tumor cells apoptosis. Taken together, our results suggest that the combination of NVP-BEZ235 and oridonin is a novel and potential strategy for neuroblastoma therapy.

Introduction
Neuroblastoma (NB) is a malignant paediatric tumor of the sympathetic nervous system that presents as a highly heterogeneous disease, ranging from spontaneous regression to high risk of fatality (1-3). Although the overall survival of neuroblastoma has increased greatly with the advances of diagnostic methods and therapeutic treatment over the recent years, the cure rate and life quality for high-risk neuroblastoma have only improved marginally when given aggressive conventional treatment (4,5). Due to issues like early osseous and (or) bone marrow metastasis and minimal residual disease (MRD), the effect of surgical operation in advanced neuroblastoma patients is limited. Therefore, combination chemotherapy, especially the molecular targeted agent-based combination, remains the dominant strategy in the field of neuroblastoma treatment and research.

Numerous pieces of evidence show that activation of PI3K/Akt/mTOR pathway correlates with poor prognosis and chemotherapy resistance in neuroblastoma (6-8). NVP-BEZ235 is a potent novel dual PI3K and mTOR kinase inhibitor which has shown great inhibitory efficacy on colorectal, breast, non-small cell lung carcinoma, renal cancer, and sarcoma (9-13).
This compound as a single agent has also been investigated in neuroblastoma, where it was shown ineffective in MYCN-non amplified neuroblastoma, which led us to speculate that NVP-BEZ235 might be better suited as a part of combination therapy (14). Oridonin is an ent-kaurane diterpenoid extracted from the plant Rabdosia rubescens which has been used as anti-microbial, anti-inflammation and antitumor agent in traditional Chinese medicine for thousands of years (15,16). As regards its antitumor activity, previous studies have demonstrated that oridonin as a supplement may potentiate the therapeutic effects of anticancer drugs gemcitabine and imatinib in the treatment of pancreatic cancer and Ph+ acute lymphoblastic leukemia separately (17,18). However, it is currently unknown whether oridonin can strengthen the effects of NVP-BEZ235 in neuroblastoma treatment.

In this study, we investigated the effect of combined treatment with NVP-BEZ235 and oridonin on the growth of human neuroblastoma cells in culture and in a preclinical mouse model. The data indicated that oridonin significantly enhanced the inhibitory effect of NVP-BEZ235 on neuroblastoma, leading to synergistic cell apoptosis and inhibition of NB xenograft tumor growth.

Materials and methods

Reagent. NVP-BEZ235 was purchased from Novartis (East Hanover, NJ, USA) and dissolved in dimethylsulfoxide (DMSO) to a stock concentration of 1 mmol/l. Oridonin (purity >98%) was purchased from Chengdu Must Bio-Technology Co., Ltd. (Chengdu, Sichuan, China) and dissolved in DMSO to a stock concentration of 10 mmol/l. The stock solutions were wrapped in foil and maintained at -20°C.

Cell lines and cell culture. Neuroblastoma cell lines (SHSY-5Y, SK-N-MC) were obtained from the Shanghai Institutes for Biological Sciences (Shanghai, China) and were cultured in monolayer culture in RPMI-1640 medium (Gibco-BRL, Long Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL) and 1% penicillin-streptomycin (Gibco-BRL), under standard culture conditions (37°C and 5% CO2). Cells in the logarithmic phase of growth were used in all experiments.

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Cell Counting Kit-8 assay. The cell proliferation was assessed by Cell Counting Kit-8 reagent (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). SHSY-5Y and SK-N-MC cells were seeded at a density of 5x10^3 cells/200 µl/well in 96-well culture plates and allowed to settle overnight. The cells were washed and fixed with ice-cold 75% (v/v) ethanol at -20°C for 2 h, then after washing twice with PBS, the cells were suspended with 500 µl PI solution (PI 50 µg/ml and RNase A 100 µg/ml). The samples were analyzed by FACScan flow cytometer (BD, Franklin Lakes, NJ, USA). FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, San Jose, CA, USA) was used to quantify drug-induced cell apoptosis following the manufacturer's guidelines.

Acridine orange/ethidium bromide (AO/EB) fluorescence. Neuroblastoma cells were cultivated in a 24-well plate exposed to NVP-BEZ235 and/or oridonin for 24 h in a humidified incubator (37°C, 5% CO2). After removing the culture solution and washing with PBS, the cells were stained with 200 µl mixture of (100 µg/ml) acridine orange (AO) and (100 µg/ml) ethidium bromide (EB) (Sigma, St. Louis, MO, USA) with the 1:1 AO to EB, and then incubated the plate for 3 min in the incubator. The cells were visualized by a fluorescence microscope (Nikon Eclipse Ti, Japan).

Lentiviral transduction. shRNA plKO.1 lentiviral constructs were obtained from Hanbio (Shanghai, China). Sequences are as follows: Beclin-1 #1, 5'-CCC GTG GAA TGG AAT GAG ATT-3'; Beclin-1 #2, 5'-GCT TGG GTG TTC CCA TTT-3'. Scrambled, 5'-GTG GAC TCT TGA AAG TAC TAT-3'. The plKO.1 vectors and package plasmids (pLP1-gag/pol, pLP2-Rev, pLP-VSVG) (Life Technologies, Carlsbad, CA, USA) were co-transfected into packaging 293T cells using Lipofectamine-3000 (Life Technologies). After 48 h, the viral supernatants were collected and used to infect SH-SY5Y cells. Stable clones expressing the shRNAs were obtained via 5 µg/ml puromycin dihydrochloride (Santa Cruz Biotech Inc.; sc-205821) selection.

Western blot analysis. Cells were lysed with SDS lysis buffer containing phosphatase and protease inhibitor cocktail (Roche, Mannheim, Germany) and the protein concentration was assayed with Bio-Rad Protein Assay kit (Bio-Rad). Cell lysates with equal protein content were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad). After 3 washes, the membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST; pH 7.4) for 1 h at room temperature, followed by incubation overnight at 4°C with primary antibodies to cleaved caspase-3, cleaved PARP, cyclin D1, cyclin E1, p62, LC3B and Beclin-1 (all from Cell Signalning Technology, MA, USA). After washing 3 times, the membranes were incubated for 1 h at room temperature with species-specific HRP-conjugated secondary antibodies (Cell Signalning Technology). Immunoreactive bands were visualized using Immobilon Western chemiluminescent HRP substrate (Millipore Co., Billerica, MA, USA). Each experiment was performed at least 3 times independently. As a loading control, the GAPDH contents in the samples were also immunoblotted using GAPDH horseradish peroxidase-conjugated antibody (Cell Signalning Technology).

Neuroblastoma xenografts and treatments. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University. Five- to six-week old female athymic (nu/nu) mice were...
housed under pathogen-free conditions in micro-isolator cages with laboratory chow and water ad libitum. SHSY-5Y cells at 5x10^6 suspended in 100 µl PBS were injected s.c. into the flank region of nude mice. When tumors were measurable and reached an average volume of 100 mm^3, the mice were randomized into four groups for the following treatments: vehicle control, NVP-BEZ235 (20 mg/kg/day, i.p.), oridonin (10 mg/kg/day; i.p.), and their combination. Tumor volumes were measured using caliper measurements once every 5 days and calculated with the formula \( V = \frac{\pi \times \text{length} \times \text{width} \times \text{height}}{6} \).

Mice were sacrificed after 4 weeks of interventions and tumor tissues were excised and fixed in 10% buffered formalin and embedded in paraffin.

Histopathology and immunohistochemistry. Paraffin-embedded blocks of all tumor samples were cut at 4 µm and each sample was stained with hematoxylin and eosin (H&E) for histologic analysis. For immunohistochemical detection of Ki-67, sections were deparaffinized in xylene and ethanol and pretreated with a citrate buffer solution (0.01 mol/l citric acid and 0.01 mol/l sodium citrate, pH 6.0) in a microwave oven at 750 W for two cycles of 10 min each. Endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide in methanol for 10 min at room temperature. After a blocking step with normal goat serum (Vector, Burlingame, CA, USA), the anti-Ki-67 antibodies (Cell Signaling Technology) were applied overnight in a moist chamber at 4°C. The following day, tissue sections were incubated with a secondary biotinylated anti-rabbit antibody and with an avidin-biotin-peroxidase complex (Vector). The final reaction product was revealed by exposure to 0.03% diaminobenzidine (Sigma) and nuclei were counterstained with hematoxylin. Appropriate negative controls for the immunostaining were prepared by omitting the primary antibody step and substituting it with nonimmune rabbit serum. All samples were analyzed by scoring staining intensity.

TUNEL assay. To observe the degree of apoptosis, TUNEL assay was performed as per the manufacturer's protocol (In Situ Cell Death Detection kit, POD, Roche). All samples were analyzed by scoring staining intensity.

Statistical analyses. Each sample was analyzed in triplicate, and experiments were repeated three times. In all figures, error bars are standard deviations. Statistical analyses were performed by Microsoft Office Excel 2003 (Microsoft, Albuquerque, NM, USA) and Statisca ver. 10 (StatSoft, Tulsa, OK, USA). Differences between mean values were evaluated by the unpaired t-test. Differences were considered statistically significant at P<0.05.

Results

NVP-BEZ235 inhibits proliferation but does not induce apoptosis in NB. To determine the effect of the dual kinase inhibitor NVP-BEZ235 on the proliferation of human neuroblastoma cells, SHSY-5Y and SK-N-MC were treated with varying concentrations of NVP-BEZ235 (0, 200, 500 and 1,000 nM) for 12 and 24 h and then cell viability was measured by CCK-8 assay. As shown in Fig. 1A and B, NVP-BEZ235 causes a time- and dose-dependent inhibition of cell proliferation in both SHSY-5Y and SK-N-MC cells. Based on the above results, we further investigated whether NVP-BEZ235-induced proliferation inhibition is associated with apoptosis. We detected the
apoptotic relative proteins caspase-3 and PARP cleavage in NVP-BEZ235 treated neuroblastoma cells. In contrast with the antiproliferative effects of NVP-BEZ235, it exerted little toxic effect, as indicated by the absence of cleaved caspase-3 and PARP in both treated cell lines (Fig. 1C and D).

NVP-BEZ235 induces G0/G1 cell cycle arrest in NB. As apoptosis could not have accounted for the potent inhibitory effect of NVP-BEZ235 on NB cell growth, we next analyzed the effect of NVP-BEZ235 on cell cycle progression in neuroblastoma cells by flow cytometry using PI staining of DNA content. Exposed to 500 and 1,000 nM NVP-BEZ235 for 24 h, there was a significant increase in the percentage of SHSY-5Y cells in the G0/G1 phase (84.29±1.03% 500 nM, 80.28±2.14% 1,000 nM) compared with the control group (50.83±2.76%) (Fig. 2A). The significant increase of G0/G1 phase cells was also detected in SK-N-MC cells (Fig. 2B). To further confirm NVP-BEZ235-induced G1 cell cycle arrest in neuroblastoma cells, we examined the expression of endogenous cyclins 12 and 24 h after NVP-BEZ235 treatment at 500 and 1,000 nM in SHSY-5Y and SK-N-MC cells. Cyclin D1 and cyclin E1 are well-known G1-phase cyclins, governing the G1→S phase progression and their inhibition results in G1 phase arrest. As shown in Fig. 2C and D, NVP-BEZ235 treatment caused a remarkable reduction in cyclin D1 and cyclin E1 in both cell lines. Based on the above results, we concluded that the dual PI3K/mTOR inhibitor NVP-BEZ235 inhibits NB cell proliferation through the induction of cell cycle arrest but not apoptosis.

Co-treatment with NVPBEZ235 and oridonin induces enhanced antiproliferation and autophagy in NB. It is reported that enhanced autophagy induced by combination treatment can lead to programmed cell death and autophagic cell death has been proven to be effective in cases of solid tumors (19,20). Given that NVP-BEZ235 alone can activate autophagy in neuroblastoma cells (Fig. 3A and B), we selected another autophagy related compound, oridonin, trying to combine with NVP-BEZ235 to improve its therapeutic efficacy. We first investigated the anti-proliferative effect of the combination treatment. The results showed that cell growth in both cell lines was markedly decreased following 24 h NVPBEZ235 and oridonin co-treatment when compared with either single agent alone (Fig. 3C and D). Then, we examined the autophagy activity of SHSY-5Y and SK-N-MC after the combination treatment through immunoblot analysis. As shown in Fig. 3E and F, there was substantially more LC3-II conversion after combination treatment compared with
NVP-BEZ235 or oridonin treatment alone in both cell lines. In addition, co-treatment with NVP-BEZ235 and oridonin led to a significant decrease in the levels of p62 in the tested cells. This indicated that the combination of NVP-BEZ235 and oridonin can synergistically induce enhanced autophagy.

**Co-treatment with NVP-BEZ235 and oridonin induces apoptosis in NB.** In order to examine whether the combination of NVP-BEZ235 and oridonin could synergistically induce apoptosis of NB, the treated neuroblastoma cells were stained with AO/EB. AO was able to infiltrate into the viable cells, and the nuclei were stained a bright green color. For the integrity of the cell plasma membrane, EB was unable to infiltrate into the cells which remained alive or were at early stage of apoptosis, while the late apoptotic cells or dead cells had EB inside and the nuclei were stained a bright red color. Fig. 4A and B show various morphologies of NB cells stained with AO/EB. After 500 nM NVP-BEZ235 treated, the number of viable cells in both cell lines was remarkably decreased in comparison with the control, while the cells cultivated with 2 µM oridonin were similar to the control cells. More notably, the percentage of apoptotic cells was significantly increased in cells treated with the combination of NVP-BEZ235 and oridonin compared with either single agent alone. The results indicated the cell proliferation of NB was strongly inhibited by NVP-BEZ235, but it had very slight cell cytotoxicity compared to the combination groups which commit NBs to apoptosis. For further confirming the results, we examined the cleavage of caspase-3 and PARP in the treated cells. As shown in Fig. 4C and D, when SHSY-5Y and SK-N-MC cells were treated with the combination of NVP-BEZ235 and oridonin for 24 h, there was a significant increase of the cleaved form of caspase-3 and PARP compared with either single agent alone, suggesting that apoptotic pathway was activated in response to the combination treatment.

**Enhanced autophagy is essential for the induction of apoptosis by the co-treatment with NVP-BEZ235 and oridonin.** To determine whether the enhanced autophagy is necessary for cell death induced by the combination treatment, we used shRNA to reduce the expression of Beclin-1 (Atg6), an integral component of the autophagic machinery, and examined whether its loss-of-function impacts on the combination-induced apoptosis. As expected, the expression level of Beclin-1 in SHSY-5Y cells was markedly reduced by shRNA treatment (Fig. 5A). To provide another level of confirmation, we performed flow cytometric analysis of Beclin-1 deficient cells stained with fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) to examine the population level of apoptotic response to the combination treatment. As shown in Fig. 5B, reduction of Beclin-1 protein decreased significantly the fraction of apoptotic cells in response to the co-treatment as quantified by the percentage of both early and late apoptotic cells. ShRNA-mediated knockdown of Beclin-1 led to a reduction of p62 degradation and LC3-II conversion and a significant rescue of cell death with the evidence of...
Figure 4. Effect of NVP-BEZ235 and/or oridonin on apoptosis in neuroblastoma cell lines. (A and B) Morphological changes in SHSY-5Y and SK-N-MC cells treated with NVP-BEZ235 (500 nM) and/or oridonin (2 µM) for 24 h followed by AO/EB staining (magnification, x100). (C and D) Western blot analysis for the expression of cleaved caspase-3 and cleaved PARP in SHSY-5Y and SK-N-MC cells treated with NVP-BEZ235 (500 nM) and/or oridonin (2 µM) for 24 h.

Figure 5. Enhanced autophagy is essential for the induction of apoptosis by the co-treatment with NVP-BEZ235 and oridonin. Western blot analysis for the expression of Beclin-1 in SHSY-5Y cells transfected with Beclin-1 shRNA. (B) Flow cytometric analysis of apoptosis with Annexin V-FITC/PI staining in Beclin-1 deficient cells exposed to the combination of NVP-BEZ235 (500 nM) and oridonin (2 µM) for 24 h. (C) Western blot analysis for the expression of p62, LC3-I/II, cleaved caspase-3 and cleaved PARP in Beclin-1 deficient SHSY-5Y cells treated with the combination of NVP-BEZ235 (500 nM) and oridonin (2 µM) for 24 h.
cleaved caspase-3 and PARP absence in response to the combination treatment of NVP-BEZ235 and oridonin (Fig. 5C). These data strongly suggested that autophagy played a key role in the generation of antineoplastic effects of NVP-BEZ235 co-treatment with oridonin in neuroblastoma cells.

**Co-treatment with NVP-BEZ235 and oridonin induces enhanced antitumor activity in NB xenografts.** We next determined whether the synergism also exists in a preclinical NB xenograft mouse model. We initiated a tumor with subcutaneous injection of SHSY-5Y cells on the flank of nude mice. When tumor size reached ~100 mm³, mice were intraperitoneally injected with vehicle, NVP-BEZ235 (20 mg/kg), oridonin (10 mg/kg), or their combination daily for 4 weeks and tumor sizes were measured every fifth day. As shown in Fig. 6A and B, the combination of NVP-BEZ235 and oridonin had a much more significant effect on suppression of tumor growth compared to any single drug. In addition, no significant changes in body weights or daily activities of tumor-bearing mice were observed after combination treatment, suggesting that the combination of these two drugs was well tolerated.

Furthermore, the proliferation and apoptosis index of tumor tissues were assessed by Ki-67 immunohistochemistry and TUNEL assay (Fig. 6C). Monotherapy with NVP-BEZ235 or oridonin only marginally decreased the proliferation of tumor tissues. While, consistently with the cytostatic effects induced by the combined treatment, proliferation of tumor tissues measured by Ki-67 immunostaining was also markedly reduced in co-treated mice. Quantification of TUNEL staining did not reveal any significant change in apoptosis between vehicle control and NVP-BEZ235 or oridonin monotherapy group. However, there was significantly increased apoptosis of tumor tissues in combination treated mice as compared to vehicle control.

**Discussion**

The current chemotherapy strategies for the treatment of neuroblastoma have focused on intensifying and alternating combinations of cyclophosphamide, doxorubicin, vincristine, cisplatin, and etoposide (21,22). Despite dose-intensive, multiagent induction chemotherapy, multicenter phase 3 trials
suggested that the CR/VGPR rate is still around 50% (23,24). Given that conventional chemotherapeutics are nearing their maximum potential with regards to efficacy and patient tolerance, there is an urgent need to explore more effective and better-tolerated targeted therapy.

In this study, we observed that NVP-BEZ235 as a single agent can effectively block NB cell proliferation through inhibition of cell cycle progression but not activation of apoptosis, as evidenced by significant G1 phase arrest and the absence of caspase-3 cleavage. Consistent with this observation, Manara et al showed that NVP-BEZ235 induced sarcoma stasis, by arresting cells in G1 phase of the cell cycle, without remarkable effects on apoptosis (13). It was postulated that autophagy induced by NVP-BEZ235 was a pro-survival mechanism which rendered tumor cells capable of anti-apoptosis surviving in the kinase inhibitor (25-27).

Autophagy is an evolutionarily conserved mechanism by which cellular material is delivered to lysosomes for degradation and recycling (28,29). Although previous reports suggest that the proper quantity of autophagy promotes malignant cell survival, there is emerging evidence that excessive autophagy modulates the cell death machinery in a positive way. Thus, it is conceivable that the induction of autophagy is context-dependent, and the extent of autophagy dictates the cellular outcome. For instance, there is evidence that autophagy promotes resistance of breast cancer cells to the monoclonal anti-HER2 antibody trastuzumab (30). Similarly, hypoxia-induced autophagy leads to chemoresistance of hepatocellular carcinoma cells (31,32). On the other hand, there is evidence that autophagic cell death is critical for the generation of the effects of As2O3 on acute myelogenous leukemia cells (33) and successful induction of prolonged remissions of acute lymphoblastic leukemia by the combination of vincristine and RAD001 (34), implicating autophagy as a mechanism by which certain antineoplastic agents generate their antitumor activities. Hence, enhancement of NVP-BEZ235 induced autophagy may represent a new chemotherapy strategy for advanced neuroblastoma.

Therefore, oridonin, a natural compound that has been demonstrated to induce cell apoptosis and autophagy in several types of tumor cells, was selected to deal with NB cells together with NVP-BEZ235. The findings indicated that the combined treatment of the two drugs led to programmed death of NB cells in a synergistic manner, dramatically enhancing the mechanism of apoptosis promotion by excessive autophagy we speculated that apoptosis was promoted through autophagic degradation of negative regulators that controls the apoptosis machinery. Alternately, we supposed that apoptosis induced by NVP-BEZ235 failed to reach the threshold to autophagic cell death, but it interrupted tumor intracellular homeostasis and enhanced the chemosensitivity of neuroblastoma cells to oridonin treatment. While the challenge remains to clearly understand the underlying mechanisms by which enhanced autophagy induced the combination of NVP-BEZ235 with oridonin to promote programmed cell death of NB.

These results showed that combination treatment of the two compounds synergize in the induction of NB cell death in vitro and in vivo, thus providing a novel strategy to develop combination therapies for advanced neuroblastoma patients that have failed the currently available therapies.

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