Inhibition of store-operated Ca\(^{2+}\) entry counteracts the apoptosis of nasopharyngeal carcinoma cells induced by sodium butyrate

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Abstract. Sodium butyrate (NaBu), a histone deacetylase inhibitor, has demonstrated anti-tumor effects in several cancers, and is a promising candidate chemotherapeutic agent. However, its roles in nasopharyngeal carcinoma (NPC), an endemic malignant disease in Southern China and Southeast Asia, has rarely been studied. In the present study, MTT assay, colony formation assay, flow cytometry analysis and western blotting were performed to explore the influence of NaBu on NPC cells and its underlying mechanism. NaBu induced morphological changes and inhibited proliferation in 5-8F and 6-10B cells. MTT assay revealed that NaBu was cytotoxic to 5-8F and 6-10B cells in a dose- and time-dependent manner. Furthermore, flow cytometry analysis revealed that NaBu induced obvious cell apoptosis in 5-8F and 6-10B cells due to the activation of the mitochondrial apoptosis axis. In addition, flow cytometry analysis and western blotting demonstrated that NaBu could enhance the Ca\(^{2+}\) influx by promoting store-operated Ca\(^{2+}\) entry (SOCE) in 5-8F and 6-10B cells. Inhibition of SOCE by specific inhibitors or downregulated expression of calcium release-activated calcium channel protein 1 and stromal interaction molecule 1 could counteract the apoptosis of NPC cells induced by NaBu. Thus, the current study revealed that enhanced SOCE and activated mitochondrial apoptosis axis may account for the mechanisms of cytotoxicity of NaBu in NPC cells, and that NaBu serves as a promising chemotherapeutic agent in NPC therapy.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignant disease characterized by its unique geographic distribution, with Southern China and Southeast Asia holding the highest incidence rates, with an annual incidence of 15-50 cases per 100,000 people (1-4). The main etiological factors of NPC are genetic susceptibility, environment factors and Epstein-Barr virus infection (1). Radiotherapy is the recommended treatment for non-metastatic NPC at early stage due to its complex anatomic location and high radiosensitivity (5). Combined chemoradiotherapy is more effective in treating NPC patients with locally advanced stages or lymph node metastasis, which account for the majority of NPC types (3,4). Although the treatment results of NPC have improved in the past years, its overall survival (OS) rates at 5 years are still unsatisfactory (3). Thus, it is necessary and helpful to explore new agents that could achieve better therapeutic efficacy of NPC than the current ones.

Sodium butyrate (NaBu) is the sodium salt of butyric acid, one of the short chain fatty acids, which is naturally produced by symbiotic bacteria in the gastrointestinal tract through fermenting dietary fibers (6-8). Histone deacetylases (HDACs) are key epigenetic regulators that can regulate the gene expression profile by directly interacting with other proteins such as transcription factors (6). Their abnormal overexpression has been confirmed to contribute to carcinogenesis in multiple cancers (6). Therefore, HDAC inhibitors (HDACis) serve as a promising new class of anti-cancer tumor agents, and various agents of this type have been approved (6,8). NaBu, as a potent HDACi, has a great potential in cancer treatment and

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prevention (9). Its anti-tumor abilities, including proliferation inhibition and apoptosis induction (9), have been validated in several cancers, including colorectal cancer, prostate cancer, breast cancer and lung cancer (9-11). However, NaBu's influence on NPC cells has been rarely studied.

Ca^{2+} is a ubiquitous second messenger in signaling transduction, and the fluctuation of intracellular Ca^{2+} levels is involved in the regulation of multiple physiological functions, including cell growth, survival, apoptosis and migration (12). In non-excitatory cells, store-operated Ca^{2+} entry (SOCE) is an important pathway for Ca^{2+} influx to refill the intracellular Ca^{2+} stores, mainly located in the endoplasmic reticulum (ER), which is mediated by the Ca^{2+} release-activated Ca^{2+} (CRAC) channel (13,14). Calcium release-activated calcium channel protein 1 (Orai1) and stromal interaction molecule 1 (Stim1) are two essential components of the CRAC channel (13,14). Upon activation, following oligomerization and translocation, Stim1, a single-pass transmembrane protein located in the membrane of ER, recruits Orai1, a cytomembrane protein, to form the functional CRAC channel, which stimulates Ca^{2+} influx (13,14). Under certain circumstances, including endogenous mutations and exogenous drugs stimulation, the Orai1/Stim1 CRAC channel may overexcrete, and the aberrant overloaded intracellular Ca^{2+} concentration would cause a series of pathological responses such as apoptosis (15,16). Interrupting the SOCE process by knocking down Orai1/Stim1 or blocking it with specific inhibitors could counteract the apoptosis of cells induced by anti-tumor agents such as cisplatin, gypenosides and oxaliplatin in several cancers (7,15,16). SOCE was also confirmed to be necessary for the apoptosis induced by NaBu in colon cancer cells (17).

Thus, in the present study, various experiments were performed to test the anti-tumor efficacy of NaBu in NPC cells, and the roles of SOCE in NaBu-induced apoptosis in NPC cells were explored.

Materials and methods

Cell culture and transfection. The NPC cell lines 5-8F and 6-10B were obtained from the Cancer Research Institute of Sun Yat-sen University (Guangzhou, China). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) supplemented with 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in a humidified 5% CO2 atmosphere. 5-8F and 6-10B cells were transfected with small interfering RNA (siRNA) against Orai1 and Stim1 using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Cells were collected for downstream analyses 48 h later. The sequences of the siRNAs were listed elsewhere (18).

MTT assay. MTT (Sigma-Aldrich; Merck Millipore) assay was performed to evaluate the proliferation ability of 5-8F and 6-10B cells treated with NaBu. Cells were seeded at a density of 2,000 cells/well in 96-well plates with 200 µl culture medium containing NaBu at different concentrations. Then, the cells were consecutively cultured for 72 h. Every 24 h, 20 µl 5 mg/ml MTT solution was added into the corresponding well, and the cells were cultured for another 4 h. Then, the solution was replaced with 150 µl dimethylsulfoxide (Sigma-Aldrich; Merck Millipore), followed by gentle agitation of the plates for 15 min at room temperature. Finally, the absorbance at 492 nm was measured to represent the cell viability.

Colony formation assay. Colony formation assay was performed as described earlier (19). First, 5-8F and 6-10B cells were treated with 5 mM NaBu for 48 h. Then, the treated cells were trypsinized and seeded in 6-well plates at a density of 1,000 cells/well. Untreated 5-8F and 6-10B cells were also seeded in 6-well plates similarly. Eight days later, the cultured cells were fixed with paraformaldehyde and stained with crystal violet. Colonies containing ≥50 cells were counted under an inverse microscope (Nikon Corporation, Tokyo, Japan), and the colony formation ability was subsequently analyzed.

Apoptotic analysis by flow cytometry. Apoptotic analysis by flow cytometry was conducted using the Annexin V-FITC Apoptosis Detection kit (Vazyme, Piscataway, NJ, USA) according to the manufacturer's protocol. Briefly, 2×10^6 cells, both treated and untreated with NaBu, were collected and washed by pre-cooling PBS twice. Then, cells were resuspended in staining buffer and stained with 5 µl fluorescein isothiocyanate and 5 µl propidium iodide for 10 min in the dark at room temperature. Finally, the cell samples were analyzed by FACSCalibur (BD Biosciences, San Jose, CA, USA).

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA extraction and RT-qPCR were performed according to a previous procedure (2). Briefly, total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and retrotranscribed into transcripts with the HiScript® Q RT SuperMix for qPCR kit (Vazyme). Then, qPCR was conducted on a MJ Mini™ thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using AceQ® qPCR SYBR® Green Master Mix (Vazyme). The qPCR cycle program was as follows: 95°C for 5 min, followed by 40 cycles at 94°C for 10 sec and 60°C for 30 sec. The relative messenger RNA expression levels of Orai1 and Stim1 were calculated using the 2^ΔΔCt method (20) following normalized to GAPDH. The primers for Orai1, Stim1 and GAPDH are listed in Table I.

Antibodies and western blotting. Rabbit anti-B-cell lymphoma (Bcl)-xL (cat. no. 10783-1-AP), anti-Bcl-2 (cat. no. 12789-1-AP), anti-caspase 3 (Cas3) (cat. no. 19677-1-AP), anti-Bcl-2-associated X protein (Bax) (cat. no. 23931-1-AP), anti-Orai1 (cat. no. 13130-1-AP), anti-Stim1 (cat. no. 11565-1-AP), anti-myeloid leukemia cell differentiation protein-1 (Mcl1) (cat. no. 16225-1-AP) and anti-survivin (cat. no. 10508-1-AP) polyclonal antibodies, and mouse anti-β-actin monoclonal antibody (cat. no. 66009-1-Ig), all were purchased from ProteinTech Group, Inc. (Chicago, IL, USA). Rabbit anti-cleaved (c)-Casp3 p17 (cat. no. D175) and c-poly ADP ribose polymerase (c-PARP) (cat. no. YC0101) polyclonal antibodies were purchased from ImmunoWay Biotechnology Company (Plano, TX, USA). Horseradish peroxidase
Results

NaBu induces morphological changes and inhibits the proliferation of NPC cell lines. It has been demonstrated that NaBu can induce cell morphological changes in multiple cancers (21). In the present study, obvious morphological transformations induced by NaBu were observed in both 5-8F and 6-10B cells, as demonstrated by extended pseudopodia, enlarged and compressed cell size, and increased cellular vacuoles (Fig. 1A). These transformations exhibited a dose- and time-dependent pattern, since higher concentrations and longer time exposures led to bigger changes (Fig. 1A; dose-dependent results not shown). NaBu inhibited the proliferation of 5-8F and 6-10B cells remarkably, as demonstrated by the reduced number of cells and reduced size of the cell colonies in the colony formation assay (Fig. 1B).

NaBu decreases the cell viability of NPC cell lines in vitro. The cytotoxicity of NaBu has been confirmed in multiple cancer types (11,22,23). The present study explored the NaBu’s cytotoxicity in NPC cells by MTT assay. 5-8F and 6-10B cells were treated with NaBu at different concentrations and exposure times. Under low concentrations (1 mM) and short exposure times (24 h), there were no significant differences in cell viability between the blank and the NaBu-treated group (Fig. 1C). However, the cytotoxicity of NaBu towards 5-8F and 6-10B cells increased with higher concentrations (5 and 10 mM) and longer exposure times (48 and 72 h) (Fig. 1C). Therefore, NaBu was cytotoxic to NPC cells, inducing a dose- and time-dependent decrease in cell viability, in both 5-8F and 6-10B cells.

NaBu induces NPC cells apoptosis by activating the mitochondrial pathway. Apoptotic induction is one of the common mechanisms responsible for the anti-tumor roles of NaBu (22,23). In the present study, obvious apoptosis was observed in 5-8F and 6-10B cells, as demonstrated by flow cytometry analysis (Fig. 2A and B). In accord with the results of the MTT assay, the apoptotic rate of NPC cells treated with NaBu also exhibited a dose- and time-dependent pattern (Fig. 2A and B). Subsequently, to identify the underlying mechanisms, the expression of proteins involved in the mitochondrial apoptosis pathway, an important signaling axis in apoptosis regulation, were detected. Downregulated expression of anti-apoptotic proteins, including Bcl-2, Bcl-xL, Mcl1 and survivin, was observed in 5-8F and 6-10B cells treated with 5 mM NaBu (Fig. 2C). Bax, one of the pro-apoptotic proteins, was downregulated in the NaBu-treated group (Fig. 2C). Furthermore, c-Casp3 and c-PARP, two of the apoptotic executors, were upregulated, while Casp3 was correspondingly downregulated in the NaBu-treated group (Fig. 2C).

Table I. Primer sequences for quantitative polymerase chain reaction.

<table>
<thead>
<tr>
<th>Gene names</th>
<th>Primer sequences</th>
<th>Product sizes</th>
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<tbody>
<tr>
<td>Orai1</td>
<td>Forward: 5'-GACTGGGATCGGCCAGAGTTAC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GTCCGGCTGGAGGCTTTAAG-3'</td>
<td>116 bp</td>
</tr>
<tr>
<td>Stim1</td>
<td>Forward: 5'-AGTCACAGTGAGAAGGCAC-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CAATTCGCAAAACTCTGCTG-3'</td>
<td>79 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-CAGCAAGAGCGCAAGGGA-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-ATGGTACATGACAAGGTCGG-3'</td>
<td>157 bp</td>
</tr>
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Orai1, calcium release-activated calcium channel protein 1; Stim1, stromal interaction molecule 1.

(HRP)-conjugated anti-rabbit immunoglobulin G secondary antibody was purchased from Sigma-Aldrich (Merck Millipore).

Cells were disrupted with radioimmunoprecipitation assay buffer (Vazyme), and the supernatants containing total proteins were isolated by high-speed centrifugation at 4°C and 13,000 x g for 20 min. Then, the protein concentration was determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. Following separation by 12% SDS-PAGE (40 µg/lane), the proteins were electrophoretically transferred to polyvinylidene difluoride membranes using a wet transfer system (Bio-Rad Laboratories, Inc.). Subsequently, the membranes were subjected to blocking with milk, incubation with the primary antibodies (all 1:200 dilution) at 4°C overnight, and incubation with the secondary antibody (1:5,000 dilution) for 1 h at room temperature. Finally, the immunoreactive bands were subjected to blocking with milk, incubation with the primary antibodies (all 1:200 dilution) at 4°C overnight, and incubation with the secondary antibody (1:5,000 dilution) for 1 h at room temperature. Finally, the immunoreactive bands were developed with a chemiluminescent HRP substrate (Merck Millipore).

Intracellular Ca2+ measurements. Ca2+ measurements were conducted according to a published study (17). Briefly, the cells were collected and loaded with 5 µM Fluo 3-acetoxyethyl (AM) (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 30 min at 37°C. After washing with Hanks’ balanced salt solution (Ca2+ free) for three times, the cells were incubated at 37°C for another 20 min to allow de-esterification of Fluo 3-AM. Subsequently, the cells were treated with different concentrations of agents and immediately subjected to flow cytometry analysis to detect the intracellular Ca2+ levels. 2-Aminoethoxydiphenyl borate (APB), a SOCE specific inhibitor, and ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a Ca2+ chelator, were both purchased from Sigma-Aldrich (Merck Millipore).

Statistical analyses. Statistical analyses were conducted with SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA) using the Student’s t-test, and all the experiments were independently performed in triplicate. P<0.05 was considered to indicate a statistically significant difference.
Taken together, these results indicate that NaBu could induce the apoptosis of NPC cells by activating the mitochondrial apoptosis pathway.

**SOCE inhibition can attenuate the apoptosis induced by NaBu.** 
Ca\(^{2+}\), as a key important second message, plays vital roles in cell signaling transduction processes such as apoptosis regulation (13). NaBu could activate the SOCE process in colon cancer cells to initiate apoptosis (17). In the present study, similar results were revealed in NPC cells. Influx of extracellular Ca\(^{2+}\) was observed in 5-8F cells immediately upon NaBu addition (Fig. 3), and similar outcomes were observed in 6-10B cells (data not shown). The Ca\(^{2+}\) influx was inhibited when EGTA, a Ca\(^{2+}\) chelator, or 2-APB, a SOCE inhibitor, were added together with NaBu. Correspondingly, the apoptosis induced by NaBu was attenuated by EGTA and 2-APB, as demonstrated by the lower apoptotic rates of the EGTA/2-APB-treated group compared with those of the NaBu alone-treated group (Fig. 4A and B), and by the increased levels of Bcl-xL and decreased levels of c-PARP in the EGTA/2-APB-treated group compared with those in the NaBu alone-treated group in western blot analysis (Fig. 4C). Therefore, the SOCE process may be involved in the NaBu-induced apoptosis of NPC cells.

**Disruption of the CRAC channel can also attenuate the apoptosis induced by NaBu.** The CRAC channel is one of the major pathways of SOCE progression (14). The present study successfully knocked down the expression of Orai1 and Stim1, two key components of the CRAC channel, in 5-8F and 6-10B cells (Fig. 5). Then, the Ca\(^{2+}\) influx induced by NaBu was analyzed, and it was observed that the Ca\(^{2+}\) influx was attenuated in 5-8F cells with downregulated Orai1 and Stim1 expression (Fig. 3). Similar results were obtained in 6-10B cells with downregulated Orai1 and Stim1 expression (data not shown).
The apoptotic rates were decreased in NPC cells with downregulated Orai1 and Stim1 expression compared with those in the control group (Fig. 4A and B). Furthermore, the extent of fluctuations in Bcl-xL and c-PARP expression levels were decreased in NPC cells with downregulated Orai1 and Stim1 expression compared with those in the control group (Fig. 4C). Thus, based on these results, the SOCE process appears to be necessary for the apoptosis induced by NaBu in NPC cells.

**Discussion**

It is well known that epigenetic aberrations contribute substantially to the onset and progression of human diseases, particularly cancers. HDACs, which are key epigenetic regulators, have been demonstrated to be involved in cancer progression (6,8). Overexpression of HDACs displayed a negative correlation with disease-free survival and OS, and could serve as a biomarker for prognosis prediction in multiple cancer types, including prostate (24), colorectal (25) and breast cancer (26). Thus, HDACis, which can inhibit the enzymatic activities of HDACs, are promising agents for cancer treatment. Currently, three HDACis, vorinostat, romidepsin and belinostat, have been approved for the treatment of cutaneous T-cell lymphoma and peripheral T-cell lymphoma (8).

NaBu, as a potent HDACi, has been confirmed to be capable of exerting anti-tumor roles in various cancers. For
example, NaBu treatment could induce growth inhibition and morphological transformation in prostate cancer (21,23), breast cancer (27) and colorectal cancer (9). NaBu has been demonstrated to inhibit interferon-γ-induced indoleamine 2,3-dioxygenase expression in NPC cells (28). However, its cytotoxicity towards NPC cells was rarely studied. In the present study, NaBu exhibited significant cytotoxicity in NPC cells in a dose- and time-dependent manner, and inhibited their proliferation ability. Furthermore, NaBu treatment also induced morphological changes in NPC cells. Consequently, NaBu was concluded to be cytotoxic to NPC cells, in accordance with its known functions in other cancers.

Apoptosis induction is one of the common mechanisms of anti-tumor agents (29). Apparent cell apoptosis both through the death receptor (extrinsic)- and the mitochondrion (intrinsic)-mediated pathways, has been observed in multiple cancer cells treated with NaBu (9,22,30). For example, NaBu can activate mitochondrial apoptosis, as demonstrated by the expression changes in the Bcl-2 family of proteins, including upregulation of the pro-apoptotic Bcl-2 homologous antagonist/killer and Bax, and downregulation of the anti-apoptotic Bcl-2 and Bcl-xL (9). In the present study, flow cytometry analysis revealed that NaBu can also induce significant apoptosis in NPC cells. Furthermore, the decreased expression of anti-apoptotic proteins, including Bcl-2, Bcl-xL, Mcl-1 and survivin, in combination with the increased expression of pro-apoptotic proteins such as Bax and apoptotic executors such as c-Casp3 and c-PARP, confirmed the activation of the mitochondrial apoptosis axis in NPC cells treated with NaBu.

Therefore, the present results revealed that the activation of mitochondrial apoptosis involves the anti-tumor roles of NaBu in NPC.

The important roles of Ca²⁺ and its ion channels in apoptotic regulation have been illustrated in numerous studies (31-33). A sustained increase in cytosolic Ca²⁺ activity triggers apoptosis (34). The roles of SOCE, a major calcium-entry pathway for non-excitatory cells, and those of the CRAC channel, a key channel in mediating SOCE, in apoptotic regulation appear to be paradoxical (7,16,17,34,35). Indeed, SOCE can serve as a pro-apoptotic or an anti-apoptotic factor in cancerous cells under different conditions (7,16,17). For example, enhanced SOCE resulted from upregulated Orai1 and Stim1 expression, which was observed in drug-resistant cancer cells, and impairing SOCE by using specific inhibitors or by knocking down Orai1 and Stim1 can sensitize the drug-resistant cells to chemotherapy (34,35). In addition, enhanced SOCE commonly occurs during the cell apoptosis induced by anti-tumor or chemotherapeutic agents (17). The use of SOCE inhibitors or knocking down the expression of Orai1 or Stim1 can counteract the apoptosis induced by anti-tumor agents (17). The use of SOCE inhibitors or knocking down the expression of Orai1 or Stim1 can counteract the apoptosis induced by anti-tumor agents (17).

In the present study, SOCE appeared to serve as a pro-apoptotic factor in NPC cells treated with NaBu, and inhibition of SOCE could attenuate the NaB-induced apoptosis in NPC cells, in accordance with previous results in colon cancer (17). Differences of SOCE in apoptosis may contribute to the dual roles of Ca²⁺ in signaling transduction:
Figure 4. Inhibition of store-operated Ca\(^{2+}\) entry could attenuate the apoptosis induced by NaBu in NPC cells. (A and B) Flow cytometry analyses revealed that the NaBu-induced apoptotic rates were decreased by 2-APB, EGTA, and downregulation of Orai1 and Stim1. *P<0.05, experimental groups compared with blank groups. The numbers in the quadrants correspond to percentages. (C) Western blot analysis demonstrated that NaBu-induced apoptosis was attenuated, as demonstrated by the lesser extent of fluctuations in Bcl-xL and c-PARP expression levels in NPC cells with downregulated Orai1 and Stim1 expression compared with those in the control group. NPC, nasopharyngeal carcinoma; FITC, fluorescein isothiocyanate; NaBu, sodium butyrate; Orai1, calcium release-activated calcium channel protein 1; Stim1, stromal interaction molecule 1; si, small interfering; 2-APB, 2-aminoethoxydiphenyl borate; EGTA, glycol-bis(\(\beta\)-aminoethyl ether)-N,N,N',N'-tetraacetic acid; c-PAPR, cleaved poly ADP ribose polymerase; Bcl-xL, B-cell lymphoma-extra large.

Figure 5. Construction of nasopharyngeal carcinoma cells with downregulated Orai1 and Stim1 expression. 5-8F and 6-10B cells with downregulated Orai1 and Stim1 expression were obtained by RNA interference. Orai1, calcium release-activated calcium channel protein 1; Stim1, stromal interaction molecule 1; si, small interfering.
On one hand, a physiological Ca\(^{2+}\) concentration is necessary to activate signaling pathways that promote cell growth, survival and metastasis, which are the hallmarks of cancers; on the other hand, overloaded Ca\(^{2+}\) concentrations would also induce apoptosis through the activation of apoptotic pathways (12,13,31,33). Therefore, the anti-apoptotic role of enhanced SOCE in untreated cancer cells is to maintain their own characteristics, which must be regulated and controlled by upstream proteins (13,34). However, the enhanced SOCE observed in the pro-apoptosis of cancer cells treated with anti-tumor agents may be due to the impaired upstream control signaling disrupted by anti-tumor agents, which leads to a sustained increase in intracellular Ca\(^{2+}\) levels and triggers apoptosis.

In conclusion, the present study demonstrated that NaBu was able to trigger hyperactivated SOCE and subsequently induce the mitochondrial apoptosis pathway, which may underlie the mechanisms of its cytotoxicity on NPC cells. The results of the present study suggested that HDACs like NaBu may serve as promising chemotherapeutic or adjuvant chemotherapeutic agents in NPC therapy.

Acknowledgements

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