The combination of NVP-BEZ235 and rapamycin regulates nasopharyngeal carcinoma cell viability and apoptosis via the PI3K/AKT/mTOR pathway

HUI LUO[1*], YU-YU YU[1*], HONG-MEI CHEN[2], WEI WU[1], YONG LI[1] and HONG LIN[1]

[1]Department of Oncology, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006; [2]Department of Oncology, The First Affiliated Hospital of Gannan Medical University, Ganzhou, Jiangxi 341000, P.R. China

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Abstract. Nasopharyngeal carcinoma (NPC) is a rare malignancy with a remarkable geographical distribution. Regarding NPC treatment, improving the survival rate of advanced patients seems promising. Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway deregulation is closely associated with tumorigenesis. In the present study, the NPC cell line SUNE1 was divided into four groups: Control, NVP-BEZ235, rapamycin, and NVP-BEZ235+rapamycin. SUNE1 cells in the NVP-BEZ235 group were incubated with NVP-BEZ235; cells in the rapamycin group were incubated with rapamycin, whereas the NVP-BEZ235+rapamycin group refers to SUNE1 cells incubated with a mixture of NVP-BEZ235 and rapamycin. The control group was treated with the same amount of vehicle. Morphological, MTT, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling and flow cytometry assays demonstrated that NVP-BEZ235 and rapamycin caused morphological changes, inhibited cell viability and induced cellular apoptosis. In addition, reverse transcription-quantitative polymerase chain reaction and western blot revealed that the combination of NVP-BEZ235 and rapamycin affected the activation of the PI3K/AKT/mTOR pathway. The combination of NVP-BEZ235 and rapamycin significantly improved the effect of the drug therapy. The potential underlying mechanism may comprise the joint effects of inhibiting cell viability, promoting cellular apoptosis and reducing relative signal protein expression levels in SUNE1 cells. These findings provided novel evidence that NVP-BEZ235 suppresses NPC development, and indicated a promising potential application of combination drug therapy (NVP-BEZ235+rapamycin) for the clinical treatment of NPC.

Correspondence to: Dr Hong Lin, Department of Oncology, The First Affiliated Hospital of Nanchang University, 17 Yongwaizheng Street, Nanchang, Jiangxi 330006, P.R. China
E-mail: linhong973@163.com

*Contributed equally

Abbreviations: NPC, nasopharyngeal carcinoma; PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; FACS, fluorescence-activated cell sorting

Key words: nasopharyngeal carcinoma, NVP-BEZ235, rapamycin, cell viability, cell apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive and metastatic head and neck cancer (1). NPC is associated with many factors, including Epstein-Barr virus infection, genetic and environmental factors (2). Although radiotherapy is effective for early-stage tumors, patients with advanced NPC succumb to skull base and neck lymph node invasion and metastasis (3). It is reported that the median survival of patients with metastatic or advanced NPC is only 5-11 months (4). The poor prognosis is mainly due to high recurrence and metastasis rates (5). Consequently, there is an urgent requirement to explore new therapies against NPC.

Phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling is commonly associated with tumorigenesis (6). During the development of tumors, PI3K pathway deregulation commonly occurs via the inactivation of the tumor suppressor phosphatase and tensin homolog (7), and mTOR is activated and associated with cell growth, proliferation, differentiation and apoptosis (8). Thus, the PI3K/AKT/mTOR pathway provides a promising target for cancer therapy. NVP-BEZ235 is an imidazo [4,5-c]quinoline derivative and suppresses the activities of PI3K and mTOR (9). Previous reports have demonstrated that NVP-BEZ235 exerts an anti-cancer function in breast cancer (10), ovarian cancer (11) and prostate cancer (12). Although NVP-BEZ235 has been used in patients with advanced NPC for phase I/II clinical trials, the outcomes of these trials have been unsatisfactory (13).

Rapamycin, as an mTOR signaling inhibitor, reduces cell proliferation in NPC (1). The main aim of the present study was to investigate the role of the combination of NVP-BEZ235 and rapamycin on NPC cell viability, cellular apoptosis and PI3K/AKT/mTOR signaling. The results indicated that both

NVP-BEZ235 and rapamycin caused morphological changes, inhibited cell viability, induced cellular apoptosis and affected the activation of the PI3K/AKT/mTOR pathway. The combination of NVP-BEZ235 and rapamycin significantly improved the effects of the drug therapy. Therefore, the present study identified potential novel anti-NPC drugs with more efficiency and less toxicity.

Materials and methods

Cell culture. The NPC cell line SUNE1 was obtained from Wuxi Innovate Biomedical Technology Co., Ltd. (Wuxi, China). SUNE1 cells were incubated with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C. SUNE1 cells were seeded into a 24-well plate at the density of 80%, and then were incubated with 100 nM NVP-BEZ235 (cat. no. A506167) (14), 100 nM rapamycin (cat. no. A606203; both Sangon Biotech Co., Ltd., Shanghai, China) (15), or 100 nM NVP-BEZ235 and 100 nM Rapamycin for 48 h at 37˚C. The control group was administered the same amount of dimethyl sulphoxide (DMSO; Sangon Biotech Co., Ltd.). Cell morphology following treatment was observed with an inverted phase contrast microscope (Olympus Corporation, Tokyo, Japan; magnification, x100).

Cellular apoptosis detected by terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay and flow cytometry. SUNE1 cells from the four groups were fixed with 4% paraformldehyde at room temperature for 5 min, washed with PBS and incubated with 50 mL TUNEL detection solution (2 ml terminal deoxynucleotidyl-transferase enzyme and digoxigenin-dUTP reaction buffer) at 37˚C for 1 h. Following washing with PBS, SUNE1 cells were stained with DAPI (1 mg/ml) in PBS for 10 min at room temperature. A total of 5 non-overlapping fields were captured with a fluorescence inverted microscope (Bio-Rad Laboratories, Inc., Hercules, CA, USA; magnification, x100).

For the flow cytometry assay, SUNE1 cells from the four groups were washed with PBS twice, and incubated with 400 ml 1X binding buffer and 5 ml fluorescein isothiocyanate-labeled Annexin-V from the Annexin V-FITC/PI kit (cat. no. APOAF-20TST; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 15 min in the dark at room temperature. Then, SUNE1 cells were incubated with 10 ml propidium iodide for 5 min in the dark at room temperature. Cellular apoptosis was detected with fluorescence-activated cell sorting (FACS) using FCSExpress version 3.0 (De Novo Software, Glendale, CA, USA) in a flow cytometer (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Cell viability examined by MTT assay and flow cytometry. SUNE1 cells in the exponential growth phase were collected and seeded in a 96-well plate at $5x10^3$ cells/well in 100 ml culture medium. Cells were incubated with NVP-BEZ235 and/or rapamycin at final concentrations of 100 nM for 48 h at 37˚C, in the groups detailed above. Following incubation, the medium was removed and 100 ml fresh medium containing 10% MTT (5 mg/ml) was added into each well. Following 4 h of incubation at 37˚C, DMSO (200 ml) was added to cells for 10 min and the absorbance was measured at 570 nm using a multifunctional microplate reader (Thermo Fisher Scientific, Inc.). To calculate the relative cell viability, 6-well replication was used. Data were analyzed in three independent samples.

SUNE1 cells from the four groups were digested with trypsin and fixed with 70% ethanol overnight at 4˚C. The following morning, SUNE1 cells were washed with PBS and subsequently stained with propidium iodide (50 mg/ml) and RNase A (100 mg/ml; cat. no. B300068; Sangon Biotech Co., Ltd.) for 1 h in the dark. The cell cycle was analyzed FACS using FCSExpress software in BD FACSCalibur flow cytometer.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from SUNE1 cells using TRIzon Reagent (Beijing ComWin Biotech, Co., Ltd., Beijing, China). The quality and quantity of RNA was examined with a NanoDrop-2000 ultramicrospectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA) at wavelengths of 260 and 280 nm. Subsequently, 1 mg RNA was reverse transcribed into cDNA using the SuperRT cDNA Synthesis kit (Beijing ComWin Biotech, Co., Ltd.) according to the manufacturer's protocol, and qPCR was carried out with SYBR Green qPCR SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.) in CFX96™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc.). The thermocycling conditions were as follows: 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 sec and 60˚C for 30 sec, and a final extension at 72˚C for 5 min. The relative expression of genes was normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method (16). The primers for RT-qPCR were as follows: GAPDH forward, 5'-TGACTT CAACAGCGACACCCA-3' and reverse, 5'-CACCCTGTT GCTGTAGCCAAA-3'; PI3K forward, 5'-GCCCAGGCT TACTACAGAC-3' and reverse, 5'-AAGTAGGGAGGCATC TCG-3'; AKT forward, 5'-CTCATTCCAGACCCACGAC-3' and reverse, 5'-ACAGCCCGAAGTCCGTTA-3'; and mTOR forward, 5'-CTGGGACTCAAATGTGTGCAGT-3' and reverse, 5'-GAACAATAGGGTGAATGATCCGGG-3'.

Western blotting. Proteins were extracted using radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and centrifuged at 12,000 x g at 4˚C for 20 min. The concentration of protein was detected with a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Subsequently, 30-50 mg protein was subjected to SDS-PAGE on a 10% gel and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 h, the membranes were probed with the following mouse monoclonal antibodies: Anti-PI3K (ab86714; 1:1,000), anti-AKT (ab175354; 1:1,000), anti-phosphorylated (p)-AKT (ab105731; 1:500), anti-mTOR (ab87540; 1:1,000), anti-GAPDH (AB8245; 1:1,000) and rabbit monoclonal anti-p-mTOR (ab109268; 1:1,000; all Abcam, Cambridge, MA, USA) at 4˚C overnight. The membranes were incubated with the rabbit anti-mouse IgG (ab6728; 1:10,000) or goat anti-rabbit IgG (ab205718; 1:10,000) horseradish peroxidase-labeled secondary antibodies (both Abcam) at room temperature for 1 h. Following washing with TBST, the blot was examined with electrochemiluminescence

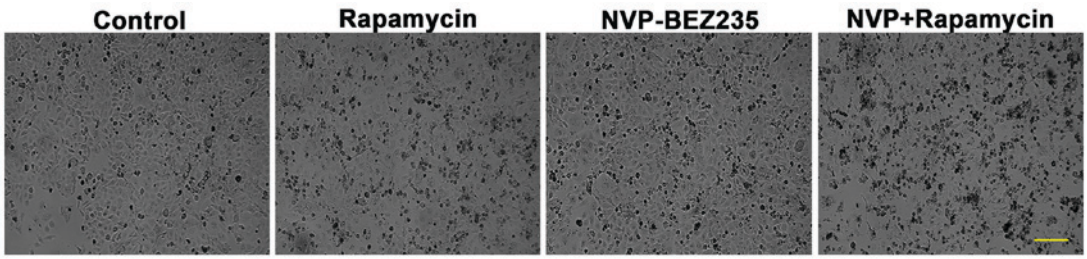


Figure 1. NVP-BEZ235 and rapamycin affected cellular morphological changes. SUNE1 cells were placed into a 24-well plate and cultured with 100 nM NVP-BEZ235, 100 nM rapamycin, or 100 nM NVP-BEZ235 and 100 nM rapamycin. The control group was administered the same amount of vehicle. Following 48 h, cells were collected for morphological analysis by inverted phase contrast microscopy. Compared with the control group, the addition of NVP-BEZ235 and rapamycin inhibited the growth of SUNE1. Scale bar, 100 μm. NVP, NVP-BEZ235.

solution (Pierce; Thermo Fisher Scientific, Inc.). The relative expression was normalized to GAPDH using Quantity One software version 4.2 (Bio-Rad Laboratories, Inc.).

Statistical analysis. Statistical analysis was performed with SPSS 17.0 (SPSS, Inc., Chicago, IL, USA). All experiments were carried out at least three times. Data are expressed as the mean ± standard deviation, and differences were analyzed using one-way analysis of variance with Tukey's post-hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Rapamycin and NVP-BEZ235 promote cellular apoptosis. As presented in Fig. 1, cells in the control group possessed clear cell contours, boundaries and round cellular nuclei, with good cell growth status. However, once SUNE1 cells were incubated with NVP-BEZ235 or rapamycin, they exhibited shrinkage and cell size reduction. Furthermore, more marked morphological changes were observed in NVP-BEZ235+rapamycin induced cells. These data suggested that the above drugs inhibited the growth of SUNE1 cells.

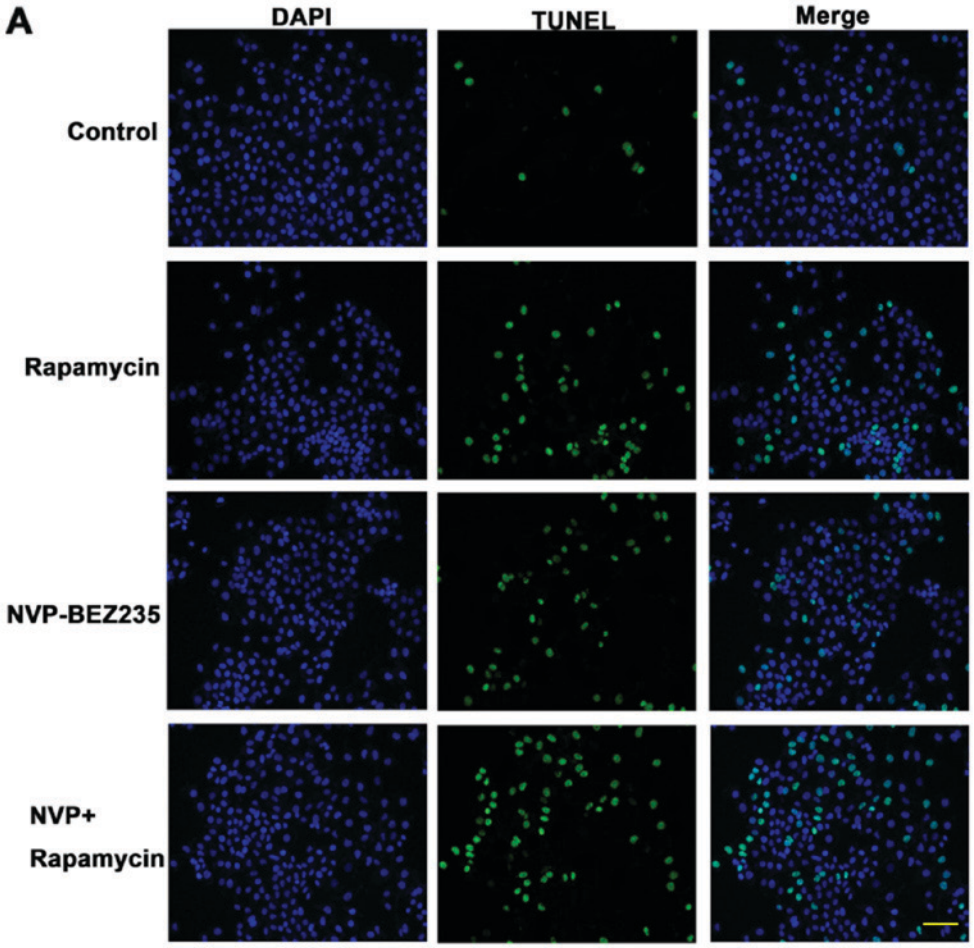
To further examine the effect of NVP-BEZ235 and rapamycin on SUNE1 cellular apoptosis, TUNEL and flow cytometry assays were performed. As presented in Fig. 2A, very few apoptotic cells were observed in the control group. However, compared with the control group, the number of TUNEL-positive cells in the NVP-BEZ235 or rapamycin groups was markedly increased, and the number of apoptotic cells in NVP-BEZ235+rapamycin was the greatest. To further validate the results, flow cytometry was performed. As presented in Fig. 2B and C, the trend in the number of early apoptotic cells (the fourth quadrant) and the late apoptotic cells (the second quadrant) was as follows: NVP-BEZ235+rapamycin group > rapamycin group > NVP-BEZ235 group > control group. These data were in accordance with the TUNEL assay results. These results suggested that NVP-BEZ235 and rapamycin induced SUNE1 cellular apoptosis, and the combination enhanced this promoting effect.

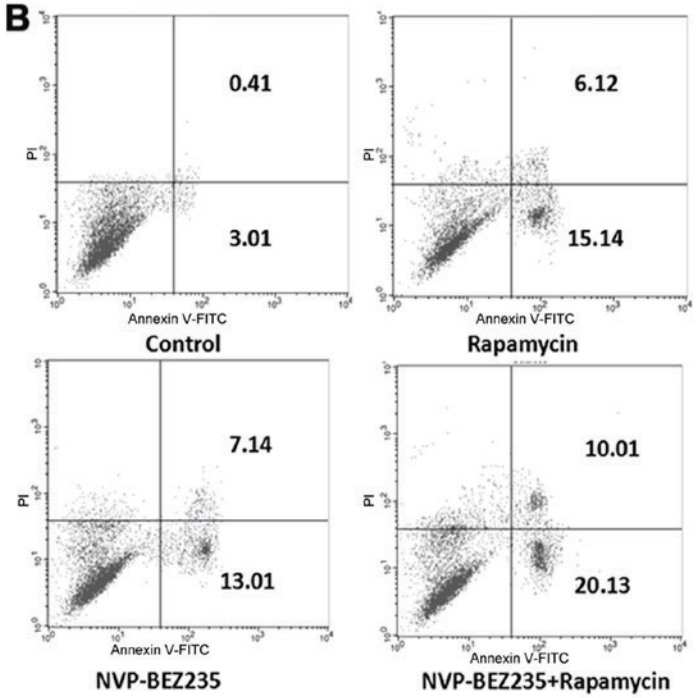
Rapamycin and NVP-BEZ235 suppress cell viability. To further explore the role of rapamycin and NVP-BEZ235 on cell viability, MTT and cell cycle distribution assays were performed following the different drugs treatments (Fig. 3). As presented in Fig. 3A, compared with the control group, the cell viability in the NVP-BEZ235 group (66.76±2.16%) and the rapamycin group (63.92±2.52%) was significantly decreased compared with the control group ($P<0.01$). In addition, NVP-BEZ235+rapamycin cell survival (38.15±1.94%) was markedly decreased compared with NVP-BEZ235 or rapamycin alone. In Fig. 3B, compared with the control group, the addition of NVP-BEZ235 or rapamycin markedly enhanced the percentage of cells at the G1 phase. The percentage of cells at G1 phage was greatest in the NVP-BEZ235+rapamycin group. These data indicated that NVP-BEZ235 and rapamycin arrested the cell cycle at the G1 phase, and that the drug combination heightened the intensity of the effect.

Rapamycin and NVP-BEZ235 suppress the activation of PI3K/AKT/mTOR pathway. To explore the mechanism by which NVP-BEZ235 and rapamycin affect cellular apoptosis and viability, the expression of PI3K, AKT and mTOR were evaluated (Fig. 4). As presented in Fig. 4A-C, compared with the control group, NVP-BEZ235 significantly suppressed the mRNA levels of PI3K, AKT and mTOR ($P<0.01$); rapamycin only significantly decreased the expression of mTOR ($P<0.01$), but had no significant effect on the mRNA levels of PI3K and AKT ($P>0.05$). The combination of NVP-BEZ235 and rapamycin exerted more marked inhibition on the mRNA levels of PI3K, AKT and mTOR compared with the control group ($P<0.01$). Similarly, the protein levels of PI3K, AKT, p-AKT, mTOR and p-mTOR were detected following the different drugs treatments. As presented in Fig. 4D-K, it was demonstrated that rapamycin suppressed the protein level of p-AKT, mTOR, p-mTOR, p-AKT/AKT, and p-mTOR/mTOR compared with the control group ($P<0.01$), but had no significant effect on the expression of AKT and PI3K ($P>0.05$). However, NVP-BEZ235 alone significantly repressed the protein levels of PI3K, AKT, p-AKT, mTOR, p-mTOR, p-AKT/AKT and p-mTOR/mTOR compared with the control group ($P<0.01$). NVP-BEZ235+rapamycin induced more marked inhibition of PI3K, AKT, p-AKT, mTOR, p-mTOR, p-AKT/AKT and p-mTOR/mTOR. Together, these findings suggested that rapamycin and NVP-BEZ235 inhibited the activation of the PI3K/AKT/mTOR pathway, and the combination of the two drugs promoted the inhibition.

Discussion

Despite the progress that has been made in researching NPC, the molecular mechanism underlying NPC remains elusive.





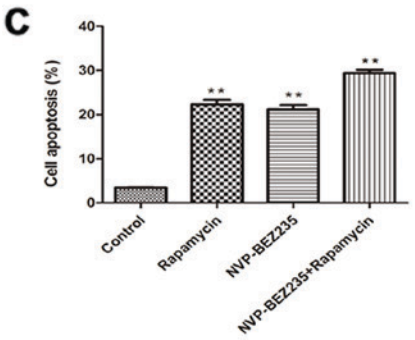


Figure 2. NVP-BEZ235 and rapamycin promoted cellular apoptosis. SUNE1 cells were cultured with 100 nM NVP-BEZ235, 100 nM rapamycin, or 100 nM NVP-BEZ235 and 100 nM rapamycin. The control group was administered the same amount of vehicle. Following 48 h, cells were collected for further experiments. (A) NVP-BEZ235 and rapamycin induced cellular apoptosis, as detected by TUNEL. TUNEL-positive cells were marked green, and nuclei were stained with DAPI in blue. Scale bar, 100 μm. (B) Cellular apoptosis was detected via flow cytometry. The fourth and second quadrants indicated the early apoptotic cells and the late apoptotic cells, respectively. (C) The percentage of apoptotic cells from different groups is presented. Few apoptotic cells were observed in the control group. The addition of NVP-BEZ235 and rapamycin increased the number of apoptotic cells, and NVP-BEZ235+rapamycin enhanced the effects. **P<0.01 vs. control. TUNEL, terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling; NVP, NVP-BEZ235.

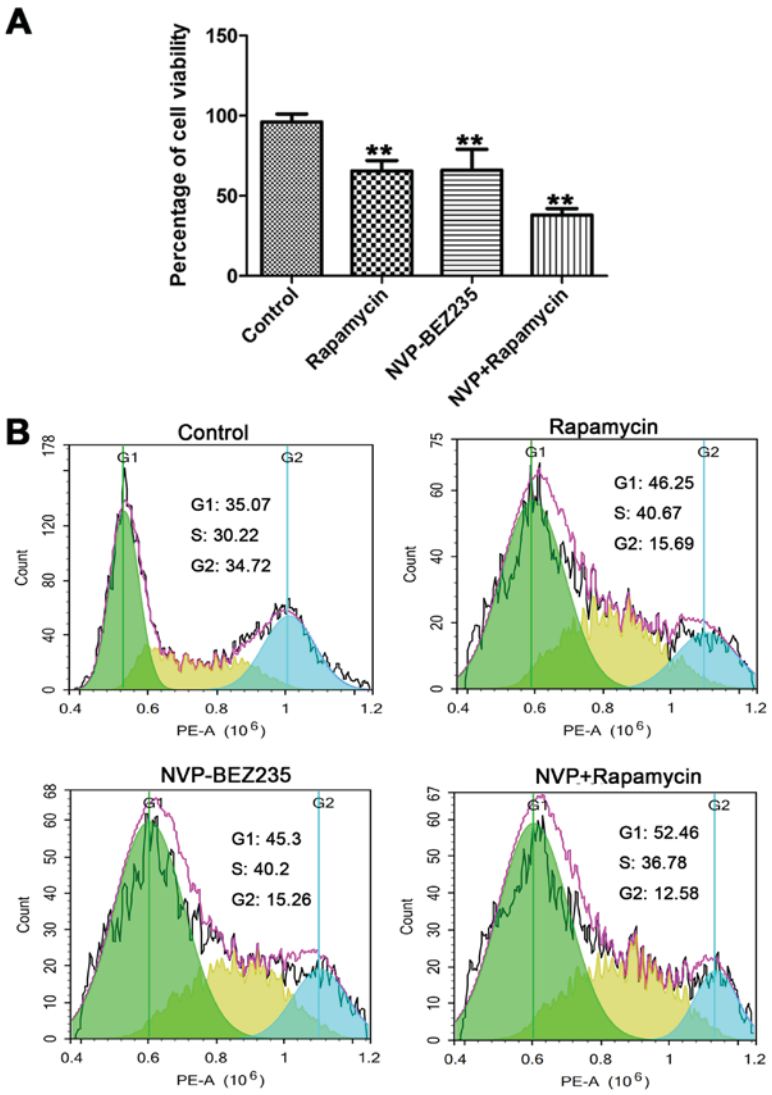


Figure 3. NVP-BEZ235 and rapamycin blocked cell viability. SUNE1 cells were cultured with 100 nM NVP-BEZ235, 100 nM rapamycin, or 100 nM NVP-BEZ235 and 100 nM Rapamycin for 48 h. The control group was administered the same amount of vehicle. (A) NVP-BEZ235+rapamycin significantly blocked cell survival as evaluated by MTT assay. (B) NVP-BEZ235+rapamycin induced cell cycle arrest at G1 phase, as detected by flow cytometry. **P<0.01 vs. control. NVP, NVP-BEZ235.

A previous study demonstrated that NVP-BEZ235 significantly inhibited cell proliferation in NPC via inhibition of the PI3K/AKT/mTOR pathway (17). However, the outcomes for NVP-BEZ235 in phase I/II clinical trials in advanced patients have been unsatisfactory (13). In the present study, the role of the combination of NVP-BEZ235 and rapamycin on NPC cellular apoptosis, cell viability, and PI3K/AKT/mTOR signaling was investigated, which might provide a novel drug for NPC therapy. The findings suggested that the combination of NVP-BEZ235 and rapamycin significantly induced cellular apoptosis, blocked cell viability and inhibited the activation of PI3K/AKT/mTOR pathway.

The PI3K/AKT/mTOR signaling pathway is associated with various biological events, including proliferation and apoptosis (18), and provides a good target for cancer treatment (19). NVP-BEZ235 is a synthetic low molecular mass compound and blocks PI3K catalytic activity by competing at its ATP-binding site (10), and represses the catalytic activity of mTOR (20). It has been revealed that AKT acts as a key regulator in PI3K/AKT/mTOR signaling, regulates cell proliferation by monitoring the expression of the cell cycle proteins c-myc and cyclin D1, and mediates cell survival by regulating a cascade of pro-apoptotic and anti-apoptotic proteins (21). However, the application of NVP-BEZ235 significantly suppresses the activation of AKT, S6 ribosomal protein and 4EBP1 in breast cancer (19). In the present study, it was demonstrated that the administration of NVP-BEZ235 significantly promoted cellular apoptosis, repressed cell viability, and modulated the expression of PI3K, AKT, p-AKT, mTOR, p-mTOR, p-AKT/AKT and p-mTOR/mTOR in SUNE1 cells. These data revealed that NVP-BEZ235 regulated NPC development via regulation of the PI3K/AKT/mTOR signaling pathway, which was in accordance with a previous study (9).

mTOR is a serine/threonine kinase which is associated with the PI3K/AKT/mTOR pathway (22). A number of studies have indicated that mTOR regulates the synthesis of key proteins for cell growth and proliferation (23-25). Rapamycin, as an inhibitor of mTOR, significantly inhibits NPC cell proliferation *in vitro* and the formation of tumors *in vivo* (26). In the present study, treatment with rapamycin promoted cellular apoptosis,

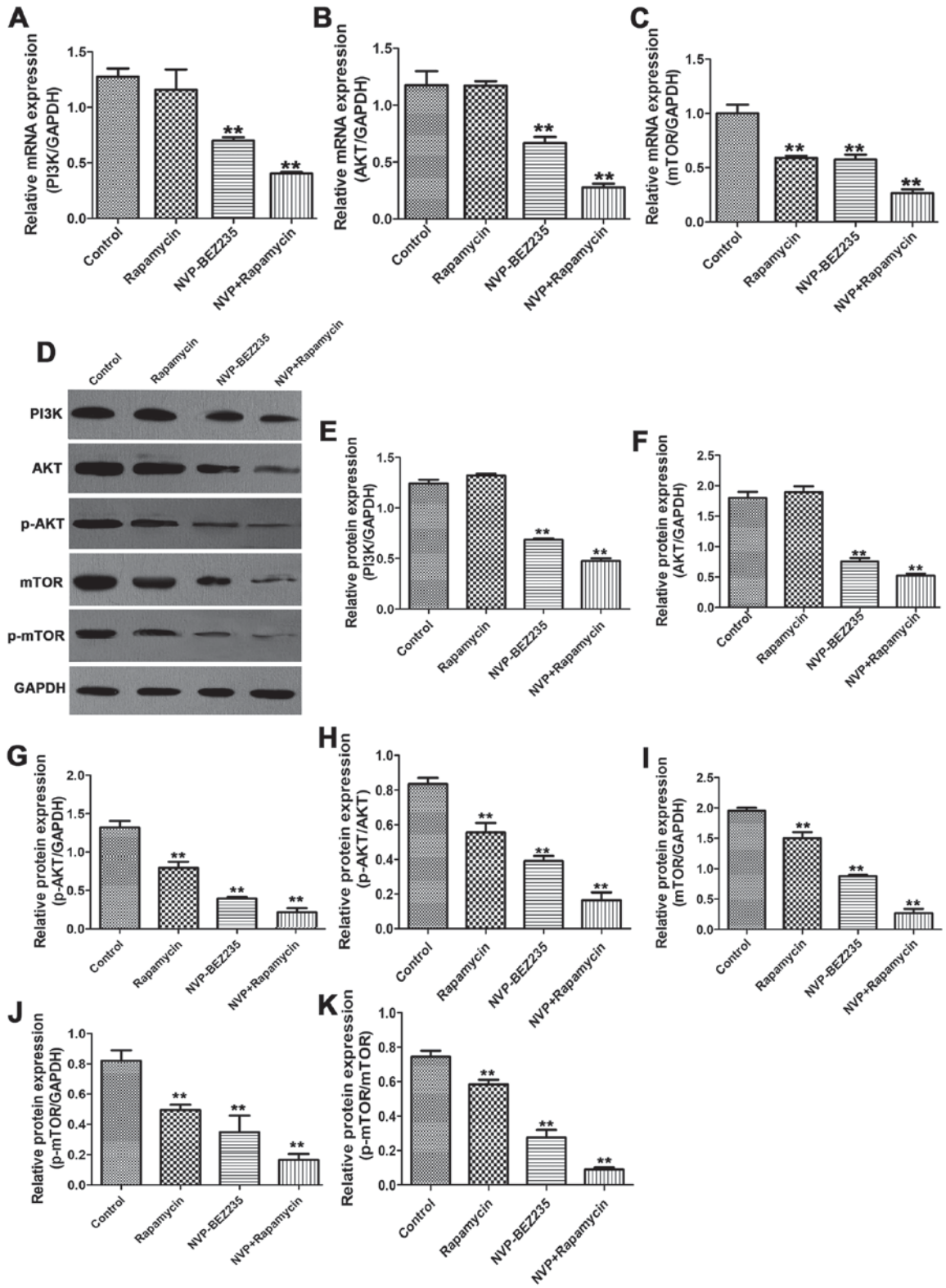


Figure 4. NVP-BEZ235 and rapamycin repressed the PI3K/AKT/mTOR pathway. SUNE1 cells were treated with 100 nM NVP-BEZ235, 100 nM rapamycin, or 100 nM NVP-BEZ235 and 100 nM rapamycin for 48 h. The control group was administered the same amount of vehicle. Total RNA from the four groups was isolated for reverse transcription-quantitative polymerase chain reaction. The mRNA levels of (A) PI3K, (B) AKT and (C) mTOR were normalized to the internal control GAPDH. (D) The protein levels of PI3K, AKT, p-AKT, mTOR and p-mTOR were detected by western blotting. (E-K) The protein density of PI3K, AKT, p-AKT, mTOR and p-mTOR was calculated relative to the internal control GAPDH, and the p-AKT/AKT and p-mTOR/mTOR ratios were calculated. **P<0.01 vs. control. PI3K, phosphoinositide 3-kinase; AKT, protein kinase B; mTOR, mammalian target of rapamycin; p, phosphorylated; NVP, NVP-BEZ235.

suppressed cell viability and blocked the expression of p-AKT, mTOR, p-mTOR, p-AKT/AKT and p-mTOR/mTOR, but had no effect on the mRNA and protein levels of PI3K and AKT. These data indicated that rapamycin participated in the inhibition of NPC development by repressing the expression of mTOR, which supported previous findings (1).

Although previous studies demonstrated that NVP-BEZ235 and rapamycin functioned as therapies for NPC (2,17), the outcome of the single drugs was unsatisfactory. The aim of the present study was to identify a more effective drug for NPC therapy, thus the effect of NVP-BEZ235+rapamycin was detected on SUNE1 cellular apoptosis and viability. In the present study, it was demonstrated that the combination of NVP-BEZ235 and rapamycin more markedly promoted cellular apoptosis and repressed cell viability than either single drug treatment (NVP-BEZ235 or rapamycin alone). Furthermore, the administration of NVP-BEZ235+rapamycin reduced the expression of PI3K, AKT, p-AKT, p-mTOR, p-mTOR, p-AKT/AKT and p-mTOR/mTOR. Thus, it was concluded that the combination of NVP-BEZ235 and rapamycin modulated cell viability and apoptosis via the PI3K/AKT/mTOR pathway in SUNE1 cells; and that the outcome with the two drugs was more positive than with either single drug. These results provided insight for exploring novel drug therapies for NPC.

However, there were certain limitations in the present study. First, the effect of NVP-BEZ235+rapamycin was evaluated in SUNE1 cells with a lack of *in vivo* animal experiments. Therefore, authors may perform further studies on the effect of NPV-BEZ235+rapamycin on animals *in vivo*, which may provide important findings for a future clinical trial. Secondly, although it was demonstrated that NVP-BEZ235+rapamycin modulated cell viability and apoptosis via the PI3K/AKT/mTOR pathway, the molecular mechanism by which NPV-BEZ235+rapamycin regulated the PI3K/AKT/mTOR pathway remains elusive. Further investigations are required to elucidate this mechanism.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HLu and Y-YY analyzed the data and were major contributors in writing the manuscript. H-MC cultured the cells, performed the cell apoptosis and cell cycle distribution detection via flow cytometry, and prepared Figs. 1, 2 and 3. WW and YL performed the RNA and protein extraction for reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively, and prepared Fig. 4. HLi directed the study and revised the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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