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ORIGINAL PAPER

Synergistic anti-proliferative and apoptotic effect of NVP-BEZ235 and curcumin on human SH-SY5Y neuroblastoma cells

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Abstract

Neuroblastoma, a tumor of the sympathetic nervous system, is one of the most common tumors found in children. Most patients develop resistance to therapy and show poor prognosis, thus there is a need of novel therapeutic agents for the treatment of neuroblastoma. NVP-BEZ235 is a dual Phosphatidylinositol 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) kinase inhibitor that induces apoptosis and suppresses the growth of cancer. Curcumin acts as an anticancer agent in certain cancers. This study investigated the synergetic effect of NVP-BEZ235 and curcumin against neuroblastoma SH-SY5Y cell line. In the current study, the synergic effect of NVP-BEZ235 and curcumin in SH-SY5Y was examined in terms of the cell growth by cell viability and colony forming assay, cell cycle and apoptotic cell death by flow cytometry and mRNA expression levels by quantitative Real Time Polymerase Chain Reaction (qRT-PCR). Curcumin, NVP-BEZ235 or a combination of both, showed cytotoxicity in a dose and time dependent manner in SH-SY5Y cells. 10 µM curcumin and 200 nM NVP-BEZ235 were chosen as combination therapy, as the combination index showed synergism. Colony forming assay showed decrease in cell growth in combination group. The cell cycle distribution for combination group demonstrated a decrease in G_0/G_1 phase at 48 h. Annexin V showed an anticancer effect in combination group when compared to control group. Moreover, qRT-PCR results showed a significant increase in caspase 3, caspase 7, Bax and p53 genes, while a decrease in Bcl-2 gene expression levels. These findings suggest that combination therapy of NVP-BEZ235 and curcumin may be a promising therapeutic candidate for treatment of neuroblastoma.

Keywords NVP-BEZ235 · Curcumin · Neuroblastoma · Combination therapy · Anti-cancer effect

Introduction

Neuroblastoma is a tumor that originates from the neural crest sympathoadrenal progenitor cells of the sympathetic nervous system and is one of the most common tumors found in children. Recent data showed that the incidence rate of neuroblastoma is 1.2 case per 100,000 and accounts for nearly 15% of childhood cancer deaths [1–3]. Despite the multimodal treatment methods, neuroblastoma is still a deadly type of cancer, as chemotherapy resistance and poor prognosis is frequently seen in patients [4]. Thus, improving the survival and long-term life quality of patients is a major challenge leading to the need of finding novel therapeutic agents for more effective treatment strategies.

PI3K/ Protein kinase B (AKT)/mTOR pathway is one of the most common altered pathways in cancer [5]. This pathway plays a critical role in controlling normal cellular functions and an abnormal activation of the pathway leads to metastasis, progression and chemoresistance in different cancers, including neuroblastoma [6, 7]. Thus, the upstream inhibition of this pathway could be an effective way in several types of cancers. A promising chemotherapeutic drug NVP-BEZ235, also called Dactolisib or BEZ235, is a dual PI3K/mTOR kinase inhibitor that

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induces the apoptosis and suppresses the growth of cancer cells [8, 9]. The mammalian/mechanistic target of rapamycin complex 1 (mTORC1) drives multiple anabolic pathways including nucleotide and protein synthesis, in addition to suppressing important catabolic processes by phosphorylating proteins, altering their activity or subcellular localization [10]. Activation of mTOR contributes to the pathogenesis of many tumor types [11]. mTORC1 inhibition is a promising treatment approach for cancer and NVP-BEZ235 inhibits TORC1 and TORC2 by linking it directly to the mTOR ATP-binding domain and preventing its catalytic activity [12]. NVP-BEZ235 shows a mitotic arrest in G0/G1 in neuroblastoma cell lines [13]. In neuroblastoma therapy, besides destroying cancer cells, safeguarding healthy cells is an important parameter [14]. This could be achieved by including multiple agents to increase treatment efficacy in cancer therapy. Recently, due to their low toxicity and effective use in cancer treatments, natural compounds and their combination strategies with anticancer drugs are becoming more attractive.

Curcumin, main component of Curcuma longa's polyphenols, shows antimicrobial, antioxidant, antiinflammation, and anticancer activities [15]. Curcumin could enhance apoptosis and inhibit proliferation of cancer cells and support the removal of reactive oxygen species [16, 17]. Treatment of curcumin induced apoptosis by stimulating caspase-3 and arrested the cells at G2/M phase on SK-N-SH neuroblastoma cell lines [18]. Moreover, curcumin exhibited anticancer activity and restrict the proliferation of LAN-5 neuroblastoma cell line through increasing ROS activity, imbalancing the mitochondrial membrane potential and suppressing the Akt signaling pathway [19]. Curcumin acts as an effective apoptotic agent against neuroblastoma cell lines and shows a decrease in tumor size in vivo [20]. Further studies focus on exploring the influence of curcumin alone or in combination on neuroblastoma. Combined therapy approach demonstrates that the apoptotic effect of doxorubicin could be doubled when it is combined with curcumin and leading to a decrease in migration on SH-SY5Y cell lines [21].

The aim of the present study was to investigate the effect of NVP-BEZ235 and curcumin as an anti-cancer treatment approach for human SH-SY5Y neuroblastoma cell line. Cell viability of SH-SY5Y and HUVEC cells were investigated under the treatment of NVP-BEZ235, curcumin or their combination. HUVEC cell line was selected as normal cell line to be able to determine the selectivity index. The synergistic effect of combined therapy on SH-SY5Y cells was studied to assess the anticancer efficacy through colony forming assay, cell cycle, apoptotic cell death and related gene expression analysis.

Materials and method

Cell lines and reagents

Human neuroblastoma cell line SH-SY5Y (CRL-2266, ATCC) and human endothelial cell line HUVEC were maintained in Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose (DMEM, Invitrogen, Gibco, UK) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Gibco, UK) and 1% penicillin, streptomycin and amphotericin (PSA, Invitrogen, Gibco, UK). Cells were washed with dulbecco's phosphate buffered saline (1X DPBS, Invitrogen, Gibco, UK) and cultured at 37 °C in a humidified incubator containing 5% CO_2. The media was changed twice a week and the cells were passaged when they reach enough confluency (~80%). Curcumin and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (USA). NVP-BEZ235 were supplied by Selleck Chemicals (USA). Curcumin and NVP-BEZ235 were dissolved in DMSO to prepare a stock solution of 100 and 2.12 mM, respectively. Aliquots were stored at − 20 °C until ready to use and freshly diluted for each experiment. The DMSO concentration was less than 0.1% in all experiments.

Cell viability assay

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (CellTiter96 AqueousOne Solution; Promega, UK). SH-SY5Y and HUVEC cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After an overnight incubation period, SH-SY5Y cells were treated with 50- 1000 nM of NVP-BEZ235, 5–40 µM of curcumin or their combination for 24 and 48 h. HUVEC cells were treated with 100–1600 nM of NVP-BEZ235, 10–40 µM of curcumin or their combination. Following post treatment, cells were subjected to 10% MTS in 1× PBS/glucose solution for 1 h and the absorbance was measured by microplate reader (Biotek, Winooski, USA) at 490 nm. Cell viability (%) was calculated by assigning non-treated cells absorbance value as 100%.

Selectivity and combination index

The 50% inhibitory concentration (IC_{50}) values of NVP-BEZ235 and curcumin were determined by calculating the 50% inhibition of SH-SY5Y cells. Selectivity index of NVP-BEZ235 and curcumin were calculated as IC_{50} of normal cell line vs IC_{50} tumor cell line [22]; where

HUVEC cell was chosen as the normal (non-neoplastic) cell line and SH-SY5Y is the tumor (neoplastic) cell line.

Drug combination was evaluated based on the principle of Chou and Talalay [23, 24]. Determination of the synergistic versus additive versus antagonistic cytotoxic effects of the combined treatment of cells with only NVP-BEZ235, only Curcumin and combination of NVP-BEZ235 and Curcumin on SH-SY5Y were assessed using the combination index (CI). The CI values < 1 indicate synergism, CI values = 1 show additive and CI value > 1 indicate antagonistic effects. The CI was calculated using the following formula: CI = [(D1)/(Dx)1] + [(D2)/(Dx)2][25] were:

(D1) and (D2) are the concentrations of NVP-BEZ235 and Curcumin in combination that inhibited cell growth by 50%, respectively. (Dx)1 and (Dx)2 are the concentrations of NVP-BEZ235 and Curcumin in single treatment that induced a 50% inhibition of cell proliferation, respectively.

Colony forming unit (CFU) assay

Colony forming assay was used to measure the proliferation capacity of SH-SY5Y cells when treated with NVP-BEZ235 and curcumin. Briefly, 300 cells/well were seeded into 6 well plates and treated with 200 nM NVP-BEZ235, 10 µM curcumin or their combination. At the end of the 14th day, cells were washed with PBS, fixed with methanol for 5 min at −20 °C following an incubation of 15 min at + 4 °C. After fixation, cells were stained with crystal violet dye (Sigma-Aldrich, USA) for 5 min and washed with PBS twice. Images of colonies were examined under an inverted light microscope (DMi1, Leica Microsystems, Germany) combined with a digital camera.

Cell cycle analysis

SH-SY5Y cells were seeded in 6 well plates at a density of 1.5×10^5 cells/well. Next day, the cells were treated with 10 µM curcumin, 200 nM NVP-BEZ235 or their combination for 48 h. Following incubation period, both adherent and non-adherent cells were collected, washed with PBS and fixed with 70% ethanol at −20 ℃ for at least 2 h. Then, the cells were centrifuged, washed with PBS and stained with 200 µL of Tali® Cell Cycle (Thermo Fisher, USA) solution containing a mixture of propidium iodide (PI), RNase A and Triton X-100 for 30 min. The prepared samples were evaluated by acquiring 20.000 events using flow cytometry (CytoFLEX, Beckman Coulter, USA).

Apoptotic cell death assay

Annexin V-FITC/PI apoptosis detection kit (Biolegend, USA) was used to examine the apoptotic cell death percentage. SH-SY5Y cells (1.5×10^5 cells/well) were seeded into 6 well plates and treated with 10 µM curcumin, 200 nM NVP-BEZ235 or their combination for 48 h. After the incubation, both adherent and non-adherent cells were collected, washed with PBS and pellet was resuspended in 500 µL of cell staining buffer. Following another washing step, the pellet was resuspended with 100 µL Annexin V binding buffer and 3 µL of Annexin V-FITC and 3 µL of propidium iodide in the dark at room temperature for 15 min. After incubation, 400 µL Annexin V binding solution was added and the apoptosis of the cells was measured using flow cytometry by acquiring 20.000 events.

qRT-PCR

Apoptosis-related gene expression levels were determined by qRT-PCR analysis. SH-SY5Y (1.5×10^5 cells/well) cells were seeded into 6-well plate and 200 nM NVP-BEZ235, 10 µM curcumin or their combination were administered. After 48 h, the cell pellets were collected for isolation of total RNA using RNA isolation kit (Invitrogen™, PureLink™ RNA Mini Kit) according to the manufacturer's instructions. cDNA was synthesized from the isolated total RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) according to the manufacturer's protocol. SYBR Green Master Mix (GeneMark, qPCR kit) was used for the qRT-PCR to quantify mRNA levels of the genes. Gene specific primers were designed using Primer-BLAST software from the National Center for Biotechnology (Bethesda, USA) and synthesized by Sentebiolab (Ankara, Turkey). Primers sequences were as follows: *Bax F: 5' TTGGAGCAGCCGCCCCAGG 3' and R: 5' CGGCCC CAGTTGAAGTTGCC 3'; Bcl2 F: 5' AGAGCAACCCAATGC CCGC 3' and Bcl2 R: 5' CAACGAGGGGCCTGAGAGG 3'; Caspase 3 F: 5' GGGAGCAAGTCAGTGGACTC 3' and Caspase 3 R: 5' CCGTACCAGAGCGAGATGAC 3'; Caspase 7 F: 5' ATGGCTGGAGAACCCACT 3' and Caspase 7 R: 5' TCAGTTAAAGTACAGTTCTTTTGTC 3'; p53 F: 5' ACG CTTCCCTGGATTGGCAGCC 3' and p53 R: 5' CCATTG CTTGGGACGGCAAGGG 3'; GAPDH F: 5' TGGTATCGT GGAAGGACTCA 3' and GAPDH R: 5' GCAGGGATGATG TTCTGGA 3'*. GAPDH was used as a housekeeping gene to ensure equal loading and the data were analyzed using GAPDH as normalization control. All qRT-PCR experiments were conducted using CFX96 RT-PCR system (Bio-Rad, Hercules, CA, United States).

Statistical analysis

GraphPad Prism version 8.2.1 (San Diego, USA) were used for all statistical analysis. All data (n = 3) were presented as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used to determine the statistical significance of intergroup

differences. Statistical significance was defined as $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****).

Results

NVP-BEZ235 and curcumin inhibited cell proliferation of SH-SY5Y and HUVEC cells

Cell proliferation MTS assay was used to determine the cytotoxic effect of NVP-BEZ235, curcumin or a combination of both on SH-SY5Y and HUVEC cell lines. As shown in Fig. 1, NVP-BEZ235 and curcumin demonstrated significant cytotoxicity on SH-SY5Y cells in time and dose-dependent manner. All doses of curcumin demonstrated significant cytotoxicity effects on SH-SY5Y cells except for the 5 µM at 48 h (Fig. 1a). On the other hand, NVP-BEZ235 increased its toxicity at all concentrations over the duration of 24 and 48 h (Fig. 1b). The IC_{50} values of NVP-BEZ235 and curcumin at 48 h were 0.72 and 30.46 µM, respectively (Table S1). The treatment of SH-SY5Y cells with combination of NVP-BEZ235 and curcumin for 24 and 48 h led to a significant decrease in cell viability compared to control. Nearly a significant decrease of $54 \pm 3\%$ seen on cell viability of SH-SY5Y cells when treated with combination of 10 µM Curcumin and 200 nM NVP-BEZ235 at 48 h (Fig. 1c). The combination index was found as 0.61, representing synergism as it is less than 1. Based on cell viability analysis, combination of Curcumin (10 µM) and NVP-BEZ235 (200 nM) was chosen as working dosage for the following cell cycle, apoptosis, colony forming and gene expression assays.

HUVEC cells showed no significant decrease ($p > 0.05$) in cell viability when treated with all doses of NVP-BEZ235 (100–1600 nM) and curcumin (10–40 µM) (Fig. 2a–b). Similarly, no significant decrease was detected in HUVEC cells when treated with combination of NVP-BEZ235 (200 nM) and curcumin (10 µM), with a cell viability of $89.53 \pm 10.59\%$ (Fig. 2c). The IC_{50} values of NVP-BEZ235 and curcumin on HUVEC cells were 2.47 µM and 26.7 µM at 48 h, respectively. The selectivity index for NVP-BEZ235 in neuroblastoma models (ratio of healthy cell line to cancer cell line) was 3.43.

NVP-BEZ235 and curcumin decreased growth of SH-SY5Y colony forming cells

CFU assay assessed the effects of curcumin, NVP-BEZ235 and their combination on colony forming capacity of SH-SY5Y cells. NVP-BEZ235 and combination treatment group drastically reduced the colony numbers and eliminated colony formation, respectively (Fig. 3a–b). The colonies were observed in the curcumin treated group in which the colony number and diameters were significantly less than control group. The number of colonies decreased significantly for curcumin, NVP-BEZ235 and combination groups when compared to control group (Fig. 3c). The diameter of the colonies for control, curcumin, NVP-BEZ235 and combination group were 1.604, 1.192, 0.144 and 0.062 mm, respectively (Fig. 3d).

NVP-BEZ235 and curcumin showed an increase in Sub G_0 and a decrease in G_0/G_1 phase for cycle distribution of SH-SY5Y cells

Flow cytometer was used to investigate the cell cycle progression of SH-SY5Y cells when treated with NVP-BEZ235 and curcumin alone and in combination. As shown in Fig. 4a-b, the cell population of SH-SY5Y cells treated with curcumin alone and combination of NVP-BEZ235 and curcumin show an increase in activity at Sub G0 phase compared to the control group. The percentage of cells at Sub G_0 phase were 0.96 ± 0.04, 4.85 ± 2.12, 4.14 ± 1.37 and $7.45 \pm 3.85\%$ for control, curcumin, NVP-BEZ235 and combination treatment group, respectively. The average G_0/G_1 cell population for control, curcumin, NVP-BEZ235 and combination were 50.73 ± 2.79, 22.64 ± 0.55, 38.76 ± 3.48 and $43.29 \pm 5.26\%$, respectively. The percentage of SH-SY5Y cells in Sub G0 phase was increased while the percentage of cells in G_0/G_1 phase was decreased when SH-SY5Y cells were treated with a combination of curcumin and NVP-BEZ235 in comparison to the control group.

NVP-BEZ235 and curcumin induced apoptosis on SH-SY5Y cells

In vitro antitumor activity was evaluated by assaying apoptosis. To compare the apoptotic response of NVP-BEZ235 and curcumin, SH-SY5Y cells were treated for 48 h and apoptotic rate was quantified by flow cytometry using an Annexin V-FITC/PI apoptosis assay detection kit. As shown in Fig. 5, very few necrotic or apoptotic cells were detected in control SH-SY5Y cells (untreated group). In contrast, NVP-BEZ235, curcumin or combination treatment caused an increase in the percentage of apoptotic cells. In the curcumin group, 25.0 and 9.5% of Annexin V-stained SH-SY5Y cells were recorded for early and late apoptosis. Similarly, SH-SY5Y cells treated with NVP-BEZ235 showed 27.6 and 11.0% of early and late apoptotic cells. In combination group (NVP-BEZ235 and curcumin), the apoptotic cell death detected in early and late apoptosis for SH-SY5Y cells were 32.47 and 18.45%. Taken together, combination of NVP-BEZ235 and curcumin showed an increase of apoptotic cell death at 48 h in SH-SY5Y cells.

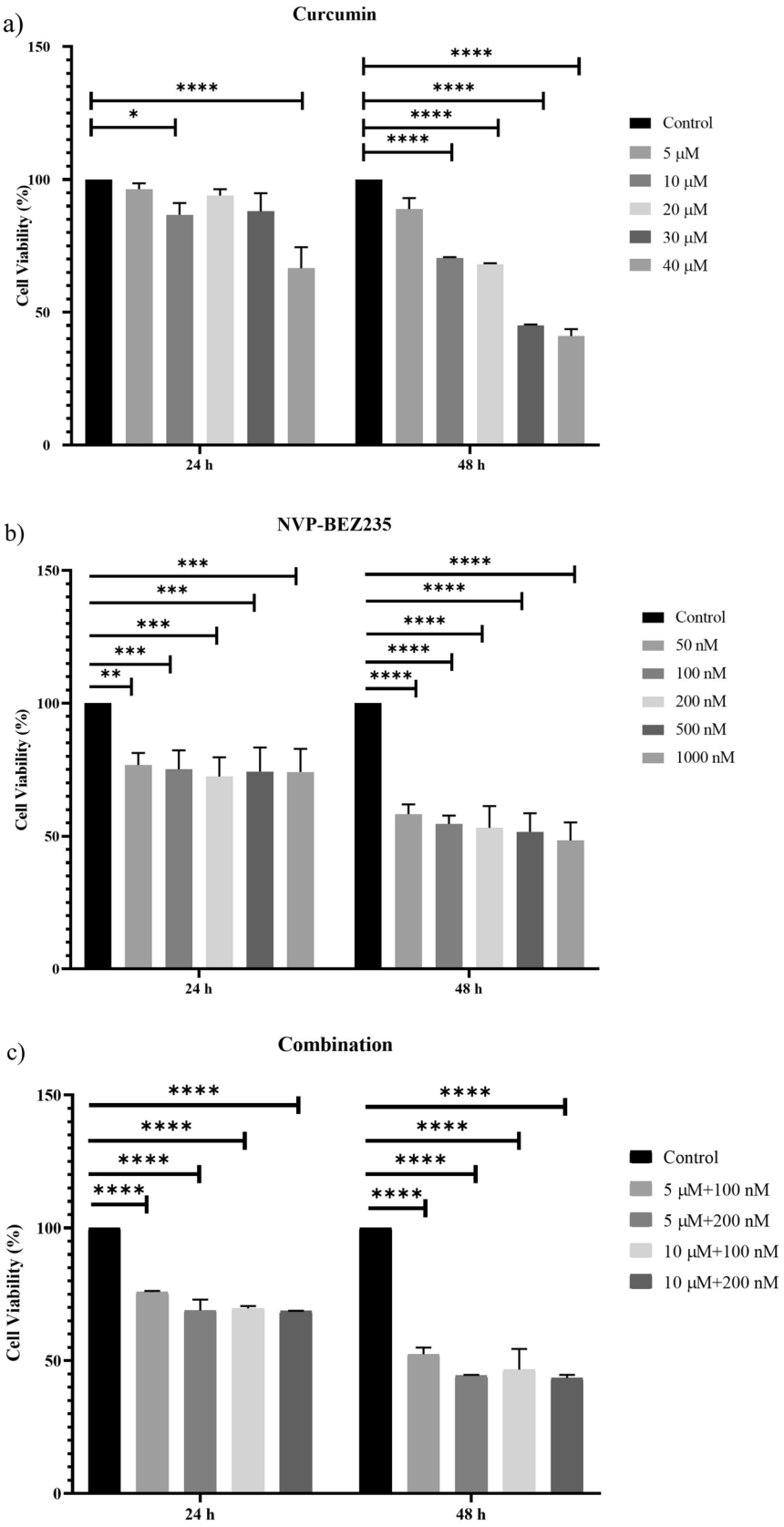

Fig. 1 The cell viability of SH-SY5Y cells after exposure to various treatment doses of NVP-BEZ235, curcumin or a combination of both. **a** 5–40 μM of curcumin, **b** 50–1000 nM of NVP-BEZ235 and **c** combination of NVP-BEZ235 and curcumin were used to treat SH-SY5Y cells for 24 and 48 h. The control group represents cells with no treatment. Cell viability was assessed by measuring the absorbance change using a microplate reader at 490 nm. Data represents average of three independent experiments ± SD ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****))

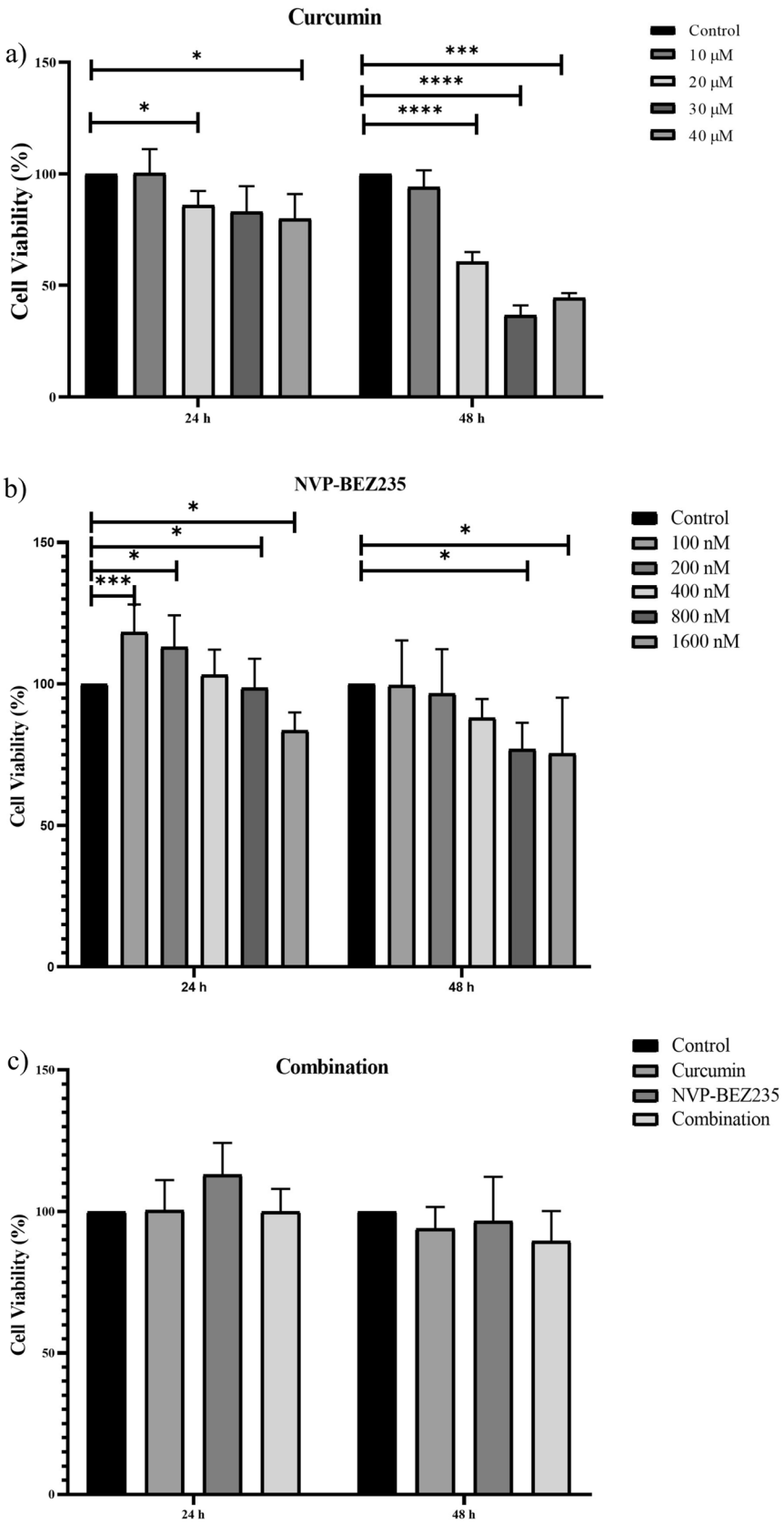

Fig. 2 The cell viability of HUVEC cells after exposure to various treatment doses of NVP-BEZ235, curcumin or a combination of both. **a** 10–40 µM of curcumin, **b** 100–1600 nM NVP-BEZ235 and **c** combination of NVP-BEZ235 and curcumin for 24 and 48 h. The control group represents cells with no treatment. Cell viability was assessed by measuring the absorbance change using a microplate reader at 490 nm. Data represents average of three independent experiments ± SD ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****))

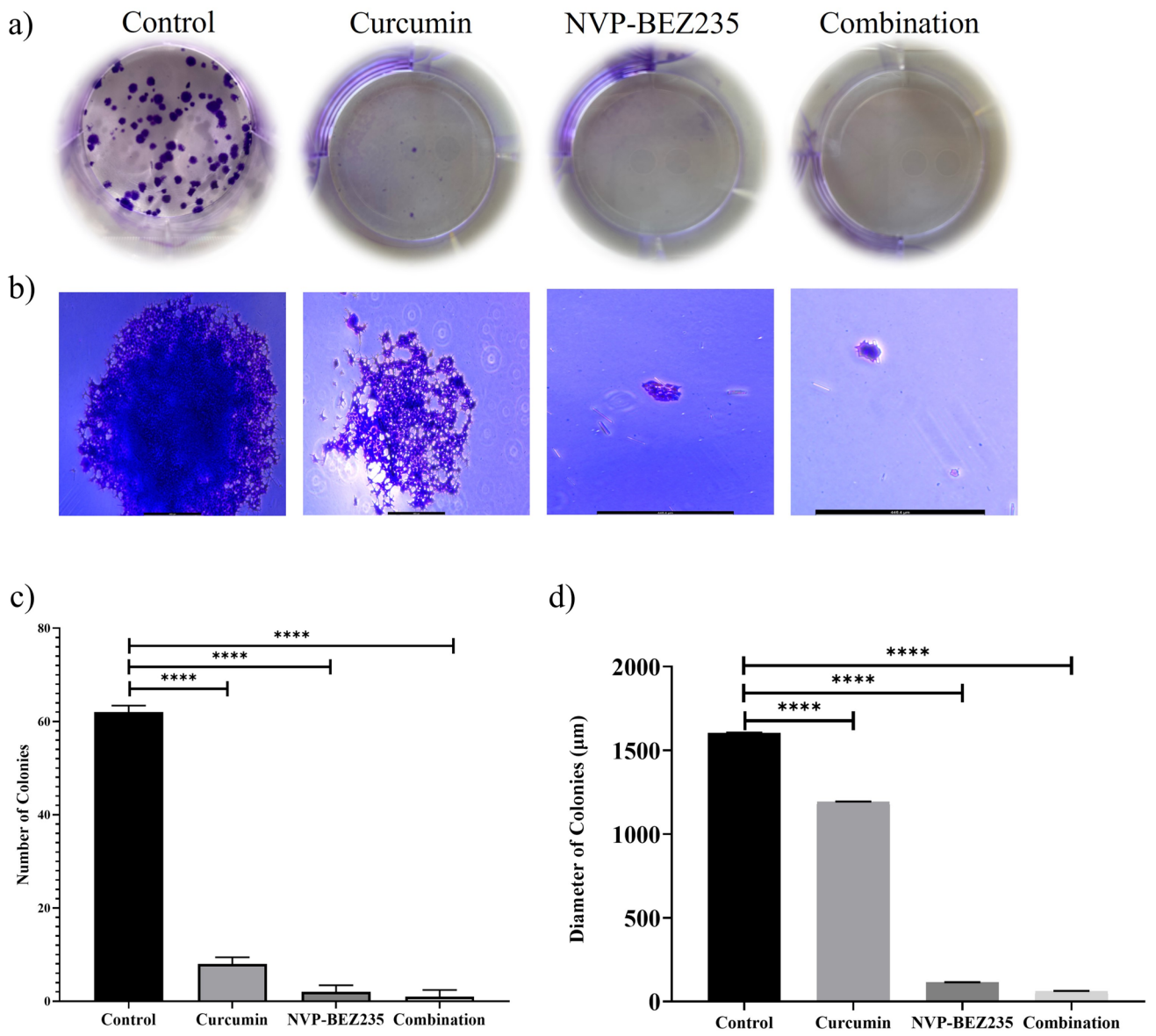

Fig. 3 NVP-BEZ235 and curcumin decreases SH-SY5Y cells colony formation. **a** Well images of colonies and **b** under light microscopy at 20X magnification for non-treated (control), Curcumin (10 µM), NVP-BEZ235 (200 nM) and combination (10 µM Curcumin and 200 nM NVP-BEZ235) groups. Graphical representation of **c** number of colonies and **d** diameter of colonies. Statistical analyses were given as: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). The scale bar: 446,4 µm

NVP-BEZ235 and curcumin influenced expression of apoptotic related genes on SH-SY5Y cells

The gene expression levels associated with proliferation and apoptosis after treatment with curcumin and NVP-BEZ235 were analyzed by qRT-PCR. In SH-SY5Y cells, curcumin and NVP-BEZ235 in combination significantly increased the expression levels of p53 compared to control group which is 62.23 ± 8.7 fold. Moreover, curcumin and NVP-BEZ235 in combination significantly increased the expression levels of BAX, Caspase 3 and Caspase 7, which were apoptosis-related genes, to 36.12 ± 9.61, 18.01 ± 5.44 and 7.57 ± 2.18 folds compared to control group, respectively. Bcl-2 expression was down-regulated significantly in combination group, while NVP-BEZ235 and curcumin alone did not cause any significant change in Bcl-2 gene expression level in SH-SY5Y compared to the control group (Fig. 6).

Discussion

Neuroblastoma is a heterogeneous tumor of infancy, originating from the sympathetic nervous system, which accounts for 15% of childhood cancer related deaths [26]. Current

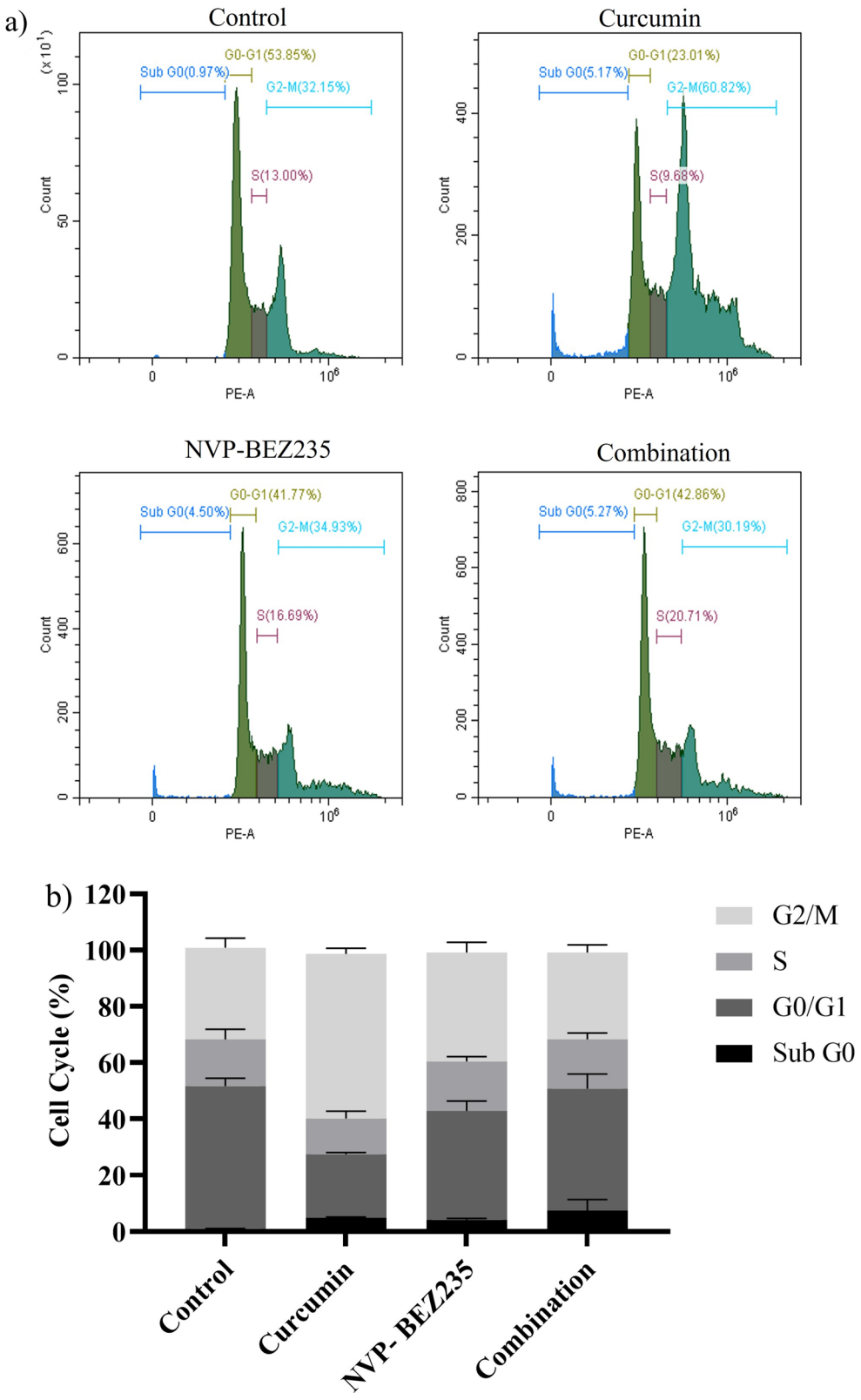

Fig. 4 Cell cycle profiles of SH-SY5Y cells were examined by flow cytometry analysis after treatment of curcumin, NVP-BEZ235 or a combination of both for 48 h. **a** The representative histogram plots and **b** average percentage of events in Sub G0, G0/G1, S and G2/M phases for the mean of three independent experiments

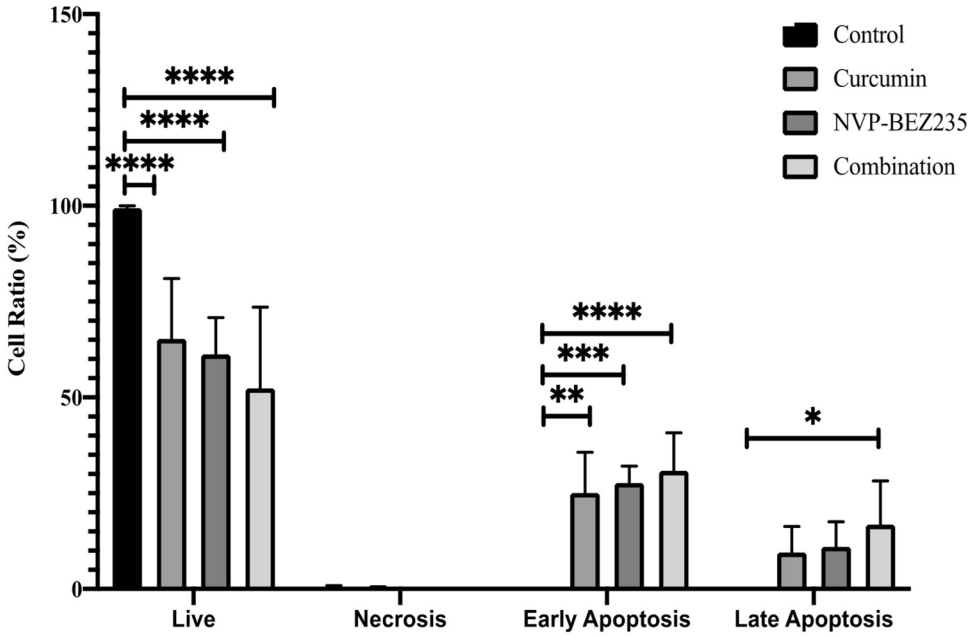

Fig. 5 Evaluation of apoptosis in SH-SY5Y cells induced by NVP-BEZ235 and curcumin using Annexin V assay. SH-SY5Y cell death was evaluated by flow cytometry using annexin V-FITC/PI staining showing graphical representation of percentage for cell death at 48 h. Data point represents the average ± SD (n = 3) ($p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****))

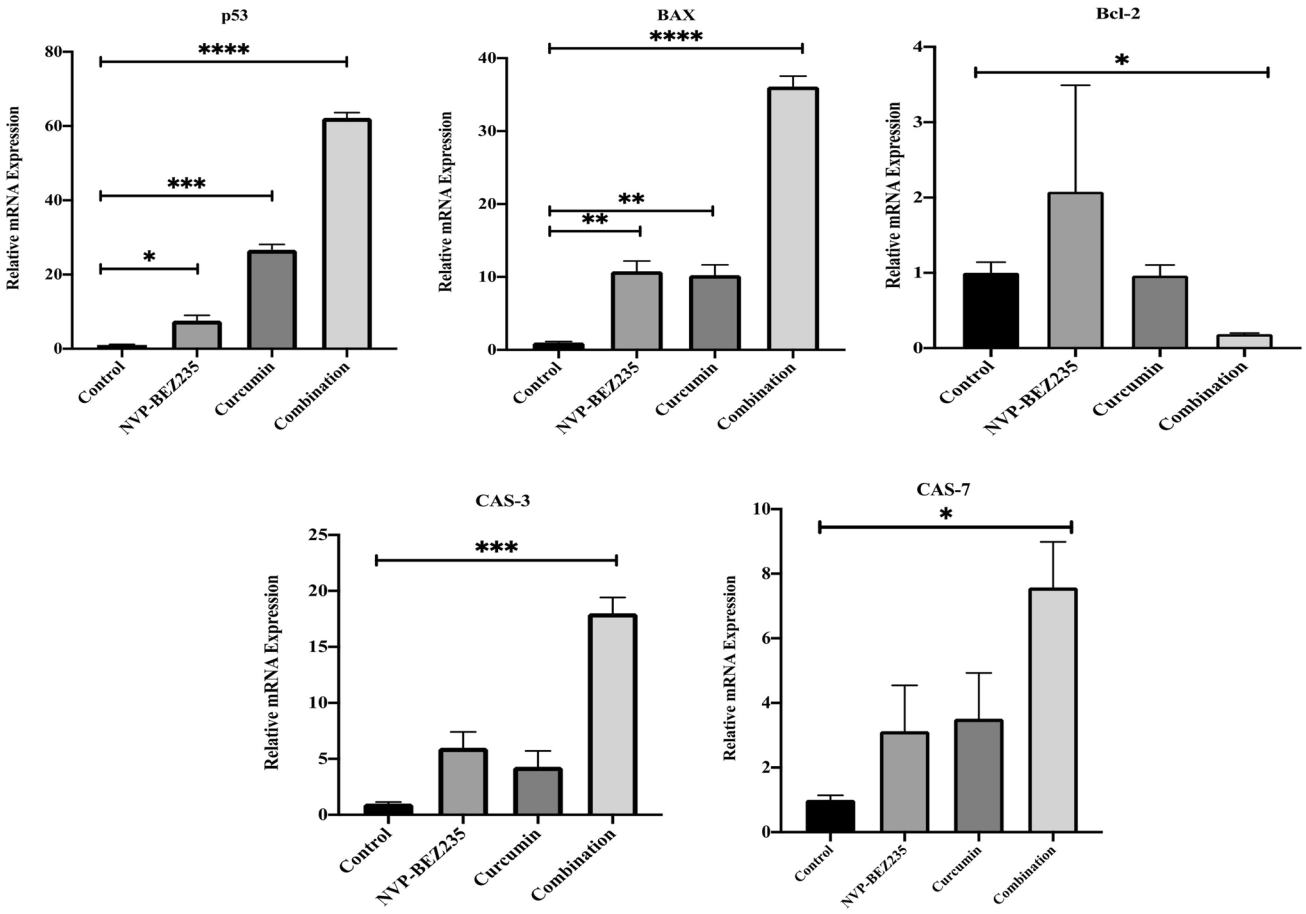

Fig. 6 Gene expression levels of p53, BAX, Bcl-2, CAS-3 (Caspase 3) and CAS-7 (Caspase 7) genes in SH-SY5Y cells treated with curcumin, NVP-BEZ235 or a combination of both for 48 h were detected by qRT-PCR analysis. GAPDH was used as internal control. Statistical analyses were given as: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****)

treatment methods include chemotherapy, radiation and surgery. Almost 50% of all cases are classified as high-risk neuroblastoma and these patients have poor prognosis as half of them relapse despite the multimodal treatment methods [27]. Combinations of chemotherapeutic drugs are current chemotherapy strategies for treatment of neuroblastoma [28]. However, neurological toxicity of chemotherapy is an important issue and limits treatment options for neuroblastoma therapy. Moreover, repetitive surgery and high radiation therapy are not applicable in neuroblastoma patients because of the location of the tumor area. Thus, neuroblastoma has remained a difficult clinical entity. To solve this problem, new effective drugs with low dosage administration to patients should be considered for neuroblastoma therapy [29, 30].

PI3K/Akt/mTOR signaling pathway plays an important role in cancer tumorigenesis [31] and regulates cellular activity, cell death mechanisms, cellular behaviors, and basic cellular activities such as growth and survival of the cell [32]. Therefore, inhibiting this signaling pathways has been an important target for cancer therapy [33]. NVP-BEZ235 is a dual PI3K- mTOR inhibitor which reduces tumorigenesis in neuroblastoma cancer cells [34–36] combining natural compounds with chemotherapeutic agents could reduce toxicity on healthy cells and increases tumor induction, providing a synergistic effect. Curcumin, a natural polyphenol compound, is an effective anti-cancer agent that induces apoptosis by stimulating caspase-3 and arrests at G2/M phase in neuroblastoma cell lines [18]. The recent studies showed that the use of curcumin with combined treatments is more effective than its use alone [34].

In this study, we focus on the synergistic effect of curcumin and NVP-BEZ235 to treat neuroblastoma SH-SY5Y cell line. The findings indicate that curcumin, NVP-BEZ235 or combination of both, showed cytotoxicity in a dose and time dependent manner in SH-SY5Y cells. IC_{50} value of NVP-BEZ235 in SH-SY5Y cells were lower than IC_{50} value in HUVEC cells. Thus, the selectivity index of NVP-BEZ235 was nearly 3.4 for HUVEC cell line, suggesting a potential agent for neuroblastoma therapy. The combination treatment of NVP-BEZ235 with curcumin in SH-SY5Y cells demonstrated synergism with a combination index of 0.61. The combination treatment group notably decreased the number of SH-SY5Y colonies compared to the control group. Thus, the colony forming assay results were parallel to our cell viability results as growth inhibition was detected in combination therapy. Our results were in consist with Zhu et al., showing that NVP-BEZ235 drastically reduced the colony numbers in lung cancer cell lines and its combination with a mTOR inhibitor eliminated the colony formation [25].

In neuroblastoma SK-N-SH cells, curcumin induces G2/M cycle arrest [18], while NVP-BEZ235 arrest SH-SY5Y and SK-N-MC neuroblastoma cells at G1 phase [13]. Some anticancer drugs inhibit the cell cycle at certain checkpoints causing arrest in that phase. Cell cycle arrest sub-G0 phase is an indication of apoptotic cell death, while G0/G1 phase arrest shows inhibition of cell proliferation [37–39]. In the current study, we observed that combination treatment of curcumin and NVP-BEZ235 decreased the percentage of SH-SY5Y cells in G1 phase. Curcumin induces apoptosis and reduce cell proliferation in neuroblastoma cells [16, 19]. In a study with Caki cell line, it was shown that combined doses of Curcumin and NVP-BEZ235 also reduced Bcl-2 protein levels and helped curcumin to inhibit both PI3K/Akt and mTOR pathways [40]. Previous studies have found that NVP-BEZ235 can induce apoptosis and block the cell cycle by regulating the PI3K-Akt signaling pathway in neuroblastoma cells [13, 36]. Similarly, we have found that the combination treatment of Curcumin and NVP-BEZ235 was much more potent on SH-SY5Y cells than either single agent, implying that the combination exerts an enhanced apoptotic effect. Bcl-2 gene expression level decreased significantly in the combination treated group. Furthermore, our study showed that there were significant increases in caspase 3, caspase 7 and proapoptotic Bax gene expression levels in the combination treatment group compared to the control group. Curcumin increases the anticancer effect of drugs in neuroblastoma by inhibiting apoptotic signaling pathways by regulating Bcl-2 protein, Nuclear factor kappa B (NF-κB) signaling pathways by regulating p-Akt level, and WNT/β-catenin, PI3K/Akt, Caspases, and p53 [19, 21, 41, 42]. Thus, our findings indicate that the combined treatment of NVP-BEZ235 and curcumin increased the anti-tumor efficacy of neuroblastoma SH-SY5Y cells in a synergistic manner. Because of p53's potential capacity to regulate one or more cell cycle check point-related genes, p53 can trigger cell growth arrest [43]. Our results shows that a significant increase in p53 gene levels were also observed in combination treatment of curcumin and NVP-BEZ235 in SH-SY5Y cells.

In summary, these results showed that combination treatment of NVP-BEZ235 and curcumin synergize in the induction of in vitro apoptotic cell death on neuroblastoma SH-SY5Y cells. Our findings demonstrate that this combination therapies could be a new strategy for neuroblastoma patients that have failed the currently available therapies.

Conclusion

Our results displayed that combination treatment of curcumin and NVP-BEZ235 have synergistic effect in human neuroblastoma SH-SY5Y cells. The combination treatment inhibits cell proliferation and colony formation of SH-SY5Y cells. The apoptotic cell death and cell cycle arrest on SH-SY5Y cells showed that combination treatment has a strong antitumor activity on neuroblastoma therapy. Changes in

caspase 3, caspase 7, BAX, Bcl-2 and p53 gene expression levels provide evidence that the combination treatment induces apoptosis in SH-SY5Y cells. Further in vivo studies will be essential to understand the anti-tumor activity of combination of curcumin and NVP-BEZ235 as potential treatments for neuroblastoma therapy.

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Author contributions FC Conceptualization, methodology, investigation of in vitro cell culture studies and writing- original draft. SK Methodology, investigation of cell viability and cell cycle studies, and writing- original draft. HA Resources, supervised data analysis, reviewing and revising the manuscript. ZBB Design the study, guided the conduct of studies, funding acquisition, writing and revising the manuscript.

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Declarations

Competing interests The authors declare no conflict of interest.

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