Apoptosis inducing effect of sodium butyrate and cisplatin alone or in combination on SH-SY5Y human neuroblastoma cells

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ABSTRACT: This study investigated the apoptotic effects of a histone deacetylase (HDAC) inhibitor, sodium butyrate (NaBu) and cisplatin as monotherapy or in combination on SH-SY5Y human neuroblastoma cells. MTT and colony formation assays were performed to investigate the anti-proliferative effects of NaBu in combination with cisplatin. Analysis of SH-SY5Y cell apoptosis was performed by Annexin V-FITC/PI and DAPI staining. The gene and protein expressions were measured by RT-PCR and Western blot. The cytotoxic effect of the combined treatment was higher than single treatments, while the combination of two did not show much higher inhibition of colony formation and induction of apoptosis compared to cisplatin alone. Treatment with cisplatin upregulated the expression of Bax and Bcl-2 and combination treatment enhanced Bax gene expression and Bax/Bcl-2 ratio. NaBu alone and combined with cisplatin enhanced the expression of nuclear factor kappa B (NF-κB) protein, however cisplatin alone significantly decreased NF-κB protein levels. p53 and phosphorylated inhibitor kappa B alpha (p-IκBα) expression did not alter following any of the treatments. The combination of NaBu with cisplatin did not show any additional benefit in SH-SY5Y human neuroblastoma cells compared to cisplatin as a conventional chemotherapeutic agent, probably due to the upregulation of antiapoptotic transcription factor NF-κB.

KEYWORDS: Histone deacetylase inhibitor; sodium butyrate; cisplatin; neuroblastoma; SH-SY5Y.

1. INTRODUCTION

Neuroblastoma is an aggressive and most common type of extra-cranial malignant tumor diagnosed in childhood and infancy and affects almost 700 children in the United States [1-5]. Approximately 8-10% of all pediatric cancers and up to 15% of cancer-related deaths among children are originated from neuroblastoma [3-6].

The clinical therapy strategy of neuroblastoma includes a platinum coordination compound, cisplatin as frontline chemotherapy [7]. However, drug resistance, various adverse effects, and poor prognosis could be observed in patients with advanced-stage neuroblastoma after cisplatin treatment [8-12]. Moreover, cisplatin has been used in combination with other antitumor approaches for treating neuroblastoma to prevent the complications of high dose cisplatin alone [8, 13-15]. There is still a need to develop novel, effective and less-toxic therapeutic strategies.

A histone deacetylase inhibitor, sodium butyrate (NaBu) is a potential new class of antineoplastic agents that induces apoptosis, cell cycle arrest and differentiation in a variety of cancer cells [16-19] and demonstrated enhanced efficacy in combination with classical chemotherapy [20, 21]. However, the precise mechanism of action of NaBu against cancer has not been established.

Induction of cancer cell apoptosis, the form of programmed cell death, is one of the main chemotherapeutic approaches [22]. Bcl-2 family members, which include anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax, regulate the mitochondrial pathway of apoptosis [23]. The ratio of Bax to Bcl-2 is a

critical determinant of apoptosis [23]. Moreover, a ubiquitous transcription factor, nuclear factor kappa B (NF-κB) mediates growth, differentiation, and apoptosis in different types of cancer [24]. In the cytoplasm, NF-κB consists of two protein subunits, p50 and p65 and these forms complex with NF-κB inhibitory subunit, inhibitor kappa B alpha (IκBα) in the resting state [25]. Phosphorylation of IκBα leads to nuclear translocation of NF-κB and induction of target gene transcription [26].

In the present study, we investigated the anticancer effects and molecular mechanisms of individual and combined treatments with NaBu and cisplatin in SH-SY5Y human neuroblastoma cells.

2. RESULTS

2.1. The effect of combination treatment with NaBu and cisplatin on the viability of SH-SY5Y cells

To explore the cytotoxic effect of NaBu or cisplatin alone or in combination in SH-SY5Y cell line, the cells were treated with serial concentrations of NaBu (0.5-40 mM) and cisplatin (0.5-15 µg/mL) for 24 and 48 h (Figure 1). Both NaBu or cisplatin alone can inhibit the proliferation of SH-SY5Y cells in a dose- and time-dependent manner.

The results showed that the viability of SH-SY5Y cells was reduced by NaBu at concentrations ≥10 mM ($p < 0.05$, Figure 1A). The treatment of SH-SY5Y cells with 5, 7.5, 10 and 15 µg/mL cisplatin induced a significant decrease in the number of viable cells ($p < 0.05$, Figure 1B). The IC_{50} values of NaBu and cisplatin at 48 h were 10 mM and 6.25 µg/mL, respectively (Figure 1A and B). As shown in Figure 1C, the combination treatment of 10 mM NaBu and 6.25 µg/mL cisplatin enhanced cytotoxicity with 20.47± 0.02% cell viability at 48 h compared with control ($p < 0.05$). Thus, the combined effect of NaBu and cisplatin on cell viability at higher dosage than IC_{50} values of each agent was not examined.
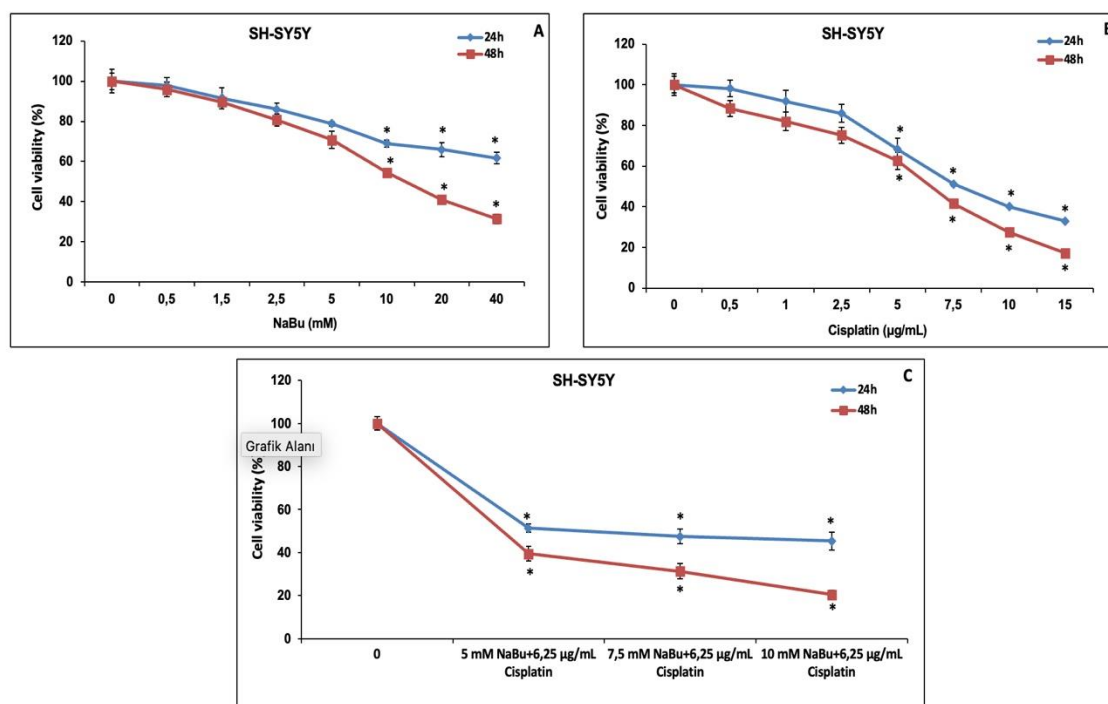

Figure 1. Effects of NaBu, cisplatin, and the combination treatment on time- and dose-dependent growth inhibition of SH-SY5Y cells (A-C). SH-SY5Y cells were treated with increasing doses of (A) NaBu (0.5-40 mM), (B) cisplatin (0.5-15 µg/mL), and (C) the combination of NaBu (5, 7.5 and 10 mM) and cisplatin (6.25 µg/mL) for 24 and 48h. Data represent the mean ± SD. *$p < 0.05$ vs control group.

2.2. The effect of NaBu combined with cisplatin on colony-forming ability of SH-SY5Y cells

The effect of NaBu combined with cisplatin on SH-SY5Y cell proliferation was evaluated by a colony formation assay. Both NaBu (10 mM) and cisplatin (6.25 µg/mL) treatment alone significantly inhibited the colony formation of SH-SY5Y cells compared to control ($p < 0.05$, Figure 2). However, combination treatment was not more effective in suppressing the number of colonies compared with cisplatin alone (Figure 2). Co-

treatment did not demonstrate a synergistic inhibitory effect on colony formation of SH-SY5Y neuroblastoma cells.
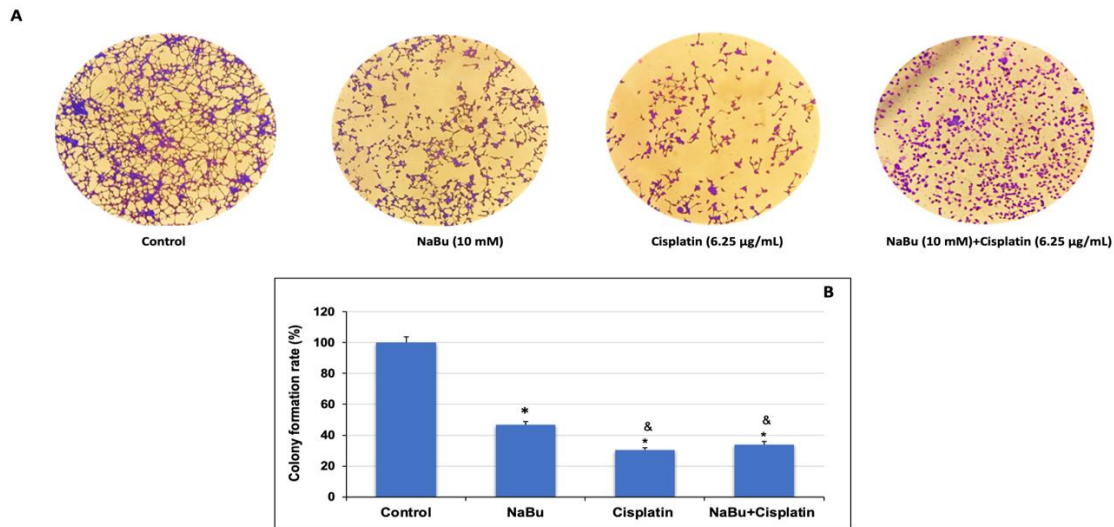

Figure 2. Effects of NaBu, cisplatin, and the combination treatment on the colony-forming ability of SH-SY5Y cells. Data in the bar graphs are expressed as mean ± SD. *$p<0.05$ vs control group, &$p<0.05$ vs NaBu-treated group.

2.3. The effect of NaBu and cisplatin on apoptosis of SH-SY5Y cells

Flow cytometric analysis and DAPI staining were performed to determine the induction of apoptosis in SH-SY5Y cells treated with NaBu (10 mM), cisplatin (6.25 µg/mL), or the combination for 48 h. The results showed that the apoptosis rate was 22.2% in SH-SY5Y cells treated with NaBu and enhanced up to 34.3% when the cells were treated with cisplatin (Figure 3A and B). However, the combination of NaBu and cisplatin did not increase apoptosis compared with cisplatin alone in SH-SY5Y cells (Figure 3A and B). These results suggested that NaBu did not enhance the effect of cisplatin on apoptosis in SH-SY5Y neuroblastoma cells. As shown in Figure 3C, nuclear condensation and fragmentation were observed in all treated groups by DAPI staining, which was more obvious in SH-SY5Y cells treated by cisplatin with/without the combination of NaBu.
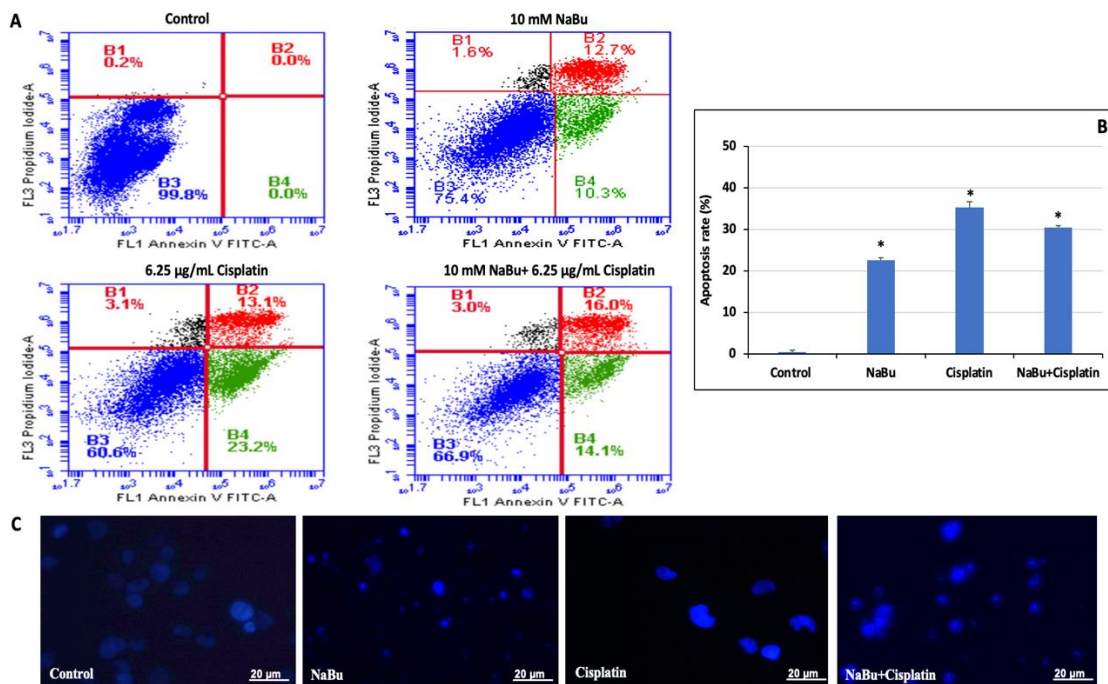

Figure 3. Effects of NaBu, cisplatin, and the combination treatment on cell apoptosis (A-C). SH-SY5Y cells were treated with NaBu (10 mM), cisplatin (6.25 µg/mL) or their combination for 48h. The apoptosis rate

and nuclear morphological changes were detected by annexin V-FITC/PI and DAPI staining, respectively. *p<0.05 vs control group.

2.4. The changes in p53 and Bax gene expression following NaBu and cisplatin treatment in SH-SY5Y cells

No significant change in p53 gene expression was observed among groups (Figure 4A). NaBu (10 mM) combined with cisplatin (6.25 µg/mL) significantly increased Bax gene expression as compared to the control in SH-SY5Y cells (p<0.05, Figure 4B).
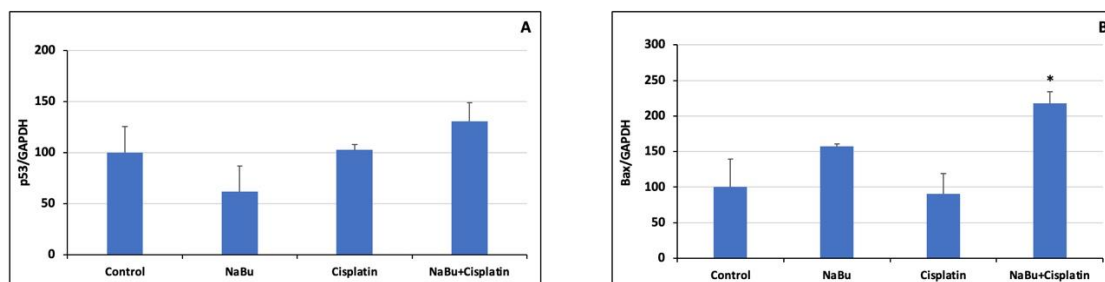

Figure 4. Effect of NaBu (10 mM), cisplatin (6.25 µg/mL) and the combination of both compounds on gene expression of (A) p53 and (B) Bax in SH-SY5Y cells. Data in the bar graphs represent the mean ± SD. p53 and Bax mRNA expressions are normalized with GAPDH values and data are expressed as fold change. *p<0.05 vs control group.

2.5. The changes in the expression of apoptotic family proteins and NF-κB signaling pathway-related proteins following NaBu and cisplatin treatment in SH-SY5Y cells

We investigated NaBu and cisplatin-mediated apoptosis by monitoring the expression levels of pro-apoptotic proteins (p53 and Bax) and anti-apoptotic Bcl-2 protein using western blotting. p53 protein expression was not affected by treatment (Figure 5A). Cisplatin at a concentration of 6.25 µg/mL significantly increased the expression of both Bax and Bcl-2 proteins in SH-SY5Y cells compared to control (p<0.05, Figure 5B and C). Moreover, SH-SY5Y cells treated with a combination of NaBu and cisplatin demonstrated lower Bax and Bcl-2 protein levels as compared to cisplatin treatment alone as shown in Figure 5B and C (p<0.05 and p<0.01, respectively). NaBu (10 mM) combined with cisplatin (6.25 µg/mL) upregulated Bax/Bcl-2 ratio compared with control and substances alone (p<0.05, Figure 5D).
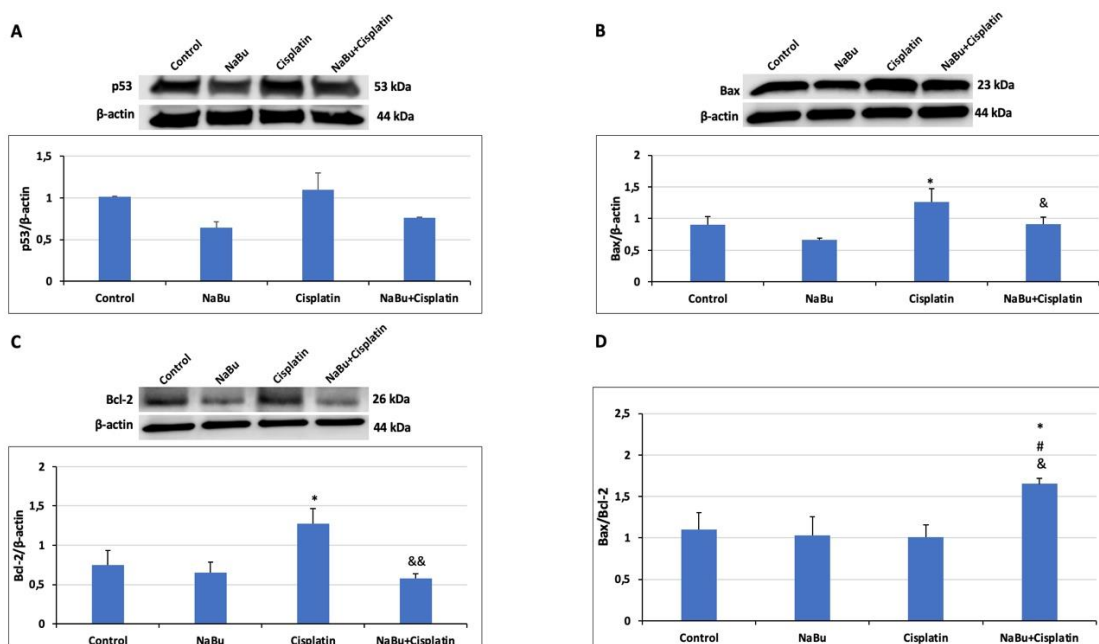

Figure 5. Effects of NaBu, cisplatin, and the combination treatment on the expression of apoptosis-related proteins. SH-SY5Y cells were treated with NaBu (10 mM), cisplatin (6.25 µg/mL) or their combination for 48 h. (A) p53, (B) Bax, and (C) Bcl-2 protein expressions and (D) the ratio of Bax/Bcl-2 were determined by

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western blot analysis. β-actin was used as a loading control. Data in the bar graphs are expressed as mean ± SD. *p<0.05 vs control group, &p<0.05 vs cisplatin-treated group, &&p<0.01 vs cisplatin-treated group, #p<0.05 vs NaBu-treated group.

Exposure of SH-SY5Y cells to 10 mM NaBu and combined treatment increased NF-κB protein expression (p<0.05 vs control, Figure 6A), whereas 6.25 µg/mL cisplatin treatment caused a statistically significant decrease in the protein expression levels of NF-κB compared to control (Figure 6A). As shown in Figure 6B, NaBu treatment alone significantly decreased the protein expressions of IκBα in SH-SY5Y cells in comparison to control group (P<0.05). The protein expression of p-IκBα and the ratio of p-IκBα/IκBα did not differ among the groups (Figure 6C and D).
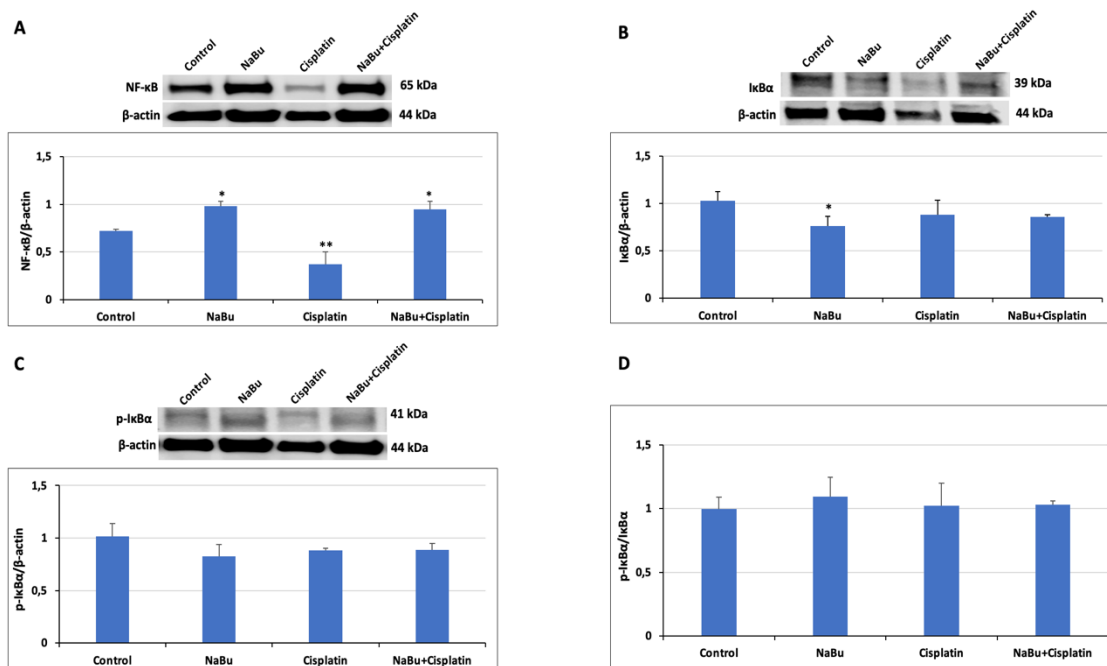

Figure 6. Effects of NaBu, cisplatin, and the combination treatment on the expression of proteins in the NF-κB signalling pathway. SH-SY5Y cells were treated with NaBu (10 mM), cisplatin (6.25 µg/mL) or their combination for 48 h. (A) NF-κB, (B) IκBα, (C) p-IκBα protein expressions and (D) the ratio of p-IκBα/IκBα were determined by western blot analysis. β-actin was used as a loading control. Data in the bar graphs are expressed as mean ± SD. *p<0.05 vs control group; **p<0.01 vs control group.

3. DISCUSSION

As a histone deacetylase inhibitor, NaBu can induce apoptosis in a variety of cancer cells [27-29]. Moreover, previous studies have demonstrated promising results with NaBu in combination with other anti-cancer drugs in many cancers [30-32]; therefore, in the current study, we aimed to investigate the anticancer effects of NaBu combined with cisplatin on SH-SY5Y human neuroblastoma cells.

In the present study, NaBu and cisplatin substances alone exerted cytotoxic effects on SH-SY5Y cells at a higher concentration of 10 mM and 5 µg/mL for 24h and 48h. Combined treatment exhibited more significant reduction in SH-SY5Y cell viability compared to substances alone in time and dose-dependent manner. Similarly, the combination of NaBu with cisplatin decreased the proliferation of bladder and gastric cancer cells in previous reports [27, 33]. Furthermore, anti-proliferative ability of single or combined NaBu and cisplatin treatment on SH-SY5Y cells was also demonstrated by inhibition of colony formation in the present study. Colony formation was inhibited in all treatment groups compared to control. In comparison with NaBu-treated group, combination treatment and cisplatin alone significantly impaired colony formation of SH-SY5Y cells. Recent studies in different cell lines also showed similar findings with NaBu or cisplatin treatment alone [31, 34]. In contrast with the results of the proliferation assay, the combination of NaBu with cisplatin did not show any effect on inhibition of colony formation compared to cisplatin alone.

It has been reported that NaBu promotes apoptosis in carcinomas by the mitochondrial apoptotic pathway [35, 36]. So we examined the expression of mitochondrial oncogene products (p53, Bax, and Bcl-2) [37]. In the present study, the apoptotic rate increased after any of the treatments, but treatment with cisplatin alone resulted in a higher rate of apoptosis compared to combination treatment and NaBu alone.

The apoptosis was also confirmed by DAPI staining which showed changes in the nuclear morphology of all treatment groups in SH-SY5Y cells. Moreover, NaBu combined with cisplatin increased Bax gene expression and Bax/Bcl-2 ratio compared to control. Treatment of SH-SY5Y cells with cisplatin resulted in increased expression of Bax and Bcl-2 proteins. However, NaBu combined with cisplatin remarkably decreased the levels of Bax and Bcl-2 as compared to cisplatin alone. The low correlation between expressions of Bax gene and protein in all treatment groups could be the result of posttranscriptional modification [38]. There was no noticeable change in the gene and protein expression of p53. Similar to our results, NaBu alone inhibited cell growth without changes in protein expression of p53, Bax and Bcl-2 compared to control in different cancer cell lines [39-41]. In contrast to our results, NaBu alone or its combination with cisplatin induced apoptosis in several cancer cells by inhibiting Bcl-2 and increasing Bax expression [33, 42]. These contradictory findings were possibly caused by the differences in cancer cell lines and sensitivity to therapeutic agents.

Previous studies reported both agonistic and antagonistic effects of NaBu on NF-κB signaling pathway [43, 44]. In the present study, SH-SY5Y cells exposed to the NaBu demonstrated significantly higher expression of NF-κB and lower expression of IκBα than control group. In addition, treatment of SH-SY5Y cells with cisplatin downregulated NF-κB protein expression. p-IκBα and the ratio of p-IκBα/ IκBα, which are an indicator of the rate of NF-κB translocation into the nucleus were unaffected by all treatments. Supportingly, a study by Mayo et al. demonstrated that transcription factor NF-κB was upregulated after treatment with NaBu in non-small cell lung cancer and HDAC inhibitors appear to enhance NF-κB-dependent gene transcription without inducing nuclear translocation [44]. Similarly, the previous study has reported decreased protein levels of NF-κB after cisplatin treatment in gastric cancer cells [45]. Cisplatin promoted apoptosis more effectively than combination treatment possibly caused by cisplatin-induced downregulation of NF-κB.

4. CONCLUSION

In conclusion, combination of NaBu and cisplatin significantly inhibited the proliferation of SH-SY5Y cells, however apoptosis and inhibition of colony-formation were more pronounced in SH-SY5Y cells exposed to cisplatin alone as compared to combination treatment. Therefore, this study does not support the use of cisplatin in combination with NaBu in neuroblastoma. The ineffectiveness of HDAC inhibitor, NaBu in combination with cisplatin to induce apoptosis of neuroblastoma cells could be associated with NaBu-mediated upregulation of antiapoptotic transcription factor NF-κB. Understanding the mechanisms which enhance the effects of NaBu over another drug are needed to consolidate the use of NaBu as a combination chemotherapy in neuroblastoma.

5. MATERIALS AND METHODS

5.1. Chemicals

NaBu and cisplatin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared as a 100 mM and 0.5 mg/mL stock solution in cell culture medium, respectively.

5.2. Cell Culture

Human neuroblastoma cells (SH-SY5Y) obtained from American Type Culture Collection (CRL-2266, ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F12; Gibco, NY, USA) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C in a humidified incubator with 5% CO_2 (Sanyo MCO-20AIC, California, USA).

5.3. Cell Viability Assay

To investigate the effect of NaBu or/and cisplatin on SH-SY5Y cell proliferation using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, cells were seeded in 96-well plates at a density of 2×10^3 cells/well with 100 µL of complete medium. Each group was treated with corresponding various concentrations of NaBu (0.5, 1.25, 2.5, 5, 10, 20 and 40 mM), cisplatin (0.5, 1, 2.5, 5, 7.5, 10 and 15 µg/mL) or in combination during 24 and 48 h according to the experimental design. Then, 20 µL of MTT solution (5 mg/mL dissolved in DMEM/F12) was added to each well. After 4 h incubation at 37°C in the dark, 100 µL of isopropanol was applied to the wells to dissolve formed formazan crystals. Optical density was measured at 590 nm by a micro-plate reader (ThermoScientific, USA). The cell viability of NaBu or/and cisplatin-treated groups was expressed relative to the control group.

5.4. Colony Formation Assay

SH-SY5Y cells were seeded in 6-well plates at a density of 3×10^3 cells/well in DMEM/F12 supplemented with 10% fetal calf serum. The cells were then treated with NaBu (10 mM) or/and cisplatin (6.25 µg/mL) for 48 h. The media was replaced every 3 days and cells were incubated under standart conditions for up to 14 days until colony formation. The colonies were fixed via 4% paraformaldehyde and stained with 0.1% crystal violet at room temperature for 15 min. The visible colonies were photographed and counted by CellCounter software (Nghia, Ho version 0.2.1).

5.5. Quantification of Apoptotic Cells by Flow Cytometry

The apoptotic cells were measured by the Annexin V-FITC Apoptosis Detection Kit (Thermo Scientific, Waltham, MA, USA). SH-SY5Y cells (1×10^6/mL) were incubated for 24 h in 25-cm² flasks and then treated with NaBu (10 mM) or/and cisplatin (6.25 µg/mL). After 24 h treatment, cells were collected via centrifugation at 1000 rpm for 5 min and resuspended in 100 µL of 1X Annexin binding buffer. 5 µL of FITC-conjugated Annexin V and 20 µL of propidium iodide (PI) were used for cell staining. Then, samples were vortexed and incubated in the dark for 30 min. 1X binding buffer was added to each tube and samples were analyzed using flow cytometry (BD Bioscience, USA) with CellQuest software.

5.6. 4', 6-diamidino-2-phenylindole (DAPI) Staining

To observe nuclear morphological changes, SH-SY5Y cells at a density of 2.4×10^4/mL were treated with NaBu or cisplatin alone, or combination of both in 6-well plates for 24 and 48 h. Then, the cells were harvested, washed with PBS, and fixed using 100 µL of 4% formaldehyde for 10 min at room temperature. After centrifugation at 3000 rpm for 2 min, the cells were stained with 2 mg/mL DAPI solution (Thermo Fisher, USA) at room temperature for 20 min in a dark place. Subsequently, DAPI was removed and cells were washed with PBS. Morphological changes in cell nuclear were immediately visualized using an inverted fluorescence microscope (Thermo Fisher EVOS M5000) with DAPI filter.

5.7. RT-PCR

SH-SY5Y cells were harvested and RNA was extracted using the RNA isolation kit (Macherey-Nagel, Düren, Germany). Complementary DNA (cDNA) was produced from equal amounts of total RNAs (200 ng) using cDNA synthesis kit (New England Biolabs, Ipswich, MA, USA) in T100 thermal cycler (Bio-rad, Hercules, CA, USA). Subsequently, newly synthesized cDNA (2 µL) was amplified in each buffer containing 10 µL qPCR master mix (New England Biolabs, Ipswich, MA, USA), 1 µL of each forward and reverse primer (0,25 µM) and 7 µL DNA-free water using ABI 7500 RT-PCR System (Applied Biosystem, Foster City, CA, USA) to detect mRNA level of p53 and Bax. The oligonucleotide sequences of these genes were designed by Primer 3 input program (Table 1). The thermocycling conditions of RT-PCR were as follow: 1 min at 95°C, 40 cycles of 15 sec at 95°C and 30 sec at 60°C. According to the amplification analysis, the relative expressions of the Bax and p53 genes were calculated using the $2^{-\Delta\Delta CT}$ method.

5.8. Western Blotting

SH-SY5Y cells were harvested and lysed with a lysis buffer containing proteinase inhibitor cocktail (Cell Signal Technology, Boston, MA). 20 µg of cell lysate protein was separated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene difluoride membrane (ThermoScientific, USA) for 60 min at 100 voltages. Blocking of non-spesific binding was performed by incubation with 10% w/v dry milk in 1× phosphate-buffered saline (PBS) at room temperature for 1.5 h at room temperature. Then, membranes were incubated overnight at 4°C with NF-κB, IκBα, phospho-IκBα (p-IκBα), β-actin (Cell Signaling Technology, Boston, MA, USA), Bax (Abcam, Cambridge, MA, USA), p53 and Bcl-2 (Santa Cruz Biotechnology, Dallas, TX, USA). Next, the membranes were washed three times in 1× PBS for 10 min and incubated with horseradish peroxidase-linked secondary antibody diluted 1:1000 in 1×PBS with 0.1% Tween 20 (PBS-T) for 1 h. After final wash, blots were visualized with Odyssey Fc system (LI-COR Biosciences, Lincoln, NE, USA) by using enhanced (ECL) kit (Millipore, Darmstadt, Germany). Band intensity of each protein was estimated by Image J software (National Institutes of Health) and the data were normalized to the intensity of corresponding loading control (β-actin).

5.9. Statistical Analysis

Statistical evaluation was carried out using one-way analysis of variance (ANOVA) test with SPSS 19.0 for Windows (SPSS, Chicago, IL, USA). Data were reported as mean±standard deviation (SD). $p \leq 0.05$ was considered statistically significant.

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