Trastuzumab enhances the anti-tumor effects of the histone deacetylase inhibitor sodium butyrate on a HER2-overexpressing breast cancer cell line

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Abstract. Trastuzumab has efficacy to improve the effect of cytotoxic drugs, such as paclitaxel and anthracyclin, against HER2-overexpressing breast cancer cells. Sodium butyrate (NaB), a histone deacetylase inhibitor, is known to have anti-tumoral properties. However, whether and how trastuzumab possesses the potential to synergize the anti-tumor effect of NaB on breast cancer cells is still equivocal. To elucidate whether combined treatment with NaB and trastuzumab exerts anti-tumor effects on a HER2-overexpressing breast cancer cell line, SKBR3 cells were treated with NaB alone or in combination with trastuzumab, and the effects on proliferation and cell cycle progression were analyzed. Combinatory treatment with NaB (4 mmol/l) and trastuzumab (20 μg/ml) significantly increased the growth-inhibitory effect on SKBR3 breast cancer cells, in comparison to NaB or trastuzumab treatment alone. The growth-inhibitory effect of the combination of NaB and trastuzumab was accompanied by elevated mRNA and protein levels of the cyclin-dependent kinase inhibitor $p27^{Kip1}$. In contrast, this effect was absent in HER2-negative HCC1937 cells. In conclusion, trastuzumab significantly improved the antitumor effect of NaB on HER2-overexpressing breast cancer cell line *in vitro*.

Introduction

Breast cancer is second only to lung cancer as a leading cause of cancer death in women worldwide (1,2). As is well known, the *HER2* (c-*ErbB-2*, *ErbB-2*) gene, a marker of increased metastatic potential and decreased overall survival (3,4), is amplified and overexpressed in 20-30% of invasive breast carcinomas. The *HER2* proto-oncogene, a member of the EGFR gene family which also includes *HER1* (*ErbB-1, EGFR*), *HER3* (*ErbB-3*) and *HER4* (*ErbB-4*), encodes a 185 kDa transmembrane glycoprotein (2-4). A recombinant humanized monoclonal antibody, trastuzumab (Herceptin), is directed against the extracellular domain of HER2. Trastuzumab alone or in combination with chemotherapy has been shown to increase both the survival and the response rate of HER2-overexpressing metastatic breast cancers (3,4). The objective response rate (ORR) of trastuzumab monotherapy ranges from 12-34% with a median duration of 9 months, which benefits the majority of breast cancer patients. Furthermore, when trastuzumab is combined with cytotoxic drugs, ORR is up to 50% in breast cancer patients (5).

However, some patients with HER2-overexpressing metastatic breast cancer who initially responded to trastuzumab will be resistant to the therapy and eventually develop disease progression. Resistance to anti-HER2 agents may occur as a result of aberrant activation of signaling pathways downstream of the receptor or the presence of truncated forms of the HER2 receptor that lack the trastuzumab binding domain. Therefore, we should overcome the resistance to develop further treatment strategies for HER2-overexpressing breast cancer (6).

Sodium butyrate (NaB), a short chain fatty acid, belongs to the histone deacetylase inhibitor (HDACi) family. Targeting the zinc-dependent histone deacetylases (Class I and II HDACs), may induce accumulation of acetylated histones, leading to the relaxation of chromatin structure and better access for transcriptional machinery proteins. NaB has potent and specific anti-cancer activities (7). Clinical studies have found that the application of HDACis treatment is effective to hematopoietic malignancies and certain solid tumors, such as epithelial ovarian cancer, primary peritoneal carcinoma, non-small cell lung cancer (NSCLC), glioblastoma multiforme and breast cancer (8).

In this study, we examined the effect of NaB and trastuzumab on proliferation, cell cycle and apoptosis of breast cancer cell lines. Furthermore, the mRNA and protein expression of $p27^{Kip1}$ was analyzed to explore the mechanism of the potential anti-tumor effects of NaB and trastuzumab.

Materials and methods

Cells culture. SKBR3 human breast cancer cells (American Type Culture Collection, Manassas, VA) were maintained in

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monolayer culture in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT). HCC1937 cells were maintained in monolayer culture in DMEM supplemented with 1% vitamins, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1% HEPES buffer (all from Life Technologies, Inc.), and 10% fetal bovine serum (Hyclone, Thremo Scientific).

Reagents. Trastuzumab was kindly provided by Genentech (San Francisco, CA). NaB and MTT were obtained from Sigma; DMSO was purchased from Biosharp; cell cycle and apoptosis analyzing kits were acquired from Invitrogen. The rabbit anti-$p27^{Kip1}$ antibody was purchased from Cell Signaling. The rabbit anti-HER2 antibody and Alexa Fluor 488 donkey anti-rabbit IgG were purchased from Molecular Probes, Inc.

MTT assay. The MTT assay was performed as previously described (7). Cells were seeded at a density of 5,000 cells/well in a 96-well plate in triplicate for each cell line. Cells were incubated with 0, 5, 15, 20 μg/ml trastuzumab, or 0, 2, 4, 6 mmol/l NaB or with the combination of 4 mmol/l NaB plus 20 μg/ml trastuzumab. Cultures were grown at 37˚C with 5% CO_2. Forty-eight hours later, 10 μl of 5 mg/ml MTT was added. Following MTT incubation for 4 h, 100 μl of 100% DMSO was added and the absorbance at 490 nm was obtained using the 96-well plate reader (Dynex Technologies).

Flow cytometry. Cell cycle distribution and apoptosis was analyzed by flow cytometry. In brief, $1x10^6$ cells were harvested, fixed for 30 min in cold ethanol (70%), and resuspended in 1 ml of DNA staining solution. After incubation for 30 min at room temperature, stained cells were analyzed with a FACSCalibur (BD Biosciences, San Jose, CA, USA), and the data were analyzed using a Modfit cell cycle analysis program.

For the apoptosis analysis, after reagent treatment, $1x10^6$ cells were harvested and resuspended in 100 μl 1X Annexin-binding buffer. In the next step 5 μl Alexa Fluor 488 Annexin V and 1 μl of 100 μg/ml PI working solution were added to each 100 μl of cell suspension. Cells were incubated at room temperature for 15 min, then 400 μl of 1X Annexin-bonding buffer was added, and the sample was mixed gently and kept on ice. The fluorescence emission at 530 nm and >575 nm was measured by a FACSCalibur, and the data were analyzed using a WinMDI analysis program.

Reverse transcription-PCR analysis. Total-RNA from the cells was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration of total-RNA was measured by a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, German). PrimeScript™ 1st Strand cDNA Synthesis kit (Takara, Japan) and GoTaq® Green Master Mix (Promega, Madison, WI, USA) were used for single-strand cDNA synthesis and PCR. All procedures were performed in accordance with the manufacturer's instructions. Total-RNA of 2 μg was tipped out for cDNA synthesis and 20 μl reaction volume was used for all further PCR. We used the following PCR conditions: a 94˚C 2 min hot start followed by 94˚C for 1 min, 57˚C for 30 sec, 72˚C for 30 sec for 34 cycles, and a final 10 min 72˚C extension. The following primer combinations were used for $p27^{Kip1}$, forward, 5'-gtcaaacgtaaacagctcgaat-3' and reverse, 5'-tgcata atgctacatccaacg-3'; HER2, forward, 5'-gcagctcatctaccagg agt-3' and reverse, 5'-ggcaacgtagccatcagtct-3'; and GAPDH, forward, 5'-caatgacccttcattgacc-3' and reverse, 5'-tggaagatggt gatgggatt-3'. The PCR products were electrophoresed on 2% agarose gels and detected by ethidium bromide staining.

Immunofluorescence. Endogenous $p27^{Kip1}$ or HER2 subcellular localization was detected in SKBR3 and HCC1937 cells. Cells ($2x10^5$) were prepared and seeded onto chamber slides, then treated with NaB at 4 mmol/l, trastuzumab 20 μg/ml or NaB at 4 mmol/l plus trastuzumab at 20 μg/ml/well for 2 days prior to staining. Cells were then fixed with methanol/acetone (1:1, v/v) at room temperature for 2 min and stained for 24 h at 4˚C with rabbit anti-$p27^{Kip1}$ antibody or rabbit anti-HER2 antibody, followed by 30 min incubation with FITC-conjugated anti-rabbit antibody. For all stainings, cells were incubated with 0.1 mg/ml of 4,6-diamidino-2-phenylindole (DAPI) (Sigma) to stain the nuclei. Immunofluorescence was detected using a BX50 fluorescence microscope (Olympus).

Statistical analysis. Data were expressed as mean ± standard error of the mean. Differences between groups were compared using ANOVA, and LSD was applied for multiple means comparisons. Differences were considered significant when $P<0.05$. Statistical analysis was conducted using the SPSS16.0 software.

Results

Trastuzumab sensitizes breast cancer cells to the growth inhibition of NaB. To investigate the effects of trastuzumab and NaB on cellular proliferation, we analyzed the inhibitory effects of increasing concentrations of reagents on SKBR3 and HCC1937 cells by the MTT assay. Inhibition of the proliferation of SKBR3 and HCC1937 cells by treatment with NaB alone were time- and dose-dependent (Fig. 1A and B). Conversely, there was a slight reduction in the proliferation of SKBR3 cells exposed to 20 μg/ml trastuzumab for 48 h (Fig. 1C), whereas, there was no significant effect on the growth of HCC1937 cells, which are HER2-negative, at all concentrations of trastuzumab between 5 and 20 μg/ml (Fig. 1D). As described in Fig. 1E, the combination of NaB and trastuzumab led to a loss of SKBR3 cells up to ~80%. However, HCC1937 cells were not more sensitive to the combination compared with the single NaB group. These results showed that NaB and trastuzumab could highly inhibit the proliferation of HER2-overexpressing SKBR3 cells.

Trastuzumab enhances the effect of NaB on the cell cycle and apoptosis. Cell cycle assays of SKBR3 and HCC1937 cells induced by NaB and trastuzumab are shown in Fig. 2. Exposure to NaB markedly induced cell arrest in the G_0/G_1 phase. In contrast, trastuzumab alone did not result in any significant differences in the distribution of cells in G_0/G_1 phase (Fig. 2) with the exception of the 20 μg/ml concentration, which resulted in 70.73% of the SKBR3 cells in the G_0/G_1 phase ($P<0.01$) vs. 71.02% in the HCC1937 cells ($P>0.05$). NaB and trastuzumab together strikingly increased the ratio of G_0/G_1 phase in SKBR3 breast cancer cells (Fig. 2) ($P<0.05$ compared to control or single reagent alone), while similar results were not found in HCC1937 cells. In addition, the fraction of proliferating

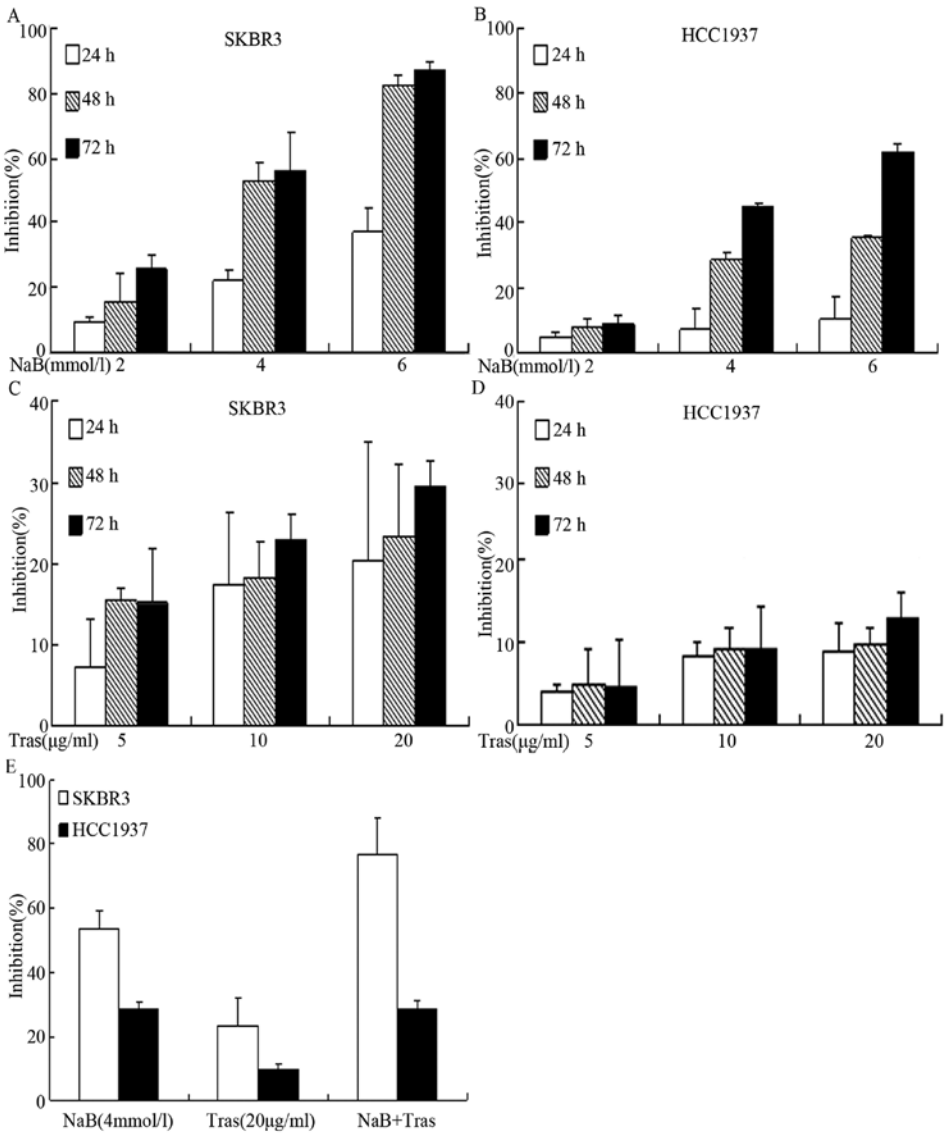

Figure 1. Effect of sodium butyrate (NaB) or/and trastuzumab (Tras) on proliferation of SKBR3 and HCC1937 cells. (A) Treated with different concentrations of NaB, the proliferation of SKBR3 cells was inhibited in a time- and dose-dependent manner. (B) The effect of NaB on the proliferation of HCC1937 cells was also time- and dose-dependent. (C) Incubation with trastuzumab, inhibited the proliferation of SKBR3 cells merely until 20 μg/ml. (D) Trastuzumab exerted a small effect on the proliferation of HCC1937 cells. (E) Compared with NaB alone, the combination of NaB (4 mmol/l) and trastuzumab (20 μg/ml) treatment significantly inhibited the proliferation of SKBR3 cells. However, no similar effect was found in HCC1937 cells. *Indicates statistical significance (P<0.05) compared to control.

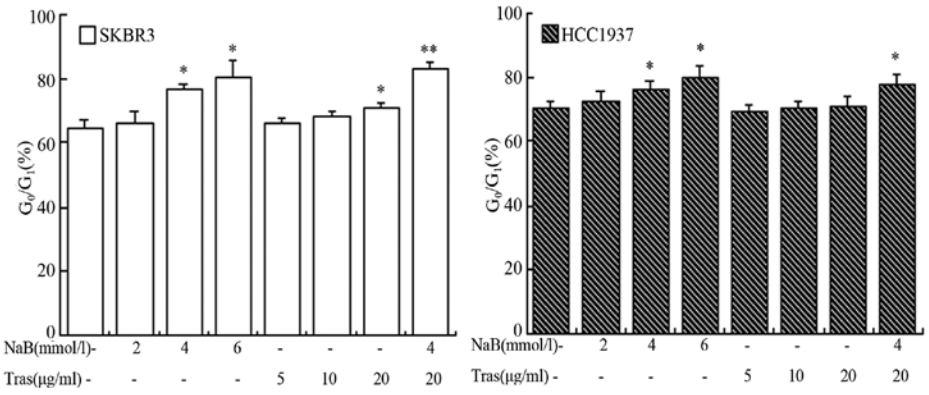

Figure 2. Flow cytometry quantitation of cell cycle of breast cancer cells incubated with sodium butyrate (NaB) or/and trastuzumab (Tras) for 48 h. NaB induced SKBR3 cells and HCC1937 cells G_0/G_1 phase arrest; only trastuzumab at 20 μg/ml had a small effect on the cell cycle arrest of SKBR3 cells, but not of HCC1937 cells. The combination of NaB and trastuzumab increased the fraction of G_0/G_1 phase in SKBR3 cells over that induced by any reagent alone. *Statistical significance (P<0.05) compared to control. **Statistical significance compared to control or any single drug.

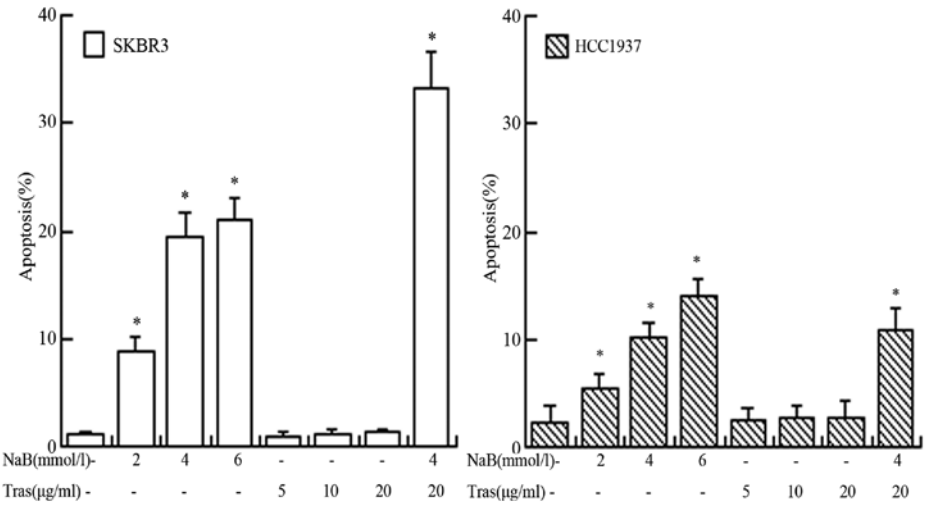

Figure 3. Flow cytometry analysis of the apoptosis rate of SKBR3 and HCC1937 cells treated with sodium butyrate (NaB) or/and trastuzumab (Tras) at the indicated concentrations. NaB induced apoptosis in a dose-dependent manner, however, there was no significant effect of trastuzumab on cell apoptosis. Incubation of trastuzumab enhanced the apoptosis rate induced by NaB in the SKBR3, but not in the HCC1937 cell line. *Statistical significance ($P<0.05$) compared to control.

Table I. $p27^{Kip1}$ mRNA content in SKBR3 and HCC1937 cells after 48 h incubation with the indicated reagents.

	Control	NaB	Trastuzumab	NaB + trastuzumab
SKBR3	80.31±3.80	134.93±9.30[a]	114.35±7.81[a]	160.63±12.36[a]
HCC1937	182.33±3.77	221.20±5.24[a]	170.93±4.94	224.04±5.66[a]

[a]Significant differences from the values obtained in the control group, $P<0.05$. Values are the means ± SE of three experiments.

(S-phase) cells in the combination group was more than either reagent alone group (data not shown). The results suggest that the combination of NaB and trastuzumab inhibited cell cycle progression in SKBR3 cells at a greater degree.

Induction of apoptosis of SKBR3 and HCC1937 cell lines by treatment with NaB alone for 48 h occured in a dose-dependent manner. At the NaB concentrations of 0, 2, 4, 6 mmol/l, the apoptosis rate was 1.1±0.30, 8.93±1.40, 19.46±2.33 and 20.9±2.27%, respectively in SKBR3 cells, vs. 2.41±1.40, 5.45±1.36, 10.36±1.38 and 13.96±1.71% in HCC1937 cells (Fig. 3). However, there was no significant effect of trastuzumab on cell apopotosis. Combination of NaB and trastuzumab for 48 h induced a higher degree of apoptosis in SKBR3 cells (Fig. 3) (33.25% in SKBR3 vs. 10.8% in HCC1937 cells).

Effects of NaB and/or trastuzumab on mRNA and protein levels of $p27^{Kip1}$. To investigate how trastuzumab improved the cell cycle and apoptotic effects of NaB on SKBR3 and HCC1937 cells, we examined the levels of $p27^{Kip1}$ mRNA and protein. First, we detected the mRNA and protein levels of HER2 in the control groups. Higher levels of HER2 mRNA and protein were detected in SKBR3 cells than in HCC1937 cells (Fig. 4A and B). On the other hand, the levels of $p27^{Kip1}$ were much lower in SKBR3 cells than in HCC1937 cells without treatment (Figs. 4C and 5) (Table I). $p27^{Kip1}$ mRNA and protein levels markedly increased in SKBR3 cells and HCC1937 cells with incubation with NaB. Trastuzumab also up-regulated the level of $p27^{Kip1}$ slightly in SKBR3 cells, but had no effect in HCC1937 cells. After co-treatment with NaB and trastuzumab, the expression of $p27^{Kip1}$ mRNA and protein increased notably in comparison to SKBR3 cells treated with NaB alone; however, no similar results were observed in HCC1937 cells, which are HER2-negative (Figs. 4C and D and 5) (Table I). In conclusion, a synergistic effect of the two reagents was found in the transcriptional and translational regulation of $p27^{Kip1}$.

Discussion

The mainstream therapeutic strategies of breast cancer include surgical resection, traditional chemotherapy, endocrinotherapy and trastuzumab-mediated targeted therapy. Previous trials have demonstrated that the sequential addition of trastuzumab to chemotherapy could obviously improve disease-free survival (DFS), overall survival (OS) and event-free survival (EFS) in HER2-positive breast cancer patients (9).

Our present study demonstrates that a combination of NaB and trastuzumab exerts greater growth inhibitory effects than either reagent individually, and that the magnitudes of such effects correlated with the levels of HER2 expression. To elucidate the molecular mechanisms underlying the growth inhibition, we examined the effects of single reagents and the combination on cell cycle and apoptosis. We found that treatment with the combination caused an obvious enhancement

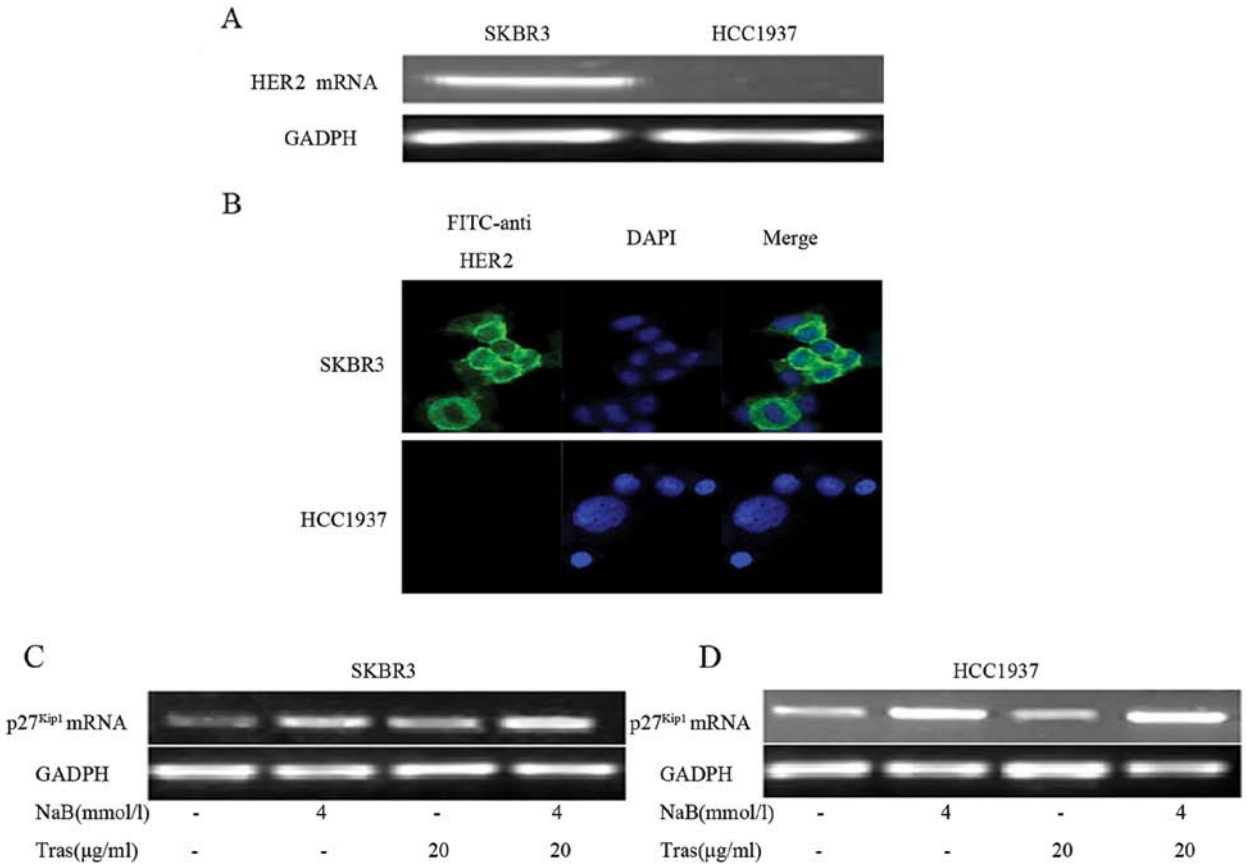

Figure 4. RT-PCR quantitation of $p27^{Kip1}$ mRNA level in SKBR3 and HCC1937 cells treated with sodium butyrate (NaB) or/and trastuzumab (Tras). (A) HER2 mRNA was detected by RT-PCR. Lane 1, SKBR3 cells; lane 2, HCC1937 cells. (B) HER2 protein expression in SKBR3 and HCC1937 cells was detected by immunofluorescence. (C and D) $p27^{Kip1}$ mRNA was detected by RT-PCR in SKBR3 and HCC1937 cell lines after various treatments. Lane 1, control; lane 2, treated with NaB; lane 3, treated with trastuzumab; lane 4, treated with NaB and trastuzumab. GAPDH mRNA expression was used as the loading control.

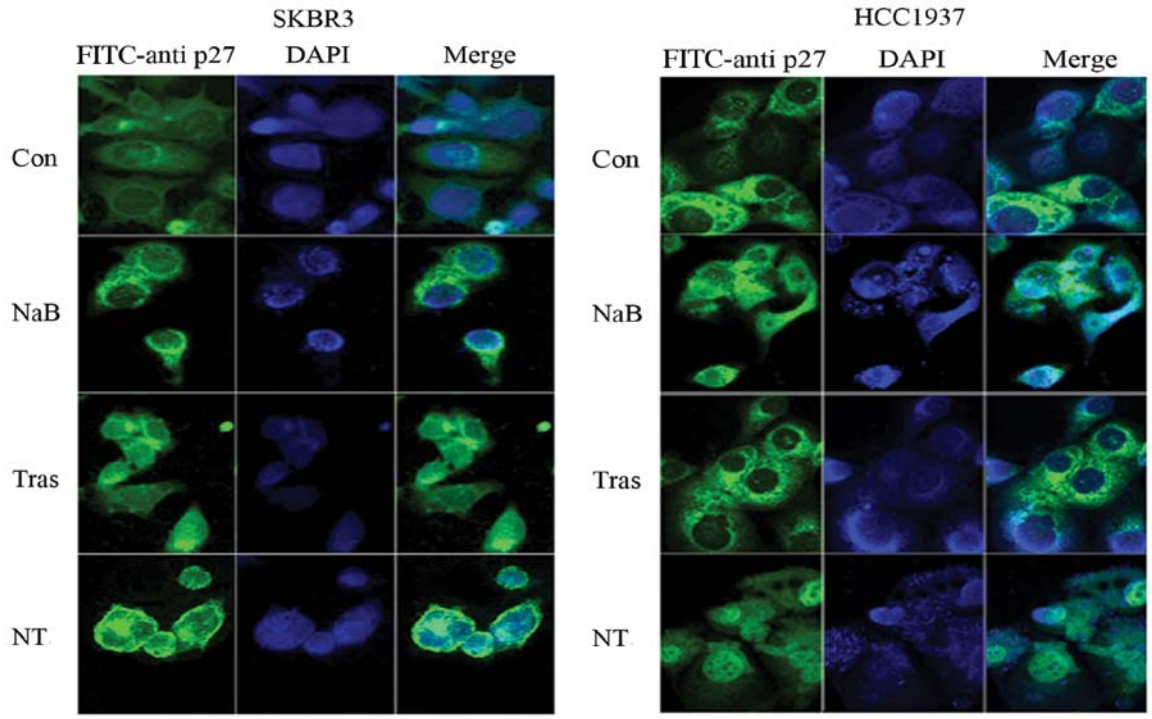

Figure 5. Immunofluorescence staining analysis of the expression of $p27^{Kip1}$ in the SKBR3 (left) and HCC1937 (right) cell lines treated with sodium butyrate (NaB) or/and trastuzumab (Tras). Semi-quantitative analysis demonstrated that treatment with 4 mmol/l NaB or 20 μg/ml trastuzumab for 48 h increased $p27^{Kip1}$ expression in SKBR3 cells, and the up-regulation of $p27^{Kip1}$ expression was more significant in the combination group. However, this effect was not observed in HCC1937 cells, which are HER2-negative.

of induction of cell cycle arrest and apoptosis, which was not observed with single trastuzumab treatment. A previous study reported that a certain concentration of trastuzumab for enough time may decelerate cell cycle progress and induce cells into quiescence by increasing expression of the genes HEC and DEEPEST (10,11), mainly in the G_2/M phase. The discrepancy here is possibly due to the fact that the continuous treatment of trastuzumab in our experiments was not long enough to result in cell cycle progression and apoptosis.

Histone deacetylation inhibitors are anti-neoplastic agents acting by net acetylation of core histones, causing the uncoiling of chromatin and activation of numerous genes implicated in the regulation of cell survival, growth, differentiation, cell cycle arrest and apoptosis (7,8,12,13). The activity of G_1 Cdks is stringently regulated by NaB through association with specific Cdk inhibitors including the CIP/KIP family ($p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p57^{Kip2}$), which could bind all G_1 cyclin-Cdk complexes (8,14). Previous studies have shown that histone deacetylation inhibitor treatment could result in down-regulation of HER2 receptor-mediated signaling, as well as increase of $p27^{Kip1}$ levels and the cyclinE-Cdk2 complex binding (14-17). Trastuzumab also blocks the HER2 downstream signaling to influence the cell cycle and apoptosis (10). In addition, the effects of NaB and trastuzumab co-treatment may affect various pathways to up-regulate $p27^{Kip1}$ or reduce its degradation.

$p27^{Kip1}$, a member of CIP/KIP family, plays a key role in cell proliferation, differentiation and apoptosis regulations. Numerous studies had demonstrated that low level expression of p27 was frequently detected in human cancers and correlated with cancer development and poor survival (18-20). We found that $p27^{Kip1}$ mRNA levels were increased abundantly in the presence of NaB alone in breast cancer cell lines and up-regulated merely in the trastuzumab-treated SKBR3 cell line (Fig. 4). $p27^{Kip1}$ mRNA was increased most strikingly when treated with the combination of the two reagents. On one hand, HDACi inhibited the activity of HDACs, which interact with sequence-specific transcriptional factors on $p27^{Kip1}$ promoter and deacetylate promoter-bound histones (21-27). Therefore NaB could directly inhibit the HDACs of the $p27^{Kip1}$ promoter to cause an accumulation of acetylated histones for the access of basal transcriptional factors to the promoter, such as activating the binding of SP1 to proximal GC boxes (22-24), NF-Y to the CCAAT box (24), ectopic E2F to 5'-TTTG/CG/CCGC-3' sequences (25,28) and repressing the binding of c-Myc to Inr elements (27), NF-κB to 5'-GGGCTTCCCC-3' sequences (27). On the other hand, HDACi may indirectly block the pathway of AKT or ERK and affect the expression of c-Myc and FoxO factors which inhibited $p27^{Kip1}$ mRNA expression (28). The faint influence of trastuzumab on the transcription level of $p27^{Kip1}$ was related to the pathways of the regulation system (28). As soon as trastuzumab binds the receptor, the PI3K/AKT or MEK/ERK pathway is blocked, and $p27^{Kip1}$ mRNA could be up-regulated. The post-transcriptional regulation is also important. In our results, the fluorescence of $p27^{Kip1}$ protein was higher in fresh HCC1937 cells than SKBR3 cells. After treatment with NaB alone, $p27^{Kip1}$ protein was up-regulated, noticeably in SKBR3 cells. Trastuzumab-treatment induced the transfer of $p27^{Kip1}$ protein certainly into the nucleus only in the SKBR3 cell lines. These results suggested that the status of HER2 was inversely correlated with expression of $p27^{Kip1}$ protein in breast cancer cells. It was likely that HER2 took part in modulation of $p27^{Kip1}$ protein in different manners: overexpression and activation of HER2 clearly decreased the half-life of $p27^{Kip1}$. Furthermore, HER2 oncogenic signals down-regulated $p27^{Kip1}$ protein to enhance ubiquitin-mediated degradation by increasing affinity with JAB1 through the HER2/Grb2/Ras/MAPK pathway (29,30), promoting $p27^{Kip1}$ protein binding to ubquitin SCF^{SKP2}, and regulating the stability of $p27^{Kip1}$ protein by MIRK/DYRK1B. It is possible that either NaB or trastuzumab may block the HER2 signals to release the restraint of $p27^{Kip1}$ protein expression and affect the proliferation of cancer cells (28,31).

In conclusion, trastuzumab significantly synergized with NaB in the inhibition of cell proliferation and the promotion of cell cycle arrest and cell apoptosis by up-regulating $p27^{Kip1}$ in a HER2-overexpressing breast cancer cell line *in vitro*. Although these data need to be verified in animal or clinical studies, our results may provide the basis for the development of better approaches for breast caner therapy.

Acknowledgements

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