Effects of NVP-BEZ235 on the proliferation, migration, apoptosis and autophagy in HT-29 human colorectal adenocarcinoma cells

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Abstract. The phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) pathway plays a significant role in colorectal adenocarcinoma. NVP-BEZ235 (dactolisib) is a novel dual inhibitor of PI3K/mTOR. The effects of NVP-BEZ235 in human colorectal adenocarcinoma are still unclear. In the present study, we aimed to explore the proliferation, migration, apoptosis and autophagy in HT-29 human colorectal adenocarcinoma cells. HT-29 human colorectal adenocarcinoma cells were treated with NVP-BEZ235 (0, 0.001, 0.01, 0.1, 1 and 3 μM) for 24 and 48 h, respectively. Cells were also treated with NVP-BEZ235 (0.1 μM), DDP (100, 300 and 1,000 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (100, 300 and 1,000 μM) respectively, and cultured for 24 h after treatment. MTT assay was utilized to evaluate the effects of NVP-BEZ235 alone or NVP-BEZ235 combined with *cis*-diamminedichloroplatinum (DDP) on proliferation of HT-29 cells. Cell wound-scratch assay was used detect cell migration. In addition, expression of microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B and LC3B) in HT-29 cells was detected by immunofluorescence at 48 h after NVP-BEZ235 (1 μM) treatment. Expression of proteins involved in cell cycle and proliferation (p-Akt, p-mTOR and cyclin D1), apoptosis (cleaved caspase-3), and autophagy (cleaved LC3B and Beclin-1) were detected by western blot analysis. NVP-BEZ235 inhibited the proliferation and migration of HT-29 human colorectal adenocarcinoma cells. NVP-BEZ235 decreased protein expression of p-Akt, p-mTOR and cyclin D1, and increased protein expression of cleaved caspase-3, cleaved LC3B and Beclin-1 as the concentrations and the incubation time of NVP-BEZ235 increased. In addition, NVP-BEZ235 and DDP had synergic effects in inhibiting cell proliferation and migration. The expression of protein involved in apoptosis (cleaved caspase-3) was higher in drug combination group compared to the NVP-BEZ235 single treatment group. NVP-BEZ235 inhibited the proliferation and migration, and induced apoptosis and autophagy of HT-29 human colorectal adenocarcinoma cells.

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Introduction

The phosphoinositide 3 kinase (PI3K)/Akt/mammalian target of the rapamycin (mTOR) pathway regulates the cell cycle and is associated with cellular proliferation and cancer. The activation of PI3K phosphorylates and activates Akt, which have numerous downstream effects including activating mTOR (1). The overactivation of this pathway stimulates proliferation and reduces apoptosis, involved in the pathogenesis of cancer.

Colorectal adenocarcinoma is the third most common type of cancer globally making up ~10% of all cases (World Cancer Report 2014). PI3K/Akt and mTOR are activated in colorectal adenocarcinoma, and they would be important targets for treatment of colorectal adenocarcinoma (2). However, direct antagonizing mTOR would activate feedback loop of PI3K/Akt/mTOR pathway and affect the anticancer effect (3,4). Hence, a dual inhibition of Akt and mTOR may be more effective to treat colorectal adenocarcinoma. In the present study, we chose NVP-BEZ235, which has dual effects on PI3K and mTOR.

NVP-BEZ235 (dactolisib) is a novel dual inhibitor of PI3K/mTOR. Its effects have been reported in tumors, such as glioma, breast cancer and Burkitt lymphoma (5-7) and Waldenström's macroglobulinemia (8). By targeting PI3K/mTOR, NVP-BEZ235 inhibits the cancer stem cells of prostate cancer, glioblastoma and colorectal adenocarcinoma (9-11). In addition, NVP-BEZ235 reduces cell proliferation in HCT-116 and DLD-1 colorectal adenocarcinoma cells after cytotoxic therapy with two inhibitors of ubiquitin-proteasome system, methyl-13-hydroxy-15-oxokaurenoate (MHOK) and bortezomib (11). However, the effects of NVP-BEZ235 alone are unclear on proliferation, migration, apoptosis and autophagy in HT-29 colorectal adenocarcinoma cells. *Cis*-diamminedichloroplatinum (DDP) binds and cross-links DNA. It is used to treat various types of cancers, such as sarcomas, lung, ovarian and cervical cancer (12-14). Nevertheless, effects of drug combination of NVP-BEZ235 and DDP in human colorectal adenocarcinoma remain elusive.

Tumor cells are hallmarked by unrestricted proliferation. Apoptosis and autophagy are involved in cancer genesis and prevention. Apoptosis is known as programmed cell death, and

autophagy is an intracellular degradation process to eliminate damaged macromolecules and organelles during cellular distress. The excessive self-cannibalistic function may be deleterious and cause cell death (15). However, it is unknown how the dual inhibition of PI3K and mTOR affects apoptosis or autophagy in colorectal adenocarcinoma. The activation of caspase-3 participates in the execution-phase of cell apoptosis (16). Microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3B and LC3B) and Beclin-1 are important proteins associated with autophagy (17). Therefore, we explored the protein expression of cleaved caspase-3, cleaved LC3B and Beclin-1 in order to explore effects of NVP-BEZ235 on apoptosis and autophagy in HT-29 colorectal adenocarcinoma cells. Akt is an essential component in PI3K/Akt/mTOR pathway and is a downstream effector of PI3K (18). mTOR is a serine/threonine kinase that regulates cellular metabolism, growth and proliferation (19). Akt and mTOR could be phosphorylated by their activating kinases. Phosphorylated Akt and mTOR are active and functional molecules that activate downstream signals of PI3K/Akt/mTOR pathway involving in cell cycle and proliferation (18,19). Cyclin D1 is required for the progression of cell cycle through G1 phase (20). Therefore, we explored the protein expression of phospho (p)-Akt, p-mTOR and cyclin D1 in order to explore effects of NVP-BEZ235 and DPP on proliferation and migration in HT-29 colorectal adenocarcinoma cells.

Materials and methods

Cells and reagents

Cells. HT-29 cells (human colorectal adenocarcinoma cell line; Cell Bank of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 supplemented with 100 ml/l fetal bovine serum (FBS), 100 kU/l penicillin and 100 mg/l chloramphenicol in a cell incubator with 5% CO_2 at 37˚C. Cells were subcultured after digestion by 0.25% trypsin, and cell growth was observed under an inverse microscope.

Drug. The powder of NVP-BEZ235 (dactolisib; Selleck Inc., Houston, TX, USA) was dissolved in dimethylformamide (DMF; Sinopharm, Inc., Shanghai, China) in a ratio of 5 mg to 10.6485 ml to make 1 mM storage solution. The powder of DDP (Shanghai Macklin Biochemical, Co., Ltd., Shanghai, China) was dissolved in dimethyl sulfoxide (DMSO). Aliquots were stored at -80˚C.

Main reagents. RPMI-1640 (Gibco, Inc., Grand Island, NY, USA); fetal bovine serum (FBS; Gibco); typsin and antibodies (Gibco); MTT Cell Proliferation and Toxicity kit (Biyuntian Inc., Shanghai, China); TUNEL apoptosis detection kit (Shanghai Yisheng Inc., Shanghai, China); primary antibodies of cleaved LC3B, p-mTOR and GAPDH (Abcam, Inc., Cambridge, MA, USA); primary antibodies of cleaved caspase-3, Beclin-1, cyclin D1 and p-Akt (Cell Signaling Technology, Inc., Danvers, MA, USA); goat anti-rabbit antibody (Invitrogen, Inc., Grand Island, NY, USA); 4% paraformaldehyde (Sigma-Aldrich, Inc., St. Louis, MO, USA); DAPI (Weiao, Inc., Shanghai, China); anti-quenching mounting medium (Sigma-Aldrich); bovine serum albumin (BSA; Amresco, Inc., Dallas, TX, USA).

Main equipments. Fluorescence microscope (Olympus, Inc., Tokyo, Japan); light microscope (Olympus); microplate reader (Shanghai Kehua, Inc., Shanghai, China); table-type refrigerated centrifuge (USTC Zonkia, Inc., Hefei, China); cell incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA); vertical and horizontal electrophoresis system (Liuyi, Inc., Beijing, China); electric thermostatic drying oven (Huyue, Inc., Shangyu, China); upright fluorescence microscope (Nikon, Inc., Tokyo, Japan).

MTT assay. MTT assay was used to detect effects of NVP-BEZ235 alone and combination of NVP-BEZ235 with DDP on proliferation of HT-29 colorectal adenocarcinoma cells. Cells were placed in 96-well plates (100 μl/well), and cultured for 24 h before adding NVP-BEZ235 to make final concentrations of 0, 0.001, 0.01, 0.1, 1 and 3 μM (control group had a concentration of 0 μM; and others were experimental groups; n=6/concentration). Cells were then cultured continuously at an incubator with 5% CO_2 at 37˚C for 24 and 48 h, respectively. Cells were treated with NVP-BEZ235 (0.1 μM), DDP (100, 300 and 1,000 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (100, 300 and 1,000 μM) respectively, and cultured for 24 h after treatment (n=5/group). A total of 20 μl of MTT solutions (5 mg/ml) was added into each well, and continue to culture at 37˚C for 4 h. Supernatant was then discarded and 150 μl DMSO was added into each well, which was shaken for 15 min to dissolve crystals. Absorbance was detect at 570 nm (A570) using ELISA microplate reader, and the inhibition rate of cell proliferation was calculated. Calculation formula: the inhibition rate of cell proliferation (%) = (1 - absorbance of experimental groups/absorbance of the control group) x 100%. The probability sum method was utilized to determine whether there was synergism combining NVP-BEZ235 and DDP (21). Formula: q = EAB/(EA + EB - EA x EB). EAB is the effect (for example, inhibition rate) when drug A and drug B are combined. EA and EB are effects after drug A and drug B treatment, respectively. If q is between 0.85 and 1.15, the effects of drug A and B are additive. Drug A and drug B are synergic if q>1.15, while antagonistic if q<0.85.

Cell wound scratch assay. HT-29 colorectal adenocarcinoma cells in logarithmic growth phase were placed on 6-well plates at a concentration of $2x10^5$/ml and cultured in a humid cell incubator with 5% CO_2 at 37˚C. Three horizontal lines were drawn at the back of 6-well plates using marker pens, and 200 μl tips were utilized to draw horizontal lines at the bottom of plates. The plates were rinsed with phosphate buffer solution (PBS) 3 times to eliminate the cells peeled off during dounding. NVP-BEZ235 was added at concentrations of 0, 0.001, 0.01, 0.1, 1 and 3 μM (control group had a concentration of 0 μM, and others were experimental groups; n=12/concentration). Cells were then cultured in humid incubator with 5% CO_2 at 37˚C for 24 and 48 h, respectively. Moreover, cells were treated with NVP-BEZ235 (0.1 μM), DDP (100, 300 and 1,000 μM) and NVP-BEZ235 (0.1 μM) combined with DDP (100, 300 and 1,000 μM) respectively, and cultured for 24 h after treatment (n=5/group). Images were taken at 0, 24 and 48 h after adding drug. The distance of migration, and migration index in each well were calculated using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). Calculation formula: the inhibition rate of cell migration (%) = (1 - migration distance of experimental groups/migration distance of the control group) x 100%.

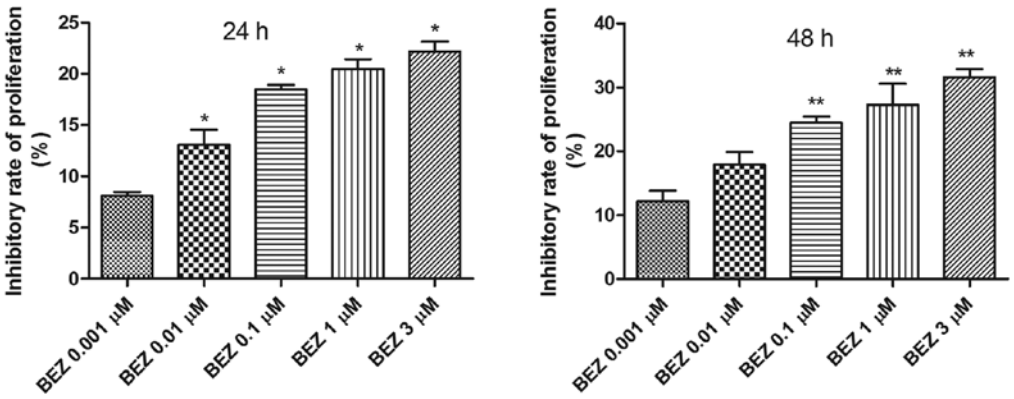

Figure 1. NVP-BEZ235 inhibited the proliferation of HT-29 colon adenocarcinoma cells at 24 and 48 h. MTT assay was used to evaluate effects of NVP-BEZ235 on proliferation of HT-29 cells. Cells were cultured in 96-well plates for 24 h before adding NVP-BEZ235 to make final concentrations of 0, 0.001, 0.01, 0.1, 1 and 3 μM, respectively. Cells were then cultured with 5% CO_2 at 37˚C for another 24 or 48 h. The inhibition rate of cell proliferation was calculated by the formula: inhibition rate of cell proliferation (%) = (1 - absorbance of experimental groups/absorbance of the control group) x 100%. The inhibitory rate of proliferation increased as the concentration of NVP-BEZ235 increased at both 24 and 48 h (mean ± SD, n=3/group). *P<0.05; **P<0.0001 compared to the 0.001 μM NVP-BEZ235 group.

Expression of LC3B detected by cell immunofluorescence. HT-29 cells were cultured in 24-well plates with coverslips at a concentration of $5x10^4$/ml. After culturing for 24 h, NVP-BEZ235 (1 μM) was added, and wells with no drug added were the control group. Cells were cultured in an incubator with 5% CO_2 at 37˚C for another 48 h. Coverslips were fixed with 4% paraformaldehyde and blocked with BSA. Anti-LC3B antibody (1:1,000; Abcam) was incubated at 4˚C overnight. After being washed with PBS, cells were incubated with goat anti-rabbit secondary antibody conjugated with FITC (1:600; OriGene Technologies, Inc., Beijing, China) in the dark at 37˚C for 30 min. Cells were stained with DAPI for 5 min, and slides were mounted with anti-quenching mounting medium. Green fluorescence at 520±20 nm and blue fluorescence at 460 nm from DAPI were detected. DAPI stained both apoptotic and necrotic cells blue.

Western blot analysis. Protein expression of cleaved caspase-3, cleaved LC3B, p-Akt, p-mTOR, cyclin D1 and Beclin-1 were detected by western blot analysis. HT-29 cells were treated with NVP-BEZ235 at different concentrations (0, 1 and 3 μM) for 48 h. HT-29 cells were then treated with 1 μM NVP-BEZ235 for 0, 4 and 24 h, respectively (n=4/group). In addition, cells were treated with NVP-BEZ235 (0.1 μM), DDP (100, 300 and 1,000 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (100, 300 and 1,000 μM) respectively, and cultured for 24 h after treatment. Protein was extracted and separated with 10% SDS-polyacrylamide gel during eletrophoresis at 120 V. Signal was transferred to polyvinylidene fluoride (PVDF) membrane at 100 V for 120 min. After blocking with 5% non-fat milk powder for 1 h, anti-cleaved LC3B antibody (1:1,000; Abcam) were incubated at 4˚C overnight. The membrane was washed with Tris-buffered saline and Tween-20 (TBST) 3 times, 10 min each time. Membrane was then incubated with goat anti-rabbit secondary antibody labeled with horseradish peroxidase (HRP, 1:3,000; Invitrogen) at room temperature for 1 h. After washing the membrane, and incubating briefly with electrochemiluminescence (ECL) solution, film was exposed in a dark room. Experiments were repeated 3 times.

Statistical analysis. Results are demonstrated as mean ± SD. One way analysis of variance (ANOVA) was utilized to compare differences among 3 or more groups, followed by Bonferroni post hoc testing for multiple comparisons. P-values ≤0.05 were regarded significant. Figures and statistical analysis were made by GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

NVP-BEZ235 inhibits proliferation of HT-29 colorectal adenocarcinoma cells. MTT assay was used to evaluate effects of NVP-BEZ235 on proliferation of HT-29 cells. Cells were cultured in 96-well plates for 24 h before adding NVP-BEZ235 to make final concentrations of 0, 0.001, 0.01, 0.1, 1 and 3 μM, respectively. Cells were then cultured at an incubator with 5% CO_2 at 37˚C for another 24 or 48 h. The inhibition rate of cell proliferation was calculated by the formula: inhibition rate of cell proliferation (%) = (1 - absorbance of experimental groups/absorbance of the control group) x 100%. The inhibitory rate of proliferation increased as the concentration of NVP-BEZ235 increased at both 24 h (P<0.0001) and 48 h (P=0.0002; Fig. 1). At 24 h after treatment with NVP-BEZ235, the inhibitory rate of cell proliferation at the concentration of 0.01 μM increased significantly compared to those at 0.001 μM (13.1±2.1 vs. 8.1±0.5%, P<0.05). The inhibitory rates of cell proliferation at the concentration of 0.1, 1 and 3 μM NVP-BEZ235 were 18.5±0.5, 20.4±1.0 and 22.2±1.4%, and they increased markedly compared to the inhibitory rate at 0.001 μM NVP-BEZ235 (P<0.05). Similarly, the inhibitory rate of cell proliferation at the concentration of 0.001 μM NVP-BEZ235 at 48 h after treatment was 12.2±2.4%. The rates increased significantly as the concentration increased to 0.1, 1 and 3 μM (24.5±1.6, 27.3±3.3 and 31.6±2.2%; P<0.05; Fig. 1).

NVP-BEZ235 inhibits the migration of HT-29 colorectal adenocarcinoma cells. HT-29 cells were placed on 6-well plates and cultured with 5% CO_2 at 37˚C until 80% confluence. Three horizontal lines were drawn at the back of 6-well plates using marker pens, and 200 μl tips were used to draw

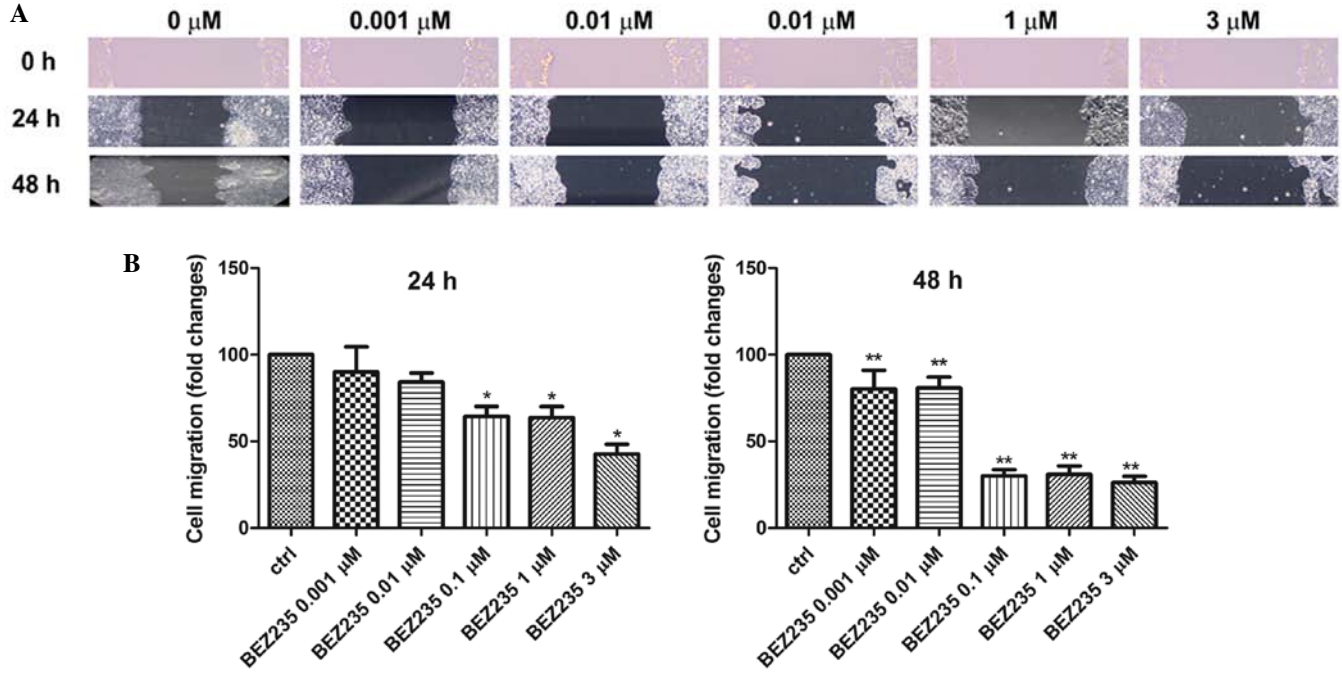

Figure 2. NVP-BEZ235 inhibits the migration of HT-29 colon adenocarcinoma cells. HT-29 cells were cultured in 6-well plates with 5% CO_2 at 37˚C until 80% confluence. Cell wound-scratch assay was performed. NVP-BEZ235 was added to make final concentrations of 0, 0.001, 0.01, 0.1, 1 and 3 μM, respectively. Cells were then cultured for another 24 or 48 h. (A) Representative images are show of the distance of cell migration, images were taken at 0, 24 and 48 h after adding the drug. (B) The fold change of cell migration after NVP-BEZ235 treatment. The distance of migration, and migration index in each well were calculated by Image-Pro Plus 6.0 software. The distance of cell migration decreased as the concentration of NVP-BEZ235 increased at both 24 and 48 h (mean ± SD, n=6/group). *P<0.05; **P<0.0001. Ctrl, control.

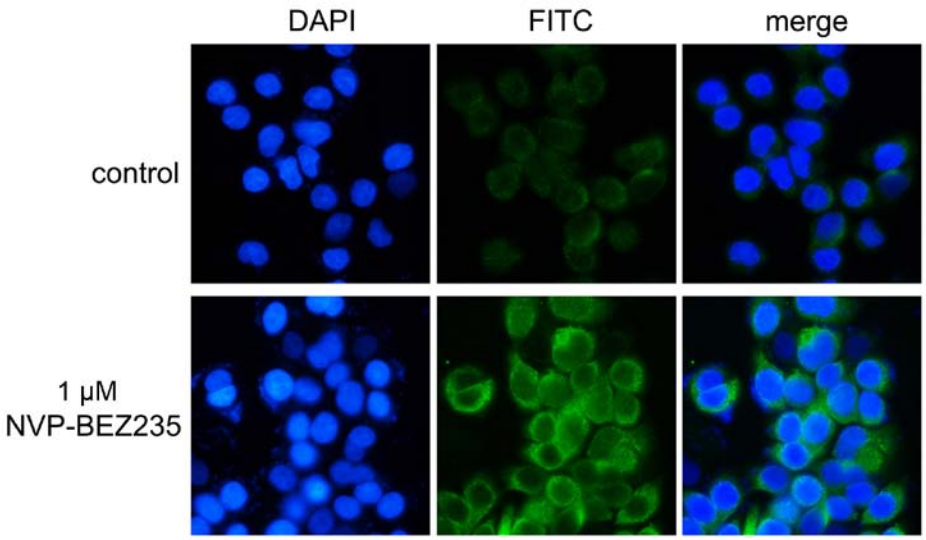

Figure 3. NVP-BEZ235 increases the expression of LC3B in HT-29 colon adenocarcinoma cells. HT-29 cells were cultured in 24-well plates with coverslips. NVP-BEZ235 (1 μM) was added 24 h later, and wells with no drug added were the control group. Cells were cultured in an incubator with 5% CO_2 at 37˚C for another 48 h. Coverslips were fixed, blocked and incubated with anti-LC3B antibody, goat anti-rabbit secondary antibody and DAPI according to the instructions described in Materials and methods. Green fluorescence indicates the signaling of LC3B and blue fluorescence indicates nuclei. NVP-BEZ235 increased the expression of LC3B in HT-29 ells. DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.

horizontal lines at the bottom of plates. NVP-BEZ235 were added at concentrations of 0, 0.001, 0.01, 0.1, 1 and 3 μM, respectively. Cells were then cultured for another 24 or 48 h. Images were taken at 0, 24 and 48 h after adding the drug. The distance of migration and migration index in each well were calculated by Image-Pro Plus 6.0 software. The distance of cell migration decreased as the concentration of NVP-BEZ235 increased at both 24 h (P<0.0001) and 48 h (P<0.0001; Fig. 2). The cell migration at the concentration of 0.1, 1 and 3 μM NVP-BEZ235 was 64.5±13.9, 63.7±15.6 and 42.7±14.0% of the control group at 24 h, and they decreased markedly compared to the control group (P<0.05; Fig. 2). In addition, the fold change of cell migration at the concentration of 0.001, 0.01, 0.1, 1 and 3 μM NVP-BEZ235 was 80.4±10.7, 80.8±15.7,

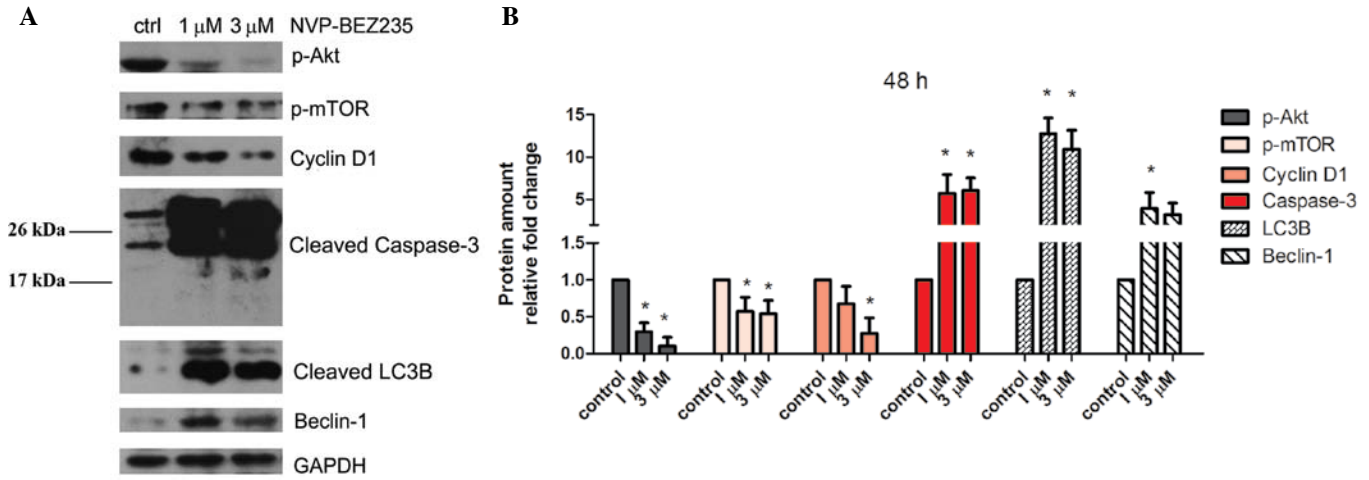

Figure 4. Protein expression of p-Akt, p-mTOR, cyclin D1, cleaved caspase-3, cleaved LC3B and Beclin-1 in HT-29 colon adenocarcinoma cells after treatment with NVP-BEZ235 at different concentrations. HT-29 cells were treated with NVP-BEZ235 at different concentrations (0, 1 and 3 μM) for 48 h. (A) Western blot results demonstrated protein expression of cleaved caspase-3, cleaved LC3B, p-Akt, p-mTOR, cyclin D1 and Beclin-1 after treatment with 0, 1 and 3 μM NVP-BEZ235. (B) Quantification of relative fold change of blot density compared to control group (mean ± SD, n=4/group). NVP-BEZ235 decreased protein expression of p-Akt, p-mTOR, and cyclin D1 and increased protein expression of cleaved caspase-3, cleaved LC3B and Beclin-1 at 48 h as the concentrations of NVP-BEZ235 increased. *P<0.05. Ctrl, control.

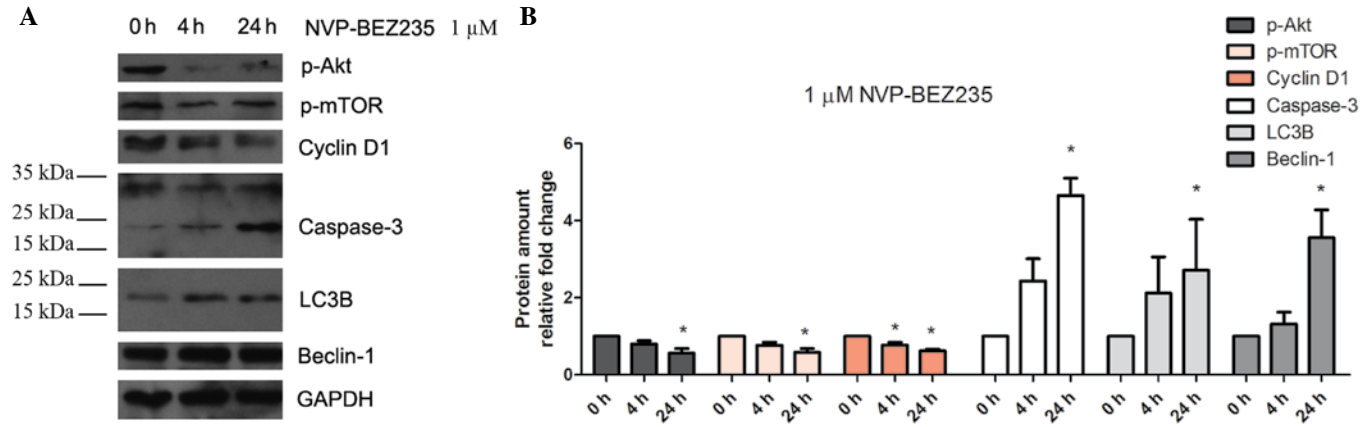

Figure 5. Protein expression of p-Akt, p-mTOR, cyclin D1, cleaved caspase-3, cleaved LC3B and Beclin-1 in HT-29 colon adenocarcinoma cells after treatment with NVP-BEZ235 at different time-points. HT-29 cells were treated with 1 μM NVP-BEZ235 for 0, 4 and 24 h. (A) Western blot results demonstrated protein expression of cleaved caspase-3, cleaved LC3B, p-Akt, p-mTOR, cyclin D1 and Beclin-1 after treatment with 1 μM NVP-BEZ235 for 0, 4 and 24 h. (B) Quantification of relative fold change of blot density compared to control group (mean ± SD, n=3/group). NVP-BEZ235 decreased protein expression of p-Akt, p-mTOR, and cyclin D1, and increased protein expression of cleaved caspase-3, cleaved LC3B and Beclin-1 as the incubation time of 1 μM NVP-BEZ235 increased. *P<0.05. Ctrl, control.

30.1±9.0, 31.0±11.8 and 26.2±8.8% of the control group at 48 h, and they decreased significantly compared to the control group (P<0.05; Fig. 2).

NVP-BEZ235 increases the expression of LC3B in HT-29 colorectal adenocarcinoma cells. The culture of HT-29 cells in 24-well plates with coverslips. After culturing for 24 h, NVP-BEZ235 (1 μM) was added, and wells with no drug added were the control group. Cells were cultured in an incubator with 5% CO_2 at 37˚C for another 48 h. Coverslips were fixed, blocked and incubated with anti-LC3B antibody, goat anti-rabbit secondary antibody and DAPI according to the instructions in Materials and methods. Green fluorescence indicates the signaling of LC3B and blue fluorescence signifies the nuclei. NVP-BEZ235 increased the expression of LC3B in HT-29 cells as shown in Fig. 3.

Protein expression of p-Akt, p-mTOR, cyclin D1, cleaved caspase-3, cleaved LC3B and Beclin-1 in HT-29 colorectal adenocarcinoma cells after being treated by NVP-BEZ235. HT-29 cells were treated with NVP-BEZ235 at different concentrations (0, 1 and 3 μM) for 48 h, and HT-29 cells treated with 1 μM NVP-BEZ235 for 0, 4 and 24 h. Protein expression of p-Akt, p-mTOR, cyclin D1, cleaved caspase-3, cleaved LC3B and Beclin-1 were detected by western blot analysis. NVP-BEZ235 decreased protein expression of p-Akt (P<0.0001), p-mTOR (P=0.0105), and cyclin D1 (P=0.0012; Fig. 4), and increased protein expression of cleaved caspase-3 (P=0.0033), cleaved LC3B (P<0.0001), and Beclin-1 (P=0.0318; Fig. 4) at 48 h as the concentrations of NVP-BEZ235 increased. In addition, NVP-BEZ235 decreased protein expression of p-Akt (P=0.0292), p-mTOR (P=0.0189), and cyclin D1 (P=0.0044; Fig. 5), and increased protein

Table I. Synergic effects of NVP-BEZ235 and DDP on cell proliferation at 24 h after treatment.

	BEZ235 0.1 μM	DDP 100 μM	DDP 300 μM	DDP 1,000 μM	B+D 100 μM	B+D 300 μM	B+D 1,000 μM
EA (BEZ235 0.1 μM)	0.215551						
EB (DDP μM)		0.079619	0.164428	0.631103			
EAB (B+D)					0.358899	0.596705	0.659406
q[EAB/(EA+EB-EA*EB)]					1.290965[a]	1.731903[a]	0.927933

Data of inhibitory rates of cell proliferation are presented as mean values. The probability sum method was utilized to determine effects of drug combination. Formula: q = EAB/(EA + EB - EA x EB). EAB is the effect when drug A and B are combined. EA and EB are effects after drug A and drug B treatment, respectively. The effects of drug A and B are additive if q is between 0.85 and 1.15. Drug A and B are synergic if q>1.15, while antagonistic if q<0.85. [a]NVP-BEZ235 and DDP had synergic effects (q>1.15). B, BEZ235: NVP-BEZ235. D, DDP, *cis*-diamminedichloroplatinum.

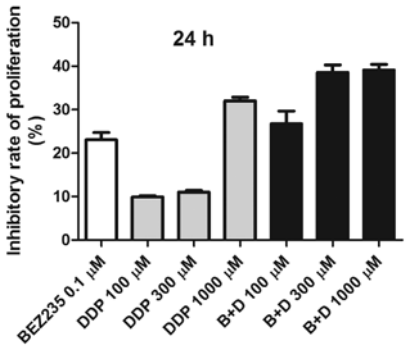

Figure 6. Synergic effects of NVP-BEZ235 and DDP on cell proliferation. Cells were treated with NVP-BEZ235 (0.1 μM), DDP (100, 300 and 1,000 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (100, 300 and 1,000 μM), respectively, and cultured for 24 h after treatment. MTT assay was used to detect cell proliferation and migration. NVP-BEZ235 and DDP had synergic effects in inhibition of cell proliferation when the concentration of DPP was 100 and 300 mM (Table I). B, BEZ235: NVP-BEZ235. D, DDP: *cis*-diamminedichloroplatinum.

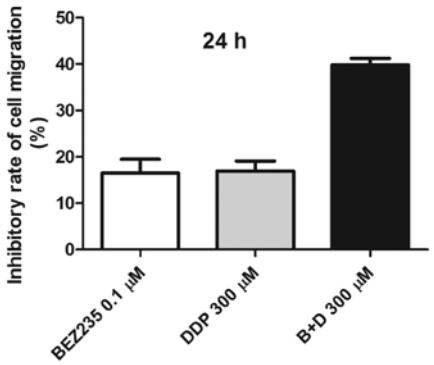

Figure 7. Synergic effects of NVP-BEZ235 and DDP on cell migration. Cells were treated with NVP-BEZ235 (0.1 μM), DDP (300 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (300 μM), respectively, and cultured for 24 h after treatment. Wound scratch assay were used to detect cell proliferation and migration. NVP-BEZ235 and DDP had synergic effects in inhibition of cell migration (Table II). B, BEZ235: NVP-BEZ235. D, DDP: *cis*-diamminedichloroplatinum.

expression of cleaved caspase-3 (P=0.0026), cleaved LC3B (P=0.0161), and Beclin-1 (P=0.0141; Fig. 5) as the incubation time of 1 μM NVP-BEZ235 increased.

Table II. Synergic effects of NVP-BEZ235 and DDP on cell migration at 24 h after treatment.

	BEZ235 1 μM	DDP 300 μM	B+D 300 μM
EA (BEZ235 1 μM)	0.165		
EB (DDP μM)		0.169	
EAB (B+D)			0.398
q[EAB/(EA+EB-EA*EB)]			1.30[a]

Inhibitory rates of cell migration are presented as mean values. The probability sum method was utilized to determine effects of drug combination. [a]NVP-BEZ235 and DDP had synergic effects (q>1.15). B, BEZ235: NVP-BEZ235. D, DDP: *cis*-diamminedichloroplatinum.

Synergic effects of NVP-BEZ235 and DDP in cell proliferation, migration and apoptosis. Cells were cultured in 96-well plates for 24 h before adding NVP-BEZ235 (0.1 μM), DDP (100, 300 and 1,000 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (100, 300 and 1,000 μM), respectively. Cells were then cultured for another 24 h. MTT assay was utilized to detect cell proliferation. We revealed that NVP-BEZ235 and DDP had synergic effects in inhibition of cell proliferation when the concentration of DDP was 100 and 300 mM (Fig. 6 and Table I). Then, we chose 300 mM DDP to combine with NVP-BEZ235, and detected cell migration and expression of proteins involved in cell proliferation and apoptosis by cell wound scratch assay and western blot analysis, respectively. NVP-BEZ235 and DDP had synergic effects in inhibition of cell migration (Fig. 7 and Table II). Western blot results demonstrated that the expression of proteins associated with cell cycle and proliferation (p-Akt and cyclin D1) decreased in drug combination group compared to the single treatment groups. The expression of protein involved in apoptosis (cleaved caspase-3) was higher in drug combination group compared to the NVP-BEZ235 single treatment group (Fig. 8).

Discussion

We have demonstrated that NVP-BEZ235 inhibited the proliferation and migration, and induced apoptosis and autophagy of

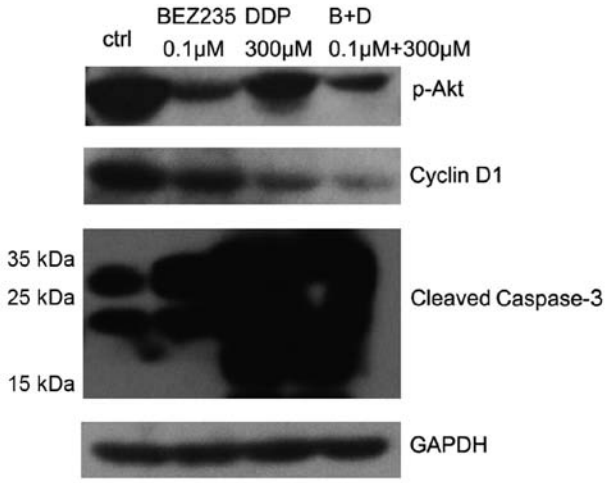

Figure 8. Protein expression of p-Akt, cyclin D1 and cleaved caspase-3 in HT-29 colon adenocarcinoma cells after treatment with NVP-BEZ235 and DDP. Cells were treated with NVP-BEZ235 (0.1 μM), DDP (300 μM), and NVP-BEZ235 (0.1 μM) combined with DDP (300 μM), respectively. Cells without treatment served as control. Cells were then cultured for 24 h after treatment. Western blot analysis was utilized to examine the expression of proteins involved in cell proliferation and apoptosis. The expression of proteins associated with cell cycle and proliferation (p-Akt and cyclin D1) decreased in drug combination group compared to the single treatment groups. The expression of proteins involved in apoptosis (cleaved caspase-3) is higher in drug combination group compared to the NVP-BEZ235 single treatment group. B, BEZ235: NVP-BEZ235. D, DDP: cis-diamminedichloroplatinum.

HT-29 human colorectal adenocarcinoma cells. NVP-BEZ235 decreased the protein expression of p-Akt, p-mTOR and cyclin D1, and increased cleaved caspase-3, cleaved LC3B and Beclin-1 in colorectal adenocarcinoma cells in a dose- and time-dependent manner. In addition, NVP-BEZ235 and DDP displayed synergic effects in cell proliferation, migration and apoptosis at certain concentrations of DDP.

The PI3K/Akt/mTOR pathway is an important intracellular signaling pathway that associated with cellular quiescence, proliferation and cancer. The activation of PI3K and Akt could activate CREB and mTOR, inhibit p27 and localize FOXO in the cytoplasm (1,22). In contrast, many factors inhibit the pathway, such as PTEN, GSK3B and HB9 (1,23,24). In addition, the PI3K/Akt/mTOR pathway promotes growth and proliferation of adult stem cells, especially neural stem cells (1). The pathway is also a necessary component in neural long-term potentiation (25). Colorectal adenocarcinoma is one of the most common cancers worldwide with high mortality. The PI3K/Akt/mTOR pathway induces cell growth and tumor proliferation, and plays a significant role in colorectal adenocarcinoma.

NVP-BEZ235 is a novel and orally available dual inhibitor of PI3K/mTOR. It displays antitumor effects in tumors such as glioma, breast cancer and Burkitt lymphoma (5-7). NVP-BEZ235 was also reported to be effective in treating Waldenström's macroglobulinemia (8). The PI3K/Akt pathway plays an important role in prostate cancer progenitors and NVP-BEZ235 inhibits the cancer stem-like cells of prostate cancer (9,26). NVP-BEZ235 inhibits the cancer stem cells of glioblastoma (10,27). Inhibition of the PI3K/Akt/mTOR pathway by NVP-BEZ235 also suppresses the proliferation of colorectal adenocarcinoma stem cells with reduced stemness indicated by expressions of CD133 and Lgr5 (28). NVP-BEZ235 was found to reduce cell proliferation and induce apoptosis in 3-D cultured HCT-116 and DLD-1 colorectal adenocarcinoma cells after cytotoxic therapy with two inhibitors of ubiquitin-proteasome system, MHOK and bortezomib (11). DDP was the first member of platinum-containing anticancer drugs. The platinum complexes react *in vivo*, bind to and cause DNA crosslinking, ultimately triggering cell apoptosis (29). However, it is unclear how NVP-BEZ235 alone affects proliferation, migration, apoptosis and autophagy in HT-29 human colorectal adenocarcinoma cells, and what the anticancer effects would be if NVP-BEZ235 and DDP are combined in treating colorectal cancer.

We revealed that NVP-BEZ235 inhibited proliferation and migration, and induced apoptosis of human colorectal adenocarcinoma cells in the present study. This can be explained by the dose- and time-dependent decrease in protein expression of p-Akt, p-mTOR and cyclin D1, and increase in cleaved caspase-3 after NVP-BEZ235 treatment as shown in the study. A number of factors enhance the PI3K/Akt pathway, such as insulin-like growth factor (IGF-1) (1), insulin (19), sonic hedgehog homolog (SHH) and epidermal growth factor (EGF) (20). The overactivation of PI3K/Akt/mTOR pathway reduces apoptosis and stimulates proliferation, which involves in the pathogenesis of cancer. Rapamycin, an mTOR inhibitor, induces apoptosis of human osteosarcoma MG63 cells (30). Quinazolinone chalcone derivative inhibits the PI3K/Akt/mTOR signaling pathway and induces mitochondrial-dependent apoptosis in human colorectal adenocarcinoma HCT-116 cells (31). We revealed for the first time that NVP-BEZ235, a dual inhibitor of PI3K and mTOR, inhibited proliferation and induced apoptosis of HT-29 human colorectal adenocarcinoma cells.

Akt is an essential component in PI3K/Akt/mTOR pathway, and is a downstream effector of PI3K. Once correctly positioned at the membrane via binding of PIP3, Akt can be phosphorylated by its activating kinases and mTOR complex 2. Activated Akt can then go on to activate or deactivate its numerous substrates via its kinase activity, such as mTOR (18). mTOR is a serine/threonine kinase, and it belongs to PI3K-related kinases family. It regulates cellular metabolism, growth and proliferation, and is a target for a number of mTOR inhibitors, including NVP-BEZ235 (19). Cyclin D is a member of cyclin protein family involving in regulating cell cycle progression. Cyclin D1 is required for the progression of cell cycle through G1 phase (20). During the G1 phase, it is rapidly synthesized and accumulates in the nucleus, and is then degraded when the cell enters S phase. Caspase-3 is a key member of the cysteine-aspartic acid protease family, and sequential activation of caspases plays a central role in the execution-phase of cell apoptosis (16). Therefore, NVP-BEZ235, a dual inhibitor of PI3K and mTOR, inhibited proliferation and migration, and induced apoptosis of human colorectal adenocarcinoma cells by downregulating p-Akt, p-mTOR and cyclin D1 and upregulating cleaved caspase-3.

We also demonstrated that NVP-BEZ235 increased protein expression of autophagy markers in human colorectal adenocarcinoma cells in a dose- and time-dependent manner. Autophagy is an intracellular degradation process for

eliminating damaged organelles and macromolecules during enhanced cellular distress. However, the self-cannibalistic function may be deleterious and lead to cell death (15). The effectiveness of combination of a selective inhibitor of epidermal growth factor receptor (EGFR) and a poly(ADP-ribose) polymerase (PARP) inhibitor has been shown to depend on the enhanced autophagy in ovarian cancer A2780 xenografts (32). Moreover, cabergoline-mediated prolactinoma shrinkage is associated with the inhibition of mTOR pathway and the induced autophagy-dependent cell death (33). Oroxylin A also induces autophagy in human malignant glioma cells via inhibiting the activation of Akt and Erk, and the phosphorylation of mTOR and STAT3 (34). We have shown for the first time that PI3K/mTOR dual inhibitor NVP-BEZ235 enhanced autophagy in human colorectal adenocarcinoma cells.

LC3B and Beclin-1 are essential components in the process of autophagy. LC3B is a subunit of neuronal microtubule-associated proteins 1A and 1B. LC3B activates extrinsic apoptosis during cigarette smoking-induced emphysema through interactions with Caveolin-1 and Fas (35). LC3B-II and Beclin-1 were reported to be increased during reperfusion in fibrillated mouse hearts (36). Beclin-1 affects every major step in autophagic pathways, from autophagosome formation to the maturation of autophagosome/endosome (15). Many of these effects are mediated through the activation of specific Beclin 1-binding proteins, including autophagic inducers and autophagic inhibitors. Cofactors, such as Bif-1, high mobility group box (HMGB) 1 and survivin, promote formation of Beclin 1-Vps34-Vps15 core complexes and induce autophagy, whereas the BH3 domain of Beclin-1 is inhibited by Bcl-2 or Bcl-XL (15). Therefore, the enhanced autophagy after NVP-BEZ235 treatment may be deleterious to colorectal cancer, and is another explanation of the decreased proliferation and migration of colorectal cancer cells after NVP-BEZ235 treatment.

DPP cross-links DNA and interferes with cell division by mitosis. Most notable changes in DNA damage are the 1,2-intrastrand cross-links with purine bases. Apoptosis induced by DDP in human colon cancer cells depends on the mitochondrial serine-protease Omi/Htra2 (37). The damaged DNA elicits DNA repair mechanisms, and it activates apoptosis if repair is impossible. In the present study, we unveiled that NVP-BEZ235 and DDP had synergic effects in inducing apoptosis in human colorectal adenocarcinoma cells. Apoptosis was greatly induced after the drug combination. It is possible that the greatly increased apoptosis and DNA changes after NVP-BEZ235 treatment exceeds cellular DNA repair mechanisms. The harmful DNA material and molecules released during NVP-BEZ235-induced apoptosis may subsequently activate apoptosis of more colorectal adenocarcinoma cells. This may result in a viscous loop, aggravating the apoptotic-inducing effects. In addition, we unveiled that NVP-BEZ235 and DDP had synergic effects in inhibiting cell proliferation and migration in colorectal adenocarcinoma cells. It was reported that DPP affected mitosis by interacting with cellular proteins, particularly HMG domain proteins (38). The synergic effects of NVP-BEZ235 and DDP in inducing apoptosis may also cause synergy in inhibiting the proliferation and migration of tumor cells. Our research findings provide rationales in combining NVP-BEZ235 and DDP during chemotherapy treating colorectal cancer, in order to enhance the anticancer effects.

In conclusion, we have demonstrated novel data suggesting that NVP-BEZ235, a dual inhibitor of PI3K/mTOR, inhibited the proliferation and migration, and induced apoptosis and autophagy of HT-29 human colorectal adenocarcinoma cells. The anticancer effects of NVP-BEZ235 are enhanced after being combined with DDP in treating colorectal adenocarcinoma. Although future research is needed to shed light on more underlying cellular and molecular mechanisms, NVP-BEZ235 may serve as a promising therapy for colorectal cancer.

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