Anticancer effects of sodium butyrate on hepatocellular carcinoma cells *in vitro*

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Abstract. In the present study, we investigated the anticancer effects of sodium butyrate (NaBu) on hepatocellular carcinoma (HCC) cells *in vitro*. As a histone deacetylase (HDAC) inhibitor, NaBu upregulated Ac-H3 and inhibited HDAC4 protein expression in a time- and dose-dependent manner. MTT assays showed that treatment with NaBu at high concentrations significantly inhibited the growth of various HCC cells. Exposure to NaBu for 24 h induced cell cycle arrest in the SMMC-7721 and HepG2 cells. NaBu also induced the apoptosis of SMMC-7721 cells. The expression levels of cell cycle- and apoptosis-related proteins were further investigated by western blot analysis using specific antibodies. The results provided a possible mechanism responsible for the inhibitory effects of NaBu on the growth of HCC cells. To further analyze the role of NaBu in cell migration, wound healing and Transwell assays were performed, indicating that NaBu significantly inhibits cell migration/invasion in HCC cells. Transforming growth factor-β1 (TGF-β1)-induced epithelial to mesenchymal transition (EMT) has been associated with tumor cell migration and invasion. The EMT markers, E-cadherin, vimentin and N-cadherin, were regulated by TGF-β1, while NaBu inhibited this process in which HDAC4 and matrix metalloproteinase (MMP)7 may be involved. Based on our findings, we propose that NaBu may be useful as an anticancer drug for HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. Liver cancer in males is the fifth most frequently diagnosed cancer worldwide and the second most frequent cause of cancer-related mortality (1). Half of these cases and deaths have been estimated to occur in China. It is also the second greatest cause of cancer-related deaths in China (2). Tumor metastasis is considered to be the main determinant in HCC patient survival; however, the mechanisms involved have not yet been fully elucidated. Epithelial to mesenchymal transition (EMT) is an important step in the invasion and metastasis of cancer and transforming growth factor-β1 (TGF-β1) induces the progression of cancer through EMT. It has been shown that TGF-β1 plays a key role in modulating HCC aggressiveness by triggering EMT (3). Therefore, effective drugs targeting the TGF-β1 signaling pathways that correlate with EMT have the potential to provide a novel approach for the prevention and treatment of the early metastasis of HCC and may thus improve prognosis. Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl from lysine residues in histones and other proteins, causing gene transcriptional repression and subsequent changes in signaling events. Eighteen HDACs, which have been shown to have unique functions, have been identified in mammals. Previous studies have demonstrated that HDAC inhibitors suppress cancer cell invasion and metastasis by regulating the subsequent gene transcription that causes invasion and migration (4,5). However, it is unclear as to which members of the HDAC family are associated with the regulation of cancer cell invasion and migration. HDAC4, a member of class II HDACs, has been shown to play an important role in regulating cell migration and invasion in ovarian cancer cells (6). A high HDAC4 expression has also been observed in liver cancer (7,8); however, it remains unclear whether HDAC4 participates in EMT in HCC cells.

Short chain fatty acids (SCFAs) have been reported as effective HDAC inhibitors with multiple effects on cell growth, differentiation and apoptosis in a variety of cancer cells (9,10). Sodium butyrate (NaBu), one of the sodium salts of SCFAs, is naturally produced in the colonic lumen as a consequence of the microbial degradation of dietary fibers (11). As an HDAC inhibitor, first reported in 1978 (12), NaBu has shown great potential in cancer treatment and prevention (13). It has been demonstrated that NaBu can cause cell cycle arrest, trigger cell apoptosis and induce cell differentiation in multiple cancer cells lines derived from the colon, breast, liver, etc. (14-18). Mechanism analysis has further demonstrated that NaBu selectively regulates gene expression by inducing chromatin

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hyperacetylation, which contributes to its diverse physiological roles. The potential effects of NaBu specifically on HCC have been investigated in a number of studies. It has been reported that NaBu suppresses cell growth and reduces the invasive ability of HCC cells (19,20). However, the mechanisms responsible for these effects remain largely illusive. This prompted us to investigate the effect of NaBu on TGF-β1-induced EMT, which may provide a reasonable explanation for the inhibition of tumor invasion and metastasis.

In the present study, we examined the possible role of NaBu in the regulation of HCC cell proliferation, apoptosis, migration and invasion. Our results show that NaBu inhibits of the proliferation, promotes apoptosis and suppresses the invasion of SMMC-7721 and HepG2 cells by inhibiting TGF-β1-induced EMT, suggesting that NaBu may have therapeutic value in HCC.

Materials and methods

Cell lines and cell culture. The human HCC cell lines, HepG2, SMMC-7721, and the L02 human normal liver cell line were purchased from the China Center for Type Culture Collection (CCTCC). The MHCC97H and MHCC97L cell lines were purchased from the Liver Cancer Institute of Fudan University (Shanghai, China). The HepG2, MHCC97H, MHCC97L and L02 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco), while the SMMC-7721 cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/ml of penicillin and 100 μg/ml of streptomycin in a 5% CO_2 incubator (Thermo Scientific, Barrington, IL, USA).

Antibodies and reagents. The anti-GAPDH antibody used to evaluate the equal loading of protein samples was purchased from Abmart, Inc., Shanghai, China. E-cadherin antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Vimentin and N-cadherin antibody were purchased from Abcam (Cambridge, UK). p21, p27, survivin, Bad, Bcl-2, Bcl-xL, caspase-9 and HDAC4 antibodies were purchased from Signalway Antibody Co., Ltd., College Park, MD, USA. Matrix metalloproteinase (MMP)7 antibody was purchased from RayBiotech (Norcross, GA, USA). The goat anti-mouse and goat anti-rabbit HRP-anchored secondary antibodies were purchased from Zhongshan Goldenbridge Biotechnology, Beijing, China. NaBu was purchased from Sigma-Aldrich (St. Louis, MO, USA), diluted with PBS to a concentration of 100 mM and then stored at 4°C until use. Recombinant human TGF-β1 was purchased from PeproTech (Rocky Hill, NJ, USA), and reconstituted at 10 μg/ml in sterile PBS containing 0.1% bovine serum albumin. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was purchased from Fluka, Inc., USA. Annexin V-FITC apoptosis detection kit, propidium iodide (PI) staining solution and Matrigel were from BD Biosciences (Franklin Lakes, NJ, USA). RPMI-1640, DMEM and FBS were from Gibco/Life Technologies, Paisley, UK.

Cell viability assay. The effect of NaBu treatment on the viability of different cells was measured by MTT assay based on the ability of live cells to cleave the tetrazolium ring to a molecule that absorbs at 570 nm. Cells ($1x10^4$) 200 μl/well were grown in 96-well plates for 24, 48 and 72 h. The cells were further incubated with various concentrations of NaBu in DMEM or RPMI-1640 supplemented with 10% FBS. After 24, 48 and 72 h, 20 μl MTT solution (5 mg/ml) were added to each well, and the cells were further incubated at 37°C for 4 h; subsequently, 150 μl of buffered DMSO were added to each well. The absorbance was recorded on a microplate reader at a wavelength of 570 nm. Untreated cells were used as the controls. At least 3 replicate experiments were performed with 6 wells per concentration.

Cell cycle and apoptosis analysis by flow cytometry. Cell cycle analyses were carried out using flow cytometry. SMMC-7721 and HepG2 cells ($1x10^6$) were seeded in 6-well plates and allowed to attach overnight. Growing cells were treated with various concentrations of NaBu and harvested by 0.25% trypsin (Beyotime Institute of Biotechnology, Haimen, China) at the indicated time points. The cells were then washed twice with PBS, centrifuged at 1,000 rpm for 5 min, and the cells were washed again with PBS, treated with 100 μg/ml RNase, and stained with 50 μg/ml PI (BD Biosciences, USA) in the dark. Flow cytometry analyses were carried out on a FACScalibur instrument using the ModFit program (Becton-Dickinson, Franklin Lakes, NJ, USA). Apoptosis was detected with an Annexin V-FITC/PI detection kit (BD Biosciences). Cells (10,000) per sample were analyzed with a FACScalibur flow cytometer.

Wound healing assay. Cells were seeded into a 6-well tissue culture dish and allowed to grow to 90% confluency in complete medium. Cell monolayers were wounded by a plastic tip (200 μl tip) that touched the plate as previously described (21). Wounded monolayers were then washed 3 times with medium to remove the cell debris and incubated in medium with various concentrations of NaBu for 24, 48, 72 or 96 h, then the distance of cell migration was determined under an inverted microscopy at various time points. The relative migration distance was calculated by the following formula: relative migration distance (%) = average migration distance of treated group/average migration distance of untreated group x100%. Experiments were repeated at least 3 times.

Transwell assay. The invasion assays were carried out using Transwell chamber with 6.5 mm diameter polycarbonate filters (8 μm pore size; Corning Costar, Rochester, NY, USA) coated with Matrigel (BD Biosciences) in a standard manner. Cells were trypsinized and suspended at a final concentration of $1x10^6$ cells/ml in DMEM or RPMI-1640 medium. Before seeding, the cells were treated with various concentrations of NaBu for 24 h. Cell suspension was loaded into each of the upper wells. Fetal calf serum (5%) was used as a chemoattractant in the lower chambers. Before seeding, the cells were incubated for 36 h at 37°C in an atmosphere of 5% CO_2/95% air, followed by a further incubation under the same conditions; all of the non-invaded cells were then removed from the upper face of the Transwell membrane with a cotton swab. The invaded cells were fixed with 100% methanol and then stained with crystal violet (Nanjing Sunshine Biotechnology, Nanjing, China). The invaded cells were counted microscopically. Ten fields were counted for each assay.

Reverse transcription PCR. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using a reverse transcription reagent kit (Fermentas, Burlington, ON, Canada). Subsequently, cDNA was used to perform PCR using specific gene primers under the condition of 95˚C (3 min), followed by 35 cycles of 94˚C (30 sec), 48˚C for vimentin, 52˚C for N-cadherin and 58˚C for E-cadherin, GAPDH (30 sec) and 72˚C (1 min), then finally 72˚C (5 min). GAPDH was employed as an endogenous control to normalize the amount of total mRNA in each sample. The PCR products were examined by 1.5% agarose gel electrophoresis, and the abundance of each mRNA was detected. The reverse transcription PCR reactions were performed in triplicate. The primer sequences were as follows: E-cadherin sense, 5'-CGTAGCAGT GACGAATGTGGTAC-3' and antisense, 5'-AACTGGAGAA CCATTGTCTGTAGC-3'; vimentin sense, 5'-CCAAACTTTT CCTCCCTGAACC-3' and antisense, 5'-GTGATGCTGAGAA GTTTCGTTGA-3'; N-cadherin sense, 5'-AGCCAACCTTAA CTGAGGAGT-3' and antisense, 5'-GGCAAGTTGATTGG AGGGATG-3'; GAPDH sense, 5'-ATGGGGAAGGTGAA GGTCG-3' and antisense, 5'-GGGTCATTGATGGCAAC AATATC-3'.

Western blot analysis. For the western blot analysis, cells were collected and lysed with 1% SDS to break down the membranes, and subsequently, whole proteins were heated at 95˚C for 10 min to inactivate the proteases. The samples were centrifuged for 15 min, and the protein concentration of each sample was assessed using BCA reagent (Beyotime Institute of Biotechnology). Equivalent amounts of protein were loaded into each lane and separated using SDS-PAGE on 10 or 12% gels (Bio-Rad, Hercules, CA, USA) and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA) by wet transfer (Bio-Rad) and blocking with 5% non-fat milk for 1 h. Finally, the membranes were incubated with the specific primary antibody overnight at 4˚C. After washing the membranes with Tris-buffered saline containing Tween-20 (TBST) 3 times for 10 min each, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h and then washed 3 times with TBST again. The target protein bands were visualized using an ECL reagent kit (Thermo Scientific) and exposed to an X-ray film (Kodak).

Statistical analysis. All values are expressed as the means ± SE. One-way ANOVA followed by the LSD t-test and SNK-q test were used to compare differences between groups. A two-sided P-value <0.05 was considered to indicate a statistically significant difference.

Results

Effects of NaBu on Ac-H3 and HDAC4 expression in HCC cells. As an HDAC inhibitor, NaBu was able to induce histone hyperacetylation. The chemical structure of NaBu is shown in Fig. 1A. In our study, we detected the activity of NaBu in regulating histone H3 acetylation. SMMC-7721 and HepG2 cells were treated with various concentrations NaBu for 24 h before being harvested and subjected to western blot analysis using specific acetylated histone H3 (Ac-H3) antibody. NaBu at a
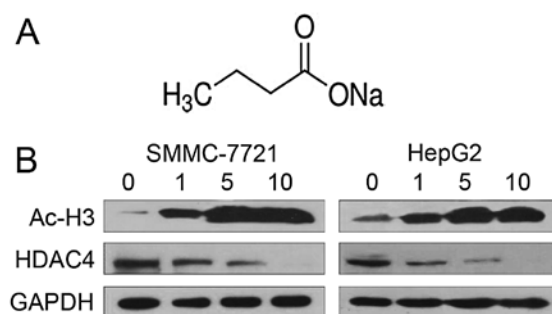
Figure 1. Sodium butyrate (NaBu) and its effect on histone H3 acetylation and HDAC4 protein expression. (A) Chemical structure of NaBu. The molecular formula of NaBu is $CH_3CH_2CH_2COONa$ and the molecular weight is 110. (B) Regulation of Ac-H3 and HDAC4 expression by different concentrations of NaBu treatment in SMMC-7721 and HepG2 cells. Western blot analysis of whole protein extracts from cells treated with 0, 1, 5 and 10 mM NaBu for 24 h. NaBu significantly inhibited HDAC4; however, it increased Ac-H3 protein expression in a dose-dependent manner. GAPDH was used as an internal control.

concentration of 0 to 10 mM (mmol/l) induced H3 acetylation in a dose-dependent manner (Fig. 1B). Western blot analysis using specific HDAC4 antibodies was performed to further analyze the expression of HDAC4 following NaBu treatment. The highly expressed HDAC4 protein in SMMC-7721 and HepG2 cells was significantly downregulated following NaBu treatment in a concentration-dependent manner.

Different inhibitory effects of NaBu on cell proliferation. To determine the effects of NaBu on HCC cells, 4 HCC cell lines, SMMC-7721, HepG2, MHCC97H and MHCC97L, were used in this study. The L02 normal liver cell line was also used. All cells were exposed to various concentrations of NaBu ranging from 0 to 10 mM for 24, 48 and 72 h, and then cell growth curves were recorded using MTT assay. NaBu significantly inhibited the proliferation of SMMC-7721, HepG2, MHCC97H and MHCC97L cells following treatment with NaBu at high concentrations (Fig. 2B-E). Surprisingly, low concentrations of NaBu (0.5 and 1 mM) had no apparent inhibitory effect on the cells at 24 h. However, at 48 or 72 h, low concentrations of NaBu inhibited hepatoma cell proliferation. Furthermore, to compare these effects, the L02 normal liver cell line was also treated with NaBu. We found that the inhibitory effect of NaBu on L02 cells was inferior to that on HCC cells (Fig. 2A). These data suggest that the same concentration of NaBu exerted a stronger inhibitory effect on hepatoma cells than L02 cells, showing low cytotoxicity against normal liver cells at 24 h after treatment at a low dose. These results demonstrate that low concentrations of NaBu have low cytotoxic effects on normal liver cells in the treatment of HCC.

Effects of NaBu on cell cycle distribution. As cell cycle progression is a critical determinant of cell growth, FACS analysis was performed to determine whether NaBu can induce cell cycle arrest. Compared to the untreated control groups, the SMMC-7721 and HepG2 cells treated with NaBu had an increased percentage of cells arresting in the G1 phase at 24 h. In addition, the S phase was significantly inhibited by treatment with 5 and 10 mM NaBu. These data suggest that
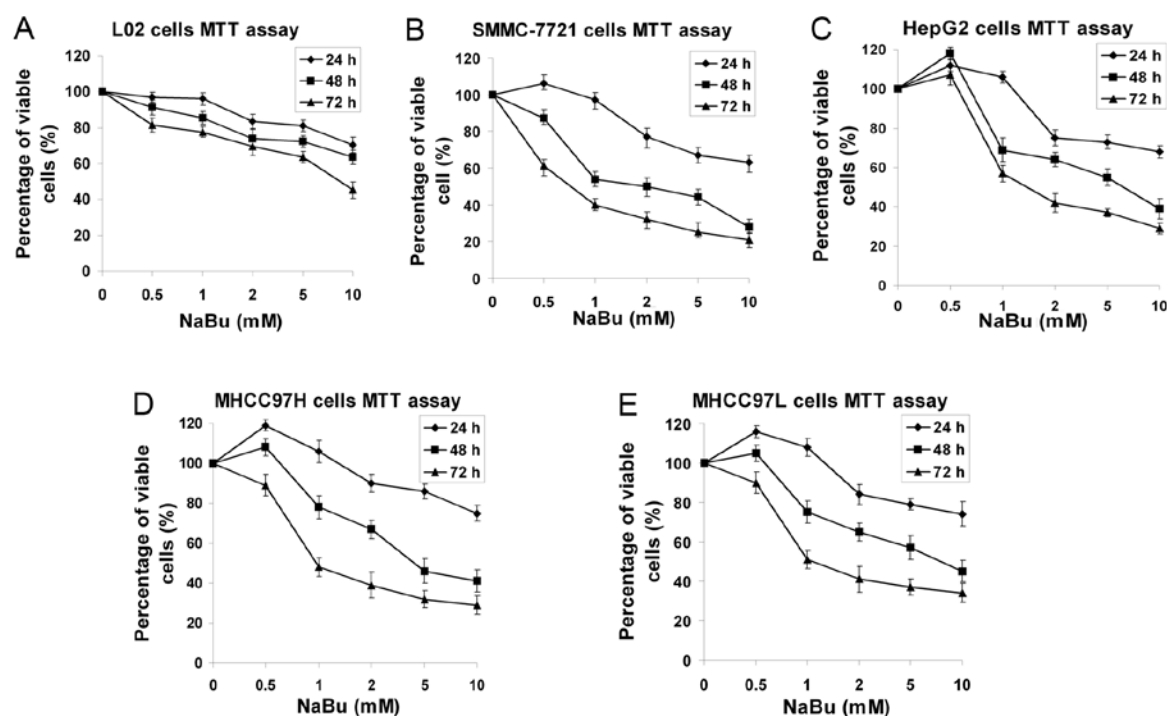
Figure 2. Different inhibitory effects of NaBu on the viability of a normal liver cell line and hepatocellular carcinoma (HCC) cell lines. (A) Inhibitory effect of NaBu on L02 normal liver cell line. (B) Inhibitory effect of NaBu on SMMC-7721 cells. (C) Inhibitory effect of NaBu on HepG2 cells. (D) Inhibitory effect of NaBu on MHCC97H cells. (E) Inhibitory effect of NaBu on MHCC97L cells. All cell lines were cultured in the presence of various concentrations of NaBu ranging from 0 to 10 mM for 72 h. The inhibitory effects of NaBu were demonstrated by MTT assays and the data are presented as the means ± SE (n=5). The percentage of viable cells is expressed as a percentage relative to the untreated control group.

NaBu induces cell cycle arrest in human SMMC-7721 and HepG2 cells (Fig. 3).

Effects of NaBu on cell apoptosis. Apoptosis induced by various concentrations of NaBu in SMMC-7721 cells was analyzed using Annexin V/PI staining. Compared with the control group, the proportion of apoptotic cells increased followed by treatment with various concentrations of NaBu (0, 1, 5 and 10 mM) for 24 h, with early apoptotic rates of 2.7, 7.8, 19.3 and 21.3%, respectively. The percentage of live cells was 93.9, 87.2, 76.1 and 75.0%, respectively (Fig. 4). These results indicated that NaBu induced the apoptosis of SMMC-7721 cells in a concentration and time-dependent manner. In conclusion, NaBu has the ability to exert significant inhibitory effects on a variety of HCC cells based on the regulation of the cell cycle and the induction of apoptosis.

Effects of NaBu on the expression of cell cycle- and apoptosis-related proteins. The expression levels of cell cycle- and apoptosis-related proteins (p21, p27, survivin, Bad, Bcl-2, Bcl-xL and caspase-9) were further investigated by western blot analysis using specific antibodies after 24 h of NaBu treatment. As shown in Fig. 5, high concentrations of NaBu caused a significant increase in p21 and p27 expression; however, 1 mM NaBu slightly reduced p21 expression. Survivin is considered to be involved not only in cell cycle regulation, but also in the inhibition of apoptosis. We observed a significant reduction in survivin protein expression following treatment with NaBu. Members of the Bcl-2 family of proteins are critical regulators of the intrinsic apoptotic pathway. In order to determine

whether NaBu inhibits Bcl-2 family proteins, Bcl-2, Bcl-xL and Bad expression was also detected by western blot analysis. We found that NaBu suppressed the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, in a dose-dependent manner; however, treatment with NaBu increased pro-apoptotic protein Bad expression in SMMC-7721 and HepG2 cells. Following NaBu treatment, caspase-9 expression was also upregulated, suggesting that the apoptotic process was activated in hepatoma cells. These results provide a possible mechanism responsible for the inhibitory effect of NaBu on the growth of HCC cells.

Effects of NaBu on HCC cell migration and invasion. To further analyze the effects of NaBu on the ability of cells to migrate and invade, wound healing and Transwell assays were performed. The wound closure was slowed down in the presence of NaBu in SMMC-7721 and HepG2 cells, as shown by wound healing assay. High concentrations NaBu significantly decreased the cell migration distance (Fig. 6A and B). Furthermore, treatment with NaBu dramatically reduced the invasive ability of the SMMC-7721 and HepG2 cells, as compared with the untreated control group, as shown by Transwell assays (Fig. 6C). Taken together, these data confer that NaBu significantly inhibits the cell migration/invasion of HCC cells.

Inhibitory effects of NaBu on TGF-β1-induced EMT. EMT has been associated with tumor cell migration and invasion. Thus, we further examined the effects of NaBu on EMT. Reverse transcription PCR and western blot analysis were employed to determine whether NaBu exerts its effects by antagonizing TGF-β1-induced EMT. E-cadherin is a universal epithelial
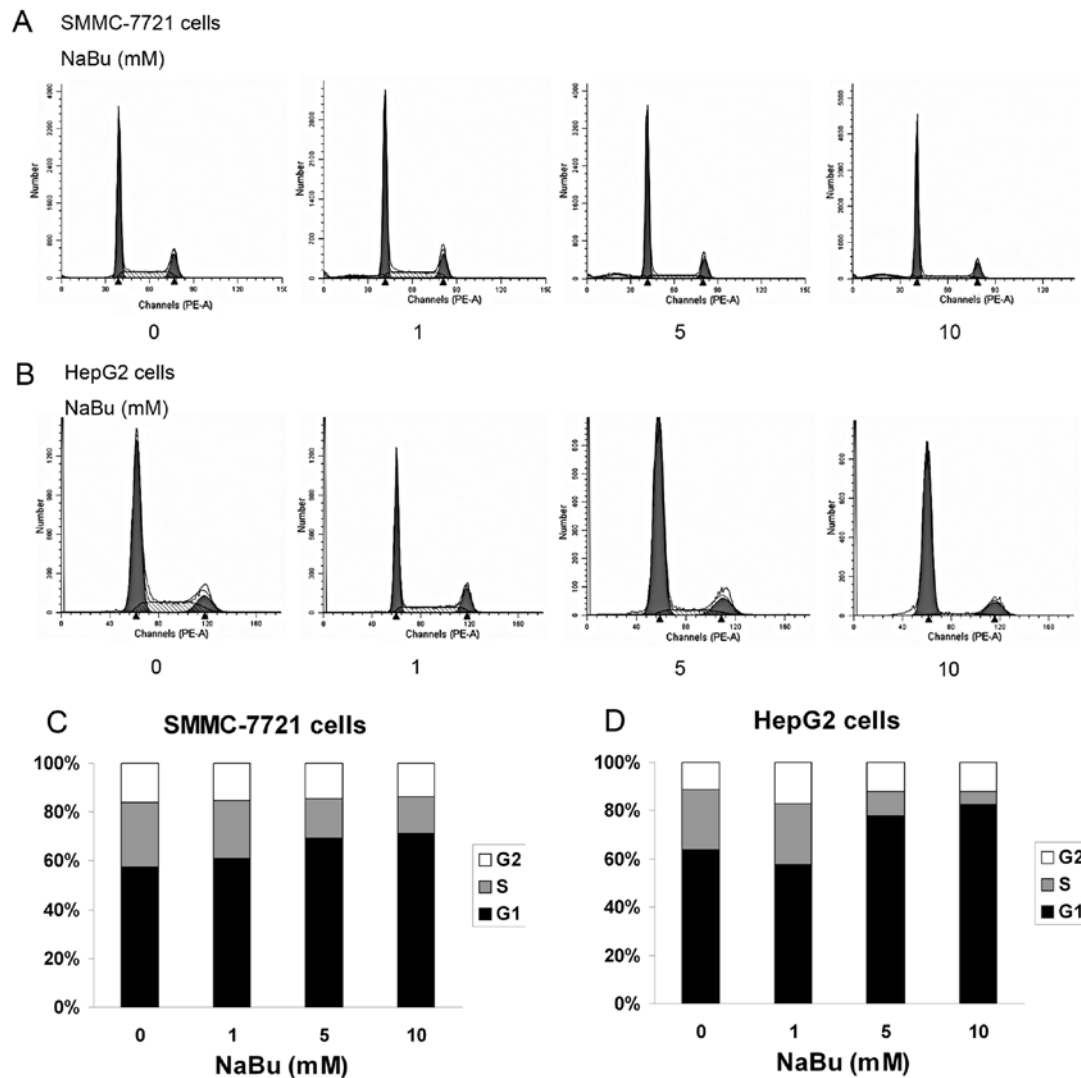
Figure 3. Effects of NaBu on cell cycle distribution. Cells were treated with 0-10 mM NaBu for 24 h before being analyzed by flow cytometry. After incubation with various concentrations of NaBu for 24 h, cells were stained with PI-staining solution containing 50 μg/ml propidium iodide and 100 μg/ml RNase A. Cells in the G1, S and G2 phase of the cell cycle were sorted based on DNA contents, and 20,000 cells were analyzed from each sample. (A) Cell cycle distribution of SMMC-7721 cells treated with various concentrations of NaBu for 24 h. (B) Cell cycle distribution of HepG2 cells treated with or without NaBu for 24 h. (C) Quantification of cell cycle distribution of the SMMC-7721 cell line. (D) Quantification of cell cycle distribution of the HepG2 cell line.
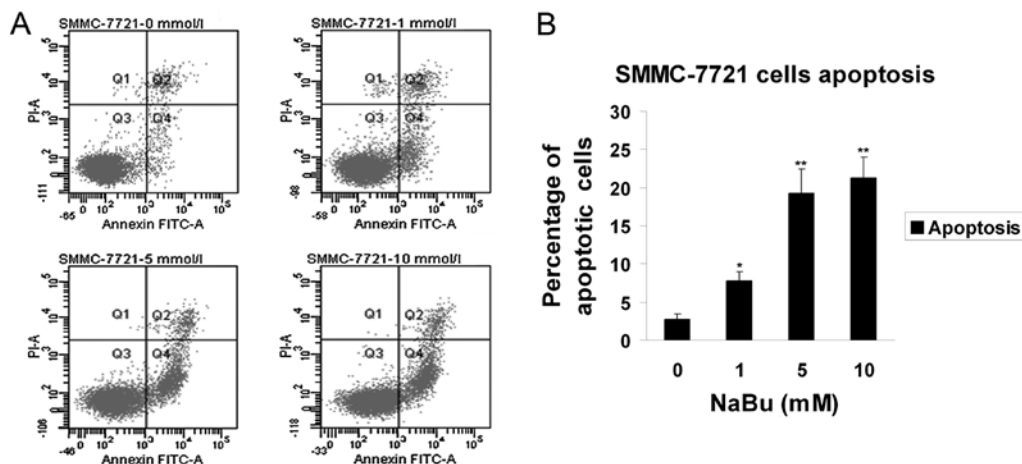

Figure 4. Effects of NaBu on cell apoptosis. After the SMMC-7721 cells were treated with various concentrations of NaBu for 24 h, cells were harvested and stained by Annexin V and propidium iodide (PI) staining. Apoptosis was then analyzed by flow cytometry. Apoptotic cells were Annexin V-positive, PI-negative cells (lower right quadrant). (A) Apoptosis of SMMC-7721 cells treated with various concentrations of NaBu for 24 h. (B) Quantification of cell apoptosis of SMMC-7721 cells following treatment with NaBu 24 h. Representative experiments were carried out at least 3 times. The percentage of apoptotic cells was statistically calculated as the mean ± SE, n=3. *P<0.05, **P<0.01 vs. untreated control group.
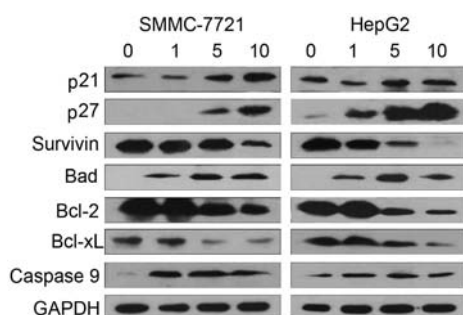
Figure 5. Effects of NaBu on cell cycle- and apoptosis-related proteins. SMMC-7721 and HepG2 cells were treated with NaBu at various concentration (0, 1, 5 and 10 mM) for 24 h. Cell lysates were analyzed by western blot analysis for the cell cycle- and apoptosis-related proteins, p21, p27, survivin, Bad, Bcl-2, Bcl-xL and caspase-9. Representative results from 3 independent experiments are shown.
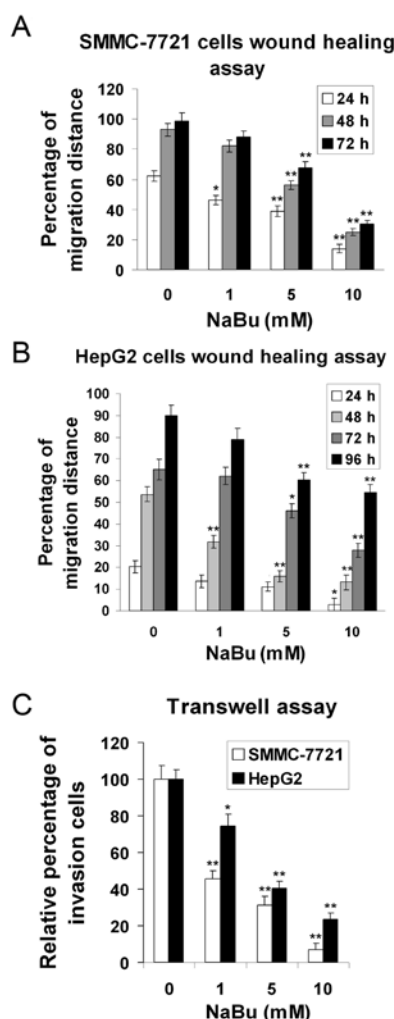

Figure 6. NaBu inhibited hepatocellular carcinoma cell migration and invasion. (A) Quantification of SMMC-7721 cell migration. Percentage of migration distance was calculated by the following formula: percentage of migration distance (%) = 1- gap distance of untreated group/gap distance of treated group x100%. Wound healing assays showed a slower wound closure rate of SMMC-7721 cells treated with higher concentrations of NaBu compared with the untreated control group (0 mM concentration) at different time points (24, 48 and 72 h). (B) The migration of HepG2 cells was also analyzed by wound healing assay. A similar effect was observed in the HepG2 cell line. (C) Cell invasion of SMMC-7721 and HepG2 cells was analyzed by transwell assay. Transwell assay showed a lower invasion rate through the Matrigel-coated membrane in SMMC-7721 and HepG2 cells treated with various concentrations of NaBu as compared with untreated (0 mM) group. The above experiments were performed 3 times independently and the result were plotted.
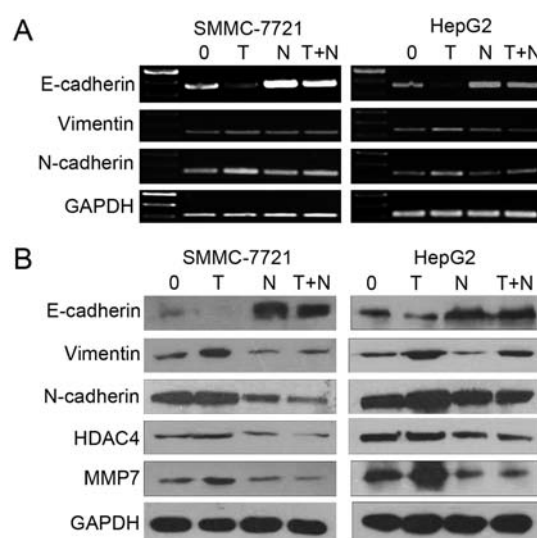

Figure 7. Effects of NaBu on EMT-related genes and protein expression. (A) Expression of E-cadherin, vimentin and N-cadherin mRNAs were analyzed by reverse-transcription PCR in the various treatment groups (0, T, N and T + N). T, 10 ng/ml TGF-β1 treatment group; and T + N, 10 ng/ml TGF-β1 combined with 1 mM NaBu treatment group. (B) Expression of E-cadherin, vimentin, N-cadherin, MMP7 and HDAC4 proteins was analyzed in the different treatment groups in SMMC-7721 and HepG2 cells. GAPDH mRNA and protein were employed as loading controls. The results revealed that the TGF-β1-induced EMT in hepatocellular carcinoma cells was inhibited by NaBu.

marker in the EMT process that plays a key role in the maintenance of cellular integrity at the cell-cell junction. In the present study, NaBu clearly upregulated E-cadherin mRNA and protein expression and aborted the repression induced by TGF-β1 (Fig. 7A). Vimentin and N-cadherin are often used to identify mesenchymal cells and the process of EMT. MMP7, an extracellular matrix protein, is also considered be related to EMT. Further experiments showed that NaBu aborted the TGF-β1-induced upregulation of vimentin, N-cadherin, MMP7 and HDAC4 in the SMMC-7721 and HepG2 cells (Fig. 7B). Taken together, these data suggest that NaBu inhibits TGF-β1-induced EMT in human hepatoma cells, suggesting that NaBu exerts an inhibitory effect on the early metastasis HCC cells.

Discussion

The modification of epigenetic markers, such as DNA methylation or histone acetylation by pharmacological agents has been hypothesized to play a role in cancer progression (22). Epigenetic regulation of gene transcription by histone acetylation has been shown to be a promising new therapeutic target in oncology (22-24). Different patterns of HDAC overexpression have been demonstrated for a variety of human cancers (25-28). HDAC4, a class II HDAC, has been associated with a number of diseases, such as neurodegeneration (29), diabetes (30) and cancer process (6,31). A recent study showed that HDAC4 mRNA was upregulated in human HCC tissues compared with paired adjacent non-cancerous hepatic tissues, as analyzed by real-time PCR (7), suggesting HDAC4 plays a role in HCC progression. An effective inhibitor of HDAC4 may therefore prove useful in the treatment of HCC. NaBu, a HDAC inhibitor, inhibits the majority of HDACs, apart from class III HDACs

and the class II HDACs, HDAC6 and HDAC10 (16). In the current study, we observed that NaBu downregulated HDAC4 expression in an dose-dependent manner in the SMMC-7721 and HepG2 cells, proving NaBu is a potent inhibitor of HDAC4 in HCC cells.

As a HDAC inhibitor, NaBu affects cell behavior through the transcriptional regulation of gene expression. Epigenetic markers, such as histone acetylation and chromatin plasticity may be modified upon NaBu treatment. In present study, NaBu induced histone H3 acetylation in a dose-dependent manner. This was observed in the HepG2 cells, providing a possible mechanism for gene expression regulation, as described above. We then analyzed the effect of NaBu on the L02 normal liver cell line and on 4 HCC cell lines by MTT. We found that NaBu exerted a significant inhibitory effect on the HCC cell lines treated with high concentrations of NaBu. However, no potent inhibitory effect was observed in the normal liver cell line following treatment with low concentrations of NaBu. This indicates that NaBu may exert weaker cytotoxic effects on normal liver cells compared to HCC cells.

We demonstrated that NaBu is highly effective in suppressing the growth of HCC cells. The arrest in the G1 phase of the cell cycle and the reduction in the S phase cell population is likely to account for this effect. The molecular mechanism involved was further examined by western blot analysis. p21 and p27 are cyclin-dependent kinase inhibitors that play important roles in blocking the cell cycle in the G1 phase (32). The protein levels of p21 and p27 were upregulated following treatment with NaBu at high concentrations, suggesting that this upregulation is one of the possible mechanisms by which NaBu inhibits the growth of HCC cells. Apoptosis is an active gene-directed mechanism of cellular suicide, important for the development and homeo-stasis of the multicellular organism. Specific therapies have been designed to enhance the susceptibility of human cancer cells to undergo apoptosis. In this study, we showed that NaBu dramatically and significantly increased the number of early apoptotic cells in the SMMC-7721 cell line. This effect was associated with a decrease in the levels of the anti-apoptotic proteins, survivin, Bcl-2, Bcl-xL and an increase in the levels of the pro-apoptotic proteins, Bad and caspase-9. The present findings raise the possibility that NaBu may prove particularly effective in the treatment of HCC. Thus, NaBu exhibits anti-proliferative activity and potently induces apoptosis in human liver cancer cells.

Tumor invasion and metastasis are the major characteristics of aggressive phenotypes of a variety of human cancers, and, therefore, the major causes of cancer-related mortality. HDAC inhibitors have been shown to be inhibitors of migration and invasion (6,33). In this study, wound healing and Transwell assays showed that a significant decrease in HCC cell migra-tion and invasion occurred following treatment with NaBu at various concentrations. However, the detailed mechanisms involved remain unclear. EMT has been associated with tumor invasion and metastasis (34,35). TGF-β1 can induce typical EMT in a number of cells, including hepatocytes and HCC cells, which has been implicated in hepatic fibrosis, cirrhosis, and HCC invasion and metastasis (36-39). It has been reported that another HDAC inhibitor, trichostatin A (TSA), suppresses the TGF-β1-induced EMT in hepatocytes (40) and renal epithelial cells (41). However, it remains unclear

whether NaBu participates in TGF-β1-induced EMT in HCC cells. Thus, we further detected EMT-related gene expression following treatment with 10 ng/ml TGF-β1 and/or 1 mM NaBu. We observed no significant cytotoxic effects on normal liver cells treated with 1 mM NaBu for 24 h. In the present study, we found that NaBu abolished the downregulation of E-cadherin mRNA and protein expression induced by TGF-β1 in SMMC-7721 and HepG2 cells. The mesenchymal markers, N-cadherin and vimentin, were upregulated by TGF-β1; however, NaBu suppressed this process. Taken together, these results demonstrate that NaBu prevented TGF-β1-induced EMT, thus providing a possible mechanism responsible for its inhibitory effect on migration and invasion. A recent study (6) revealed that apicidin, a HDAC inhibitor, inhibited human ovarian cancer cell migration via HDAC4 silencing, showing that HDAC4 may be involved in EMT in cancer. In this study, the HDAC4 inhibitor, NaBu, inhibited TGF-β1-induced EMT; therefore, we propose that HDAC4 may also participate in TGF-β1-induced EMT. In our study, we found that HDAC4 levels were elevated following treatment with TGF-β1. The upregulated HDAC4 expression was suppressed following treatment with NaBu in the SMMC-7721 and HepG2 cells. It has been substantiated that the TGF-β1 signaling pathway can activate MMP7 expression (42-44) which is able to cleave and shed digestive E-cadherin (42). In our study, we found that NaBu suppressed the TGF-β1-induced upregulation of MMP7 expression in both HCC cells, providing a new mechanism responsible for the inhibitory effect of NaBu on TGF-β1-induced EMT. Taken together, our results showed that NaBu suppressed cell migration and invasion by inhibiting TGF-β1-induced EMT, in which HDAC4 and MMP7 may be involved.

In conclusion, our study demonstrates the inhibitory effects of the HDAC inhibitor, NaBu. These effects include the inhi-bition of cell proliferation, cell cycle arrest, the regulation of TGF-β1-induced EMT and the inhibition of cell migration and invasion in HCC cells. These data suggest that such activities serve as anti-tumor mechanisms of the HDAC inhibitor, NaBu, in which HDAC4 and MMP7 may be involved. However, further research is required. In future studies, we aim to investigate the role of NaBu in animals to verify whether there is a similar anticancer effect. In conclusion, NaBu exerts anti-cancer effects on HCC cells *in vitro*; thus, NaBu may provide a novel strategy for the treatment of HCC.

Acknowledgements

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