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Effect of sodium butyrate on ABC transporters in lung cancer A549 and colorectal cancer HCT116 cells

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Abstract. Histone deacetylase (HDAC) inhibitors and DNA alkylators are effective components of combination chemotherapy. The aim of the present study was to investigate the possible mechanism of their synergism by detecting the effect of HDAC inhibitors on the expression levels of drug transporters that export DNA alkylators. It was demonstrated that the HDAC inhibitor sodium butyrate (NaB) induced the differential expression of multidrug resistant ATP-binding cassette (ABC) transporters in lung cancer and colorectal cancer cells. Specifically, NaB increased the mRNA expression levels of *ABC subfamily B member 1 (ABCB1)*, *ABCC10* and *ABCC12*, and protein expression levels of multidrug resistance-1 (MDR1), multidrug resistance-associated protein 7 (MRP7) and MRP9. Moreover, NaB decreased the expression levels of *ABCC1*, *ABCC2* and *ABCC3* mRNAs, as well as those of MRP1, MRP2 and MRP3 proteins. The molecular mechanism underlying this process was subsequently investigated. NaB decreased the expression of HDAC4, but not HDAC1, HDAC2 or HDAC3. In addition, NaB promoted histone H3 acetylation and methylation at lysine 9, as well as MDR1 acetylation, suggesting that acetylation and methylation may be involved in NaB-mediated ABC transporter expression. Thus, the present results indicated that the synergism of the HDAC inhibitors with the DNA alkylating agents may due to the inhibitory effect of MRPs by HDAC inhibitors. The findings also suggested the possibility of antagonistic effects following the combined treatment of HDAC inhibitors with MDR1 ligands.

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Introduction

Histone deacetylase inhibitors (HDACIs) have emerged as novel antitumor agents that are continuously tested for the treatment of various cancer types due to their significant antitumor activities, including inhibition of angiogenesis and induction of cell cycle arrest, differentiation and apoptosis (1). HDACIs have a promising therapeutic potential and are approved for lymphoma treatment (1). Furthermore, HDACIs have been reported to induce cell cycle arrest, to promote cell differentiation and to induce apoptosis in a variety of cancer cells, including lung cancer, colorectal cancer, nasopharyngeal carcinoma and cervical cancer cells, while having little effect on healthy cells (1,2). HDACIs can be classified into various types according to their different structure, such as aliphatic acid, hydroxamic acids, cyclopeptide and benzamides. Vorinostat [suberolanilide hydroxamic acid (SAHA)] was the first HDACI approved by the Food and Drug Administration for the treatment of cutaneous T-cell lymphoma (3). Sodium butyrate (NaB) is a structurally similar HDACI that has also exhibited potent antitumor activity (4).

HDACIs have been reported to exhibit synergistic effects in combination with a variety of antitumor agents, including the DNA alkylating agents busulfan (Bu) and melphalan (5-7). The majority of the antitumor drugs are more effective when used in combination, and the cytotoxicity produced from their combined efficacy decreases the development of chemotherapy-resistant tumor cells (5). However, the combination of antitumor drugs may also produce antagonistic effects, which highlights the requirement for the extensive investigation of their interactions.

In order to investigate the molecular mechanisms of HDACIs in combination with DNA alkylating agents, it was hypothesized that HDACIs may affect the expression of drug transporters, which are involved in the efflux of functionally and structurally irrelevant antitumor drugs, including DNA alkylating agents (8). The efflux of antitumor drugs by ATP binding cassette (ABC) transporters serves an important role in the development of the multidrug resistance (MDR) phenotype, which is a main obstacle for successful cancer treatment (8-10). Drug-resistant-related ABC transporter genes mainly include *ABC subfamily B member 1*

(ABCB1), *ABCCs* and *ABCG2*, which encode for MDR1 protein, MDR-associated proteins (MRPs) and breast cancer resistance protein (BCRP), respectively (8). Furthermore, a significant overlap has been reported in the substrate specificity of the ABC transporters. MDR1 extrudes natural toxins, antitumor drugs and drug metabolites (11,12), while MRPs export a variety of structurally diverse glutathione (GSH)-conjugates or therapeutic drugs (9). It has been revealed that MRP1-3 lead to resistance to hydrophobic and anionic compounds, including several natural compounds, whereas MRP4, MRP5 and MRP8 efflux cyclic nucleotides (13,14). BCRP, a ubiquitous ABC transporter, has been shown to transport nucleoside drugs and nucleoside-monophosphate derivatives of clinically relevant nucleoside drugs (15,16). Moreover, these ABC transporters are highly expressed in various human cancer types and are closely associated with poor prognosis (17,18).

The aim of the present study was to investigate the effects of NaB on the expression levels of MDR1, MRPs and BCRP in lung cancer and colorectal cancer cells. Since MRP1 and MRP2 export GSH-conjugated DNA alkylating agents (19), the decrease in their expression levels may contribute to the synergistic antitumor effect of NaB and DNA alkylating agents. In contrast to this hypothesis, NaB may antagonize the anticancer efficacy of drugs that are substrates for MDR1, MRP7 and MRP9. These differential effects of NaB on the expression of ABC transporters require detailed investigations in order to identify its combined antitumor action with other chemotherapeutic agents.

Materials and methods

Chemicals and reagents. NaB, 5-Carboxyfluorescein diacetate (CFDA) and 3,3'-diethyloxacarbocyanine iodide ($DiOC_2$) were obtained from Sigma-Aldrich (Merck KGaA). The primary antibody against MDR1 (cat. no. 13978) and acetylate (cat. no. 9441) were purchased from Cell Signaling Technology, Inc. The primary antibody against α-tubulin (cat. no. sc-134237) was obtained from Santa Cruz Biotechnology, Inc. The primary antibodies against MRP2 (cat. no. 24893-1-AP), p21 (cat. no. 10355-1-AP) and p27 (cat. no. 25614-1-AP) were from ProteinTech Group, Inc., while the primary antibodies against MRP1 (cat. no. BS7474) and BCRP (cat. no. BS3482) were obtained from Biogot Technology Co., Ltd. The primary antibodies against MRP3 (cat. no. ab3375), MRP7 (cat. no. ab91451) and MRP9 (cat. no. ab91453) were purchased from Abcam, and those against HDAC1 (cat. no. ET1605-35), HDAC2 (cat. no. ET1607-78), HDAC3 (cat. no. ET1610-5), HDAC4 (cat. no. ET1612-51), were from Hangzhou HuaAn Biotechnology Co., Ltd. Acetylation at lysine 9 on histone H3 (AcH3K9) (cat. no. 39917) and methylation at lysine 9 on histone H3 (2MeH3K9) (cat. no. 39239) were from Active Motif. Horseradish peroxidase (HRP)-conjugated secondary antibody (cat. nos. SH001X, SH002X and SH003X) were also purchased from DingGuo Biotechnology Co., Ltd. PrimeScript® RT reagent kit and SYBR® Premix Ex Taq™ were obtained from Takara Bio, Inc.

Cell culture. The human lung cancer cell line A549 and the colorectal cancer cell line HCT116 were obtained from the Type Culture Collection of the Chinese Academy of Sciences. HCT116 cells were maintained in Dulbecco's modified Eagle media (DMEM)/F12 culture medium (Biological Industries) supplemented with 10% fetal bovine serum (FBS), and A549 cells were cultured in RPMI 1640 culture medium (Biological Industries) supplemented with 10% FBS under a humidified 5% CO_2 atmosphere at 37˚C.

Reverse transcription-quantitative (RT-q)PCR. A549 and HCT116 cells were treated with NaB (2 mM) at 37˚C for 24 h, and total mRNA was extracted using TRIzol® reagent (Thermo Fisher Scientific Inc.). RNA concentration was determined using spectrophotometry and 500 ng RNA was used for cDNA synthesis. RT-qPCR was performed using the Takara SYBR® Premix Ex Taq™ system in order to quantify the expression levels of the target genes in an ABI 7500 thermal cycler (Thermo Fisher Scientific, Inc.). The thermocycling conditions for RT-qPCR were: Initial denaturation at 95˚C for 30 sec, followed by 45 cycles at 95˚C for 5 sec and 60˚C for 30 sec; and a melt curve stage at 95˚C for 15 sec, 60˚C for 60 sec, 95˚C for 30 sec and 60˚C for 15 sec. *GAPDH* was selected as the housekeeping gene. After normalized to *GAPDH* gene, each target gene expression were calculated using the comparative threshold cycle (Cq) method (20). The ΔCq values were calculated according to the formula ΔCq=Cq (gene of interest)-Cq (*GAPDH*) in correlation analysis, and the $2^{-\Delta\Delta Cq}$ was calculated according to the formula ΔΔCq=ΔCq (control group)-ΔCq (experimental group) for determination of relative. The sequences of the primers used in the RT-qPCR experiments are presented in Table I.

Western blot analysis. A549 and HCT116 cells were treated with NaB (2 mM) at 37˚C for 24 h. Following washing with ice-cold PBS for three times, the cells were lysed using western blotting lysis buffer. The concentration of the total protein was determined using the BCA reagent. In total, 20 μg proteins were separated by electrophoresis using 8% SDS-PAGE. The proteins were electrophoretically transferred onto PVDF membranes. Following blocking with 5% non-fat milk for 2 h at room temperature, the PVDF membranes were incubated with primary antibodies at 4˚C overnight. The antibodies against MDR1, MRP1, MRP2, BCRP, α-tubulin, HDAC1, HDAC2, HDAC3, HDAC4, p21, p27, 2MeH3K9 and AcH3K9 were used at 1:1,000 dilution, while the antibodies against MRP3, MRP7 and MRP9 were used at 1:50 dilution. The PVDF membranes were washed three times with PBS-1% Tween-20 and subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 1.5 h at room temperature. Specific immune complexes were detected using a chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Band intensity was semi-quantified via densitometry analysis using Image-Pro Plus 4.5 software (Media Cybernetics, Inc.).

MTT assay. The cell viability was measured using the MTT assay (Sigma-Aldrich; Merck KGaA). Cells ($1x10^4$) were seeded on 96-well plates and pretreated with NaB (2 mM) at 37˚C for 24 h, then treated with fluorouracil (5-FU) (2.5, 5, 10, 20 and 40 μg/ml) or chlorambucil

Table I. Primers used in reverse transcription-quantitative PCR.

Gene	Forward primer, 5'→3'	Reverse primer, 5'→3'
ABCB1	TGCTCAGACAGGATGTGAGTTG	AATTACAGCAAGCCTGGAACC
ABCC1	GCCAAGAAGGAGGAGACC	AGGAAGATGCTGAGGAAGG
ABCC2	TGGTGGCAACCTGAGCATAGG	ACTCGTTTTGGATGGTCGTCTG
ABCC3	GGTTCCCCTTGGAATCATTT	AATCCTGGTGTGCATCAAACAG
ABCC5	ACCCGTTGTTGCCATCTTAG	GCTTTGACCCAGGCATACAT
ABCC6	GTGGTGTTTGCTGTCCACAC	ACGACACCAGGGTCAACTTC
ABCC10	ATTGCCCATAGGCTCAACAC	AGCAGCCAGCACCTCTGTAT
ABCC11	GGCTGAGCTACTGGTTGGAG	TGGTGAAAATCCCTGAGGAG
ABCC12	GGTGTTCATGCTGGTGTTTGG	GCTCGTCCATATCCTTGGAA
ABCG2	TATAGCTCAGATCATTGTCACAGTC	GTTGGTCGTCAGGAAGAAGAG
GAPDH	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA

ABC, ATP-binding cassette.

(2, 5, 10, 15 and 20 μM) at 37˚C 48 h. The cells were washed twice with PBS, and 100 μl MTT solution (0.25 mg/ml) was added to the culture medium. Following incubation for 4 h at 37˚C, 100 μl DMSO was added to each well to dissolve the dark blue crystals. The absorbance was measured at 570 nm using a microplate reader.

Functional assays for MRP1 and MDR1. CFDA and $DiOC_2$ were used as fluorescent substrates to assay the MRP1 and MDR1 transport activity via fluorescence spectrophotometry. A549 cells were treated with NaB (2 mM) and/or DMSO for 24 h at 37˚C, harvested and resuspended in fresh medium with 1 μM CFDA and/or 0.5 μg/ml $DiOC_2$ at 4˚C for 20 min. The cells were centrifuged at 200 x g at 4˚C for 1 min, washed with PBS 3 times, resuspended in fresh medium and incubated at 37˚C for 40 min. The mean fluorescence intensities for CFDA and $DiOC_2$ were determined via fluorescence spectrophotometer (UV3000; Shanghai Mapada Instruments Co., Ltd.) using an excitation and emission wavelength of 488 and 525 nm, respectively.

Immunoprecipitation. A549 cells were treated with NaB (2 mM) or DMSO (equal volume) at 37˚C for 24 h, and washed three times with ice-cold PBS. The cells were harvested at 4˚C in immunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) and 20 μg protein was immunoprecipitated using an anti-MDR1 antibody (1:50) at 4˚C overnight. The immune complexes were bound to protein A/G Sepharose (Beyotime Institute of Biotechnology) and the beads were washed with lysis buffer. Then, the protein was subjected to western blotting as aforementioned with an anti-acetylate antibody (1:1,000).

Statistical analysis. Data are presented as the mean ± SD of three independent experiments, and were analyzed using a two-tailed unpaired Student's t-test. The analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of NaB on cancer cell viability. The effect of NaB on the viability of lung cancer and colorectal cells was investigated. A549 and HCT116 cells were treated with different concentrations of NaB (0-16 mM) for 24 or 48 h. The viability of cancer cells was measured using the MTT assay, and it was identified that NaB markedly suppressed proliferation of A549 and HCT116 cells within a 24-h treatment period (Fig. 1A). Furthermore, the effects of NaB on the expression of the proliferative markers were evaluated. The results demonstrated that NaB significantly increased the expression levels of p21 and p27 proteins compared with the DMSO treatment group (Fig. 1B). These results suggested that NaB could inhibit the viability of lung cancer and colorectal cancer cells.

Effect of NaB on the expression of ABC transporters. Subsequently, the effect of NaB on the expression levels of the ABC transporters was examined. A549 and HCT116 cells were treated with 2 mM NaB for 24 h, and the gene and protein expression levels of the ABC transporters were detected via RT-qPCR and western blotting, respectively. The results identified that NaB increased the mRNA expression levels of *ABCB1*, *ABCC10* and *ABCC12,* and the protein expression levels of MDR1, MRP7 and MRP9. However, NaB decreased the expression levels of *ABCC1*, *ABCC2* and *ABCC3* mRNAs, as well as MRP1, MRP2 and MRP3 proteins. In addition, NaB did not alter the expression of *ABCG2* mRNA and BCRP protein in the two cell lines (Fig. 2A and B).

Effect of NaB on the drug sensitivity of A549 and HCT116 cells. It was further investigated whether cells exposed to NaB could retain their ability to export specific drugs. CFDA and $DiOC_2$ are substrates for MRP1 and MDR1, respectively. A549 and HCT116 cells were stimulated with 2 mM NaB for 24 h. It was identified that NaB decreased the efflux of CFDA from the cells (Fig. 3A), while NaB increased $DiOC_2$ efflux (Fig. 3B). Moreover, it was evaluated whether NaB treatment could enhance the drug sensitivity of the cells to chlorambucil,

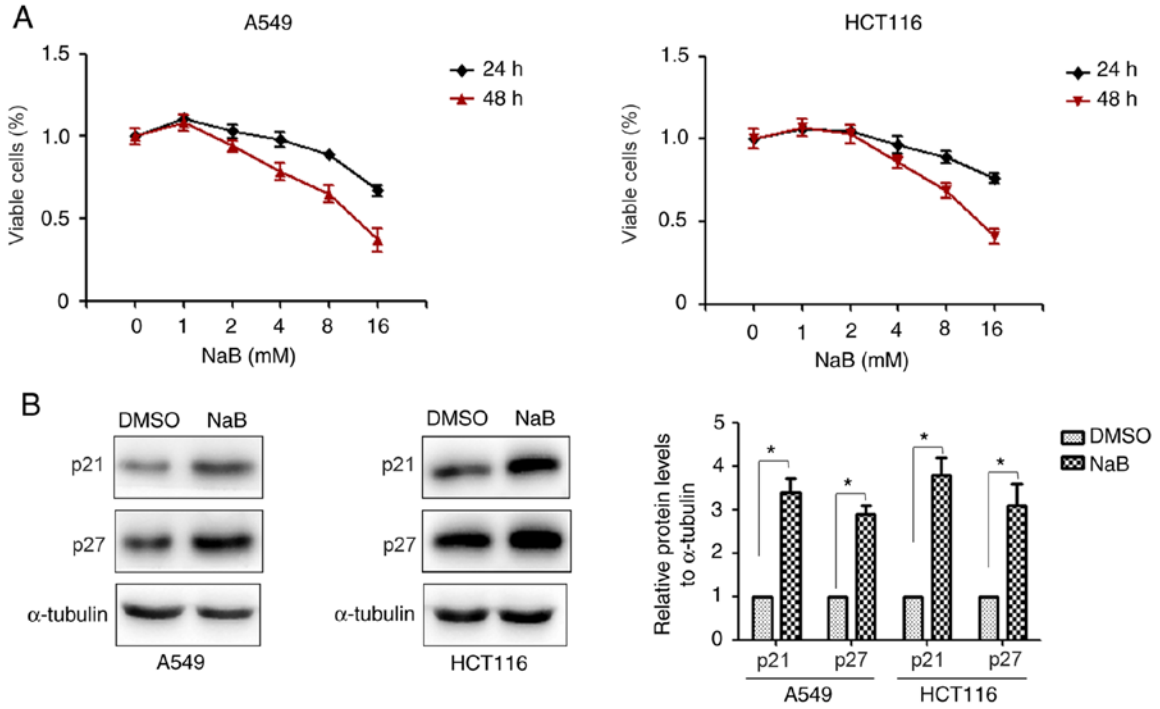

Figure 1. Effect of NaB on cell viability of lung cancer and colorectal cancer cells. (A) A549 and HCT116 cells were treated with various concentrations of NaB (0-16 mM) for 24 and 48 h. Cell viability was detected using a MTT assay. (B) A549 and HCT116 cells were treated with 2 mM NaB for 24 h, and the expression levels of p21 and p27 proteins were detected via western blotting. *P<0.05 compared with DMSO group. NaB, sodium butyrate.

a GSH-conjugated alkylator exported by MRP1 (21). A549 and HCT116 cells were pretreated with DMSO and/or NaB for 24 h and then exposed to increasing concentrations of chlorambucil. The results demonstrated that NaB significantly promoted the sensitivity of the cells to chlorambucil (Fig. 3C). Inversely, NaB increased drug resistance of A549 and HCT116 cells to 5-FU (Fig. 3D).

NaB induces acetylation of MDR1. HDACIs regulate gene transcription mainly by inhibiting the expression of HDAC enzymes, which serve crucial roles in tumor progression (22). The present study detected the effect of NaB on the expression levels of HDAC1, HDAC2, HDAC3 and HDAC4. The results indicated that NaB downregulated the expression of HDAC4, but not HDAC1, HDAC2 and HDAC3 (Fig. 4A). NaB also promoted AcH3K9 and 2MeH3K9 (Fig. 4A). Furthermore, NaB significantly increased the acetylation of MDR1 (Fig. 4B).

Discussion

Previous studies have reported the synergistic cytotoxicity of HDACIs and DNA alkylating agents in a variety of experimental models, for example HDAC inhibitor panobinostat and DNA alkylators busulfan showed synergistic cytotoxicity in lymphoma cells (5-7). In the present study, it was demonstrated that NaB could downregulate the expression levels of *ABCC1*, *ABCC2* and *ABCC3* mRNAs, and MRP1, MRP2 and MRP3 proteins in lung cancer and colorectal cancer cells. Since MRP1, MRP2 and MRP3 have been revealed to export GSH-conjugated DNA alkylating agents from the cytoplasmic regions of cancer cells, the downregulation of their expression gives rise to cellular accumulation of these agents, which leads to enhanced cytotoxicity (9,19). The present findings are consistent with the inverse association between the sensitivity of DNA alkylating agents and high expression of MRP1 (23). Cancer cells with high expression of MRP1 are more resistant to DNA alkylating agents, such as Bu and chlorambucil (23). A previous study revealed that SAHA and belinostat downregulated the expression of MRP1 in T-cell lymphoma and T-cell prolymphocytic leukemia (21). However, another study observed that the HDACI FK228 could upregulate MRP1 expression (14). It has been shown that MRP1 is not induced in all the examined cell lines that are treated with FK228, suggesting that FK228-induced MRP1 expression is cell line-dependent (24). Kim *et al* (25) also revealed that SAHA could overcome MDR export of anticancer drugs via the downregulation of MRP2 in MDR cancer cell lines. The downregulation of MRP2 that is caused by the combined treatment of paclitaxel and SAHA leads to an increase in G_2/M arrest and apoptosis (25). In contrast to SAHA, valproic acid results in upregulation of MRP2 expression (26). Therefore, it was speculated that the difference in MRP expression that was induced by HDACIs could be due to the different cancer types and the diverse structures of the HDACIs used.

Chemotherapy is one of the effective methods for the treatment of malignant tumors. However, the effectiveness of chemotherapy is limited by the acquirement of the MDR phenotype mediated by MDR1, which exports antitumor drugs out of cancer cells and reduces their intracellular accumulation (8,27). Although HDACIs are emerging as a novel class of chemotherapeutic agents, the development of the MDR phenotype is of notable concern for the efficacy of several antitumor drugs, including HDACIs (4). In the present study, it was found that NaB induced 5-FU resistance and increased the expression

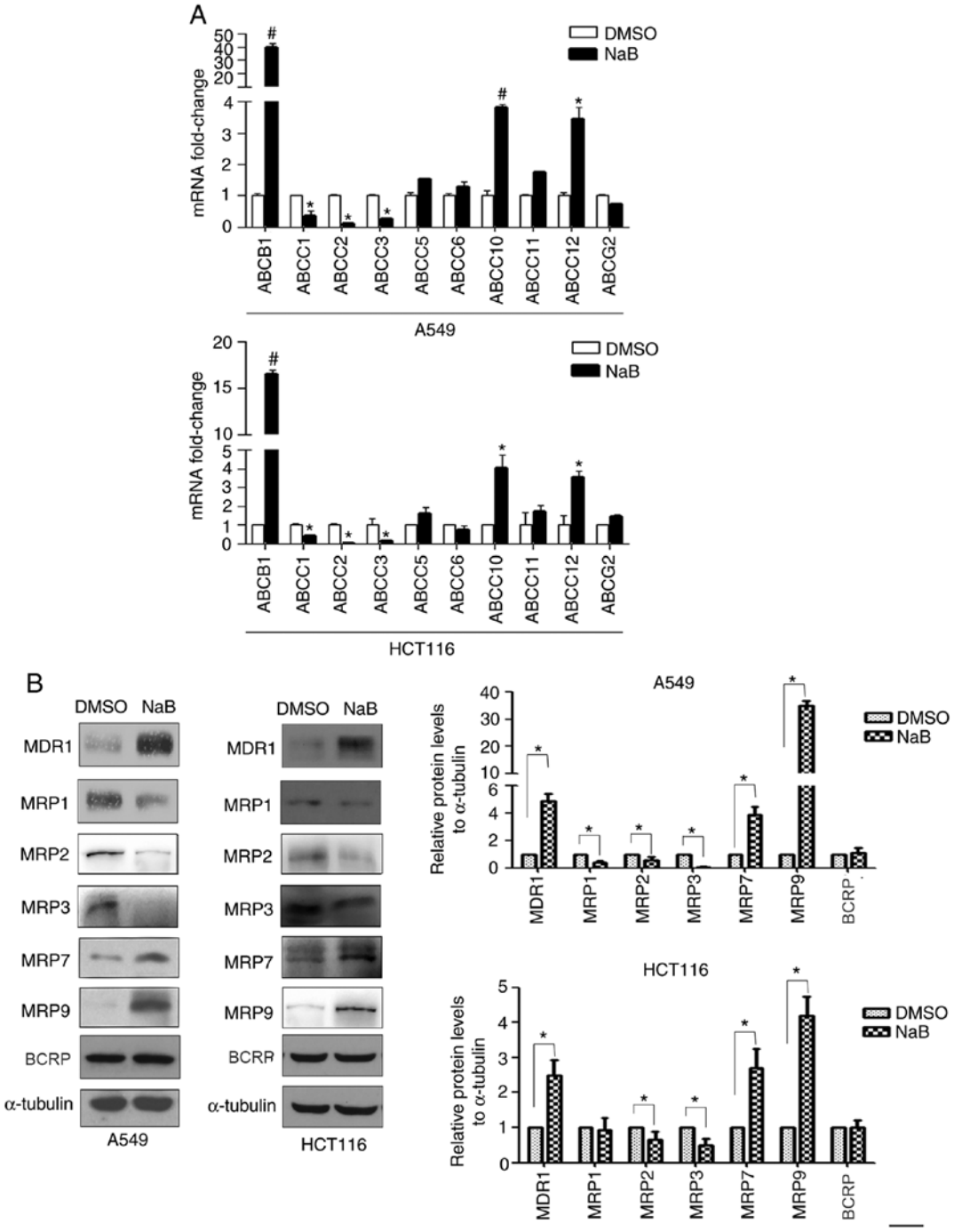

Figure 2. Effect of NaB on the expression of ABC transporters. (A) A549 and HCT116 cells were treated with NaB (2 mM) for 24 h. mRNA expression levels of *ABCB1, ABCC1, -2, -3, -5, -6, -10, -11, -12* and *ABCG2* were detected using reverse transcription-quantitative PCR. (B) A549 and HCT116 cells were treated with NaB (2 mM) for 24 h, and the protein expression levels of the MDR1, MRP1, -2, -3, -7, -9 and BCRP were detected via western blotting. #P<0.01, *P<0.05 compared with DMSO group. *ABC*, ATP-binding cassette; NaB, sodium butyrate; BCRP, breast cancer resistance protein; MDR1, multidrug resistance-1; MRP, multidrug resistance-associated protein.

of MDR1. Previous studies have reported that HDACIs could promote MDR1 expression in several cancer types. For example, treatment with the HDACI apicidine induces paclitaxel resistance and Rhodamine-123 efflux in HeLa cells (28). Colon and renal cancer cells that are treated with Trichostatin A (TSA) or depsipeptide also display increased MDR1 expression (29,30). Moreover, the same effect was observed for leukocytes isolated from patients (29,30). MDR1 induction has also been reported in human and murine cells treated with valproate (31). A recent study demonstrated that SAHA and TSA induced MDR1 expression via transcriptional activation of STAT3 and the stabilization of MDR1 mRNA in colorectal cancer (32). The present study investigated the molecular mechanism of NaB-induced MDR1 expression. Several HDAC family members are aberrantly expressed in various types of cancer, and therefore, HDACIs are considered a promising novel class of anticancer drug targets (22). Previous studies have reported that some agents can inhibit the expression and

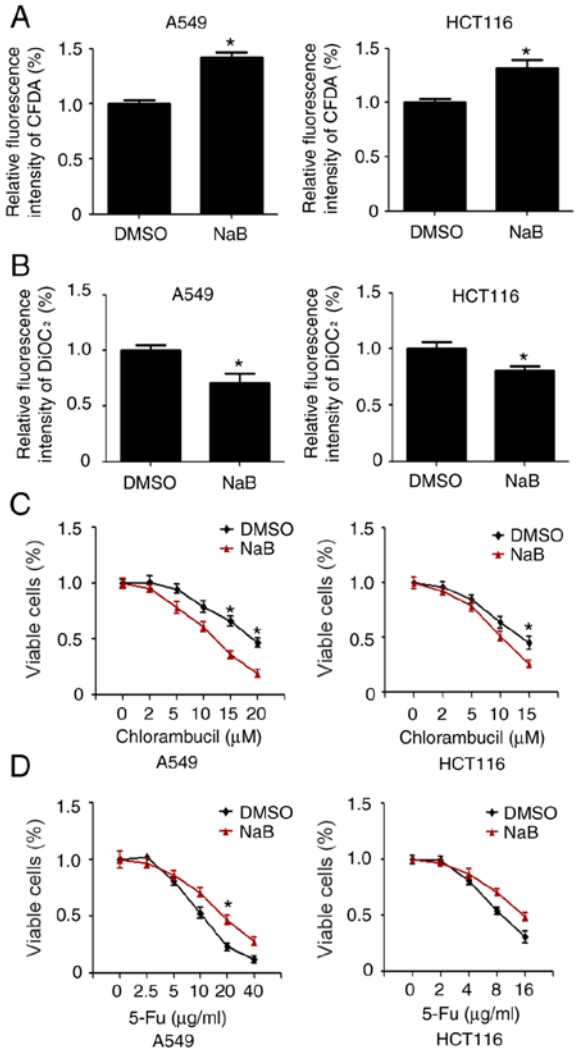

Figure 3. Effect of NaB on the drug sensitivity of A549 and HCT116 cells. (A) A549 and HCT116 cells were pretreated with NaB (2 mM) or DMSO for 24 h. The fluorescent intensity of CFDA was measured via fluorescence spectrophotometer. (B) A549 and HCT116 cells were pretreated with NaB (2 mM) or DMSO for 24 h and the fluorescent intensity of $DiOC_2$ was measured using fluorescence spectrophotometer. (C) A549 and HCT116 cells were pretreated with NaB (2 mM) or DMSO for 24 h, and the cells were exposed to various concentrations of chlorambucil for 48 h. The cell viability was detected with a MTT assay. (D) A549 and HCT116 cells were pretreated with NaB (2 mM) or DMSO for 24 h and the cells were exposed to various concentrations of 5-FU for 48 h. The cell viability was detected with a MTT assay. *P<0.05 compared with DMSO group. DiOC2, 3,3'-diethyloxacarbocyanine iodide; CFDA, 5-Carboxyfluorescein diacetate; 5-FU, fluorouracil; NaB, sodium butyrate.

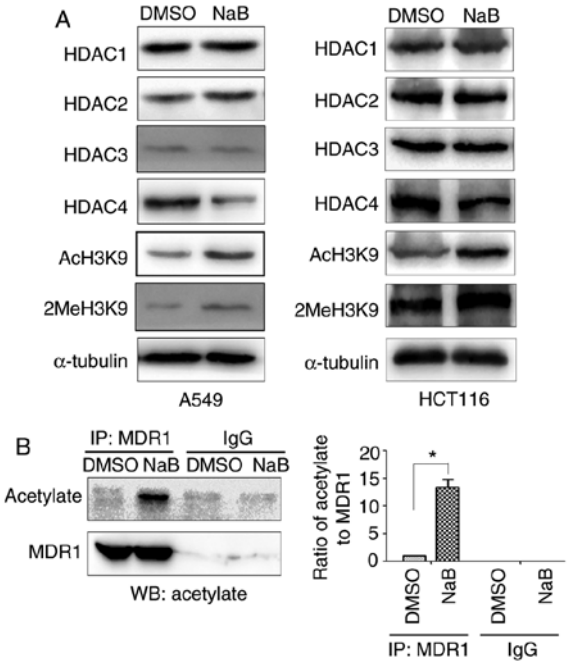

Figure 4. NaB induces acetylation of MDR1. (A) A549 and HCT116 cells were treated with NaB (2 mM) or DMSO for 24 h, and the protein expression levels of HDAC1, HDAC2, HDAC3, HDAC4, AcH3K9 and 2MeH3K9 were detected via WB. (B) A549 cells were treated with NaB (2 mM) or DMSO for 24 h and MDR1 protein was immunoprecipitated. The protein expression of MDR1 and the levels of acetylation were detected using WB. *P<0.05 compared with DMSO group. NaB, sodium butyrate; HDAC, Histone deacetylase; AcH3K9, acetylation at lysine 9 on histone H3; 2MeH3K9, methylation at lysine 9 on histone H3; MDR1, multidrug resistance-1; WB, western blotting; IP, Immunoprecipitation.

function of MDR1, MRP7 and MRP9 (33,34). Lapatinib and erlotinib are potent reversal agents for MRP7 (34). Therefore, it was suggested that a drug combination of HDACIs with these inhibitors may solve the drug resistance mediated by MDR1, MRP7 and MRP9. Thus, it is worth further evaluating the details of drug combination in future studies.

Histone acetylation and methylation serve an important role in gene expression (21). The present study demonstrated that NaB could inhibit the expression of HDAC4, promote AcH3K9 and acetylation of MDR1, which may result in the upregulation of its expression. On the other hand, NaB increased 2MeH3K9, which may result in the decreased protein expression levels of MRP1, MRP2 and MRP3. Therefore, it was speculated that different regulation of the expression levels of the ABC family may due to histone acetylation and methylation induced by NaB. Although the current study did not address the reasons for dysregulated expression levels of the target genes, the present results provide preliminary data for researchers in this field. In a future study, the molecular mechanisms underlying the different effects on MRP and HDAC protein families induced by NaB will be investigated.

In conclusion, the present study demonstrated the crucial implications for combining NaB with other antitumor drugs. NaB treatment had different effects on the expression of ABC transporters, which have various substrates. For example, MRP1 and MRP2 export GSH-conjugated DNA alkylating agent, and MDR1 exports anthracyclines (11-14). The combination of NaB with DNA alkylating agents and/or other MRP1-3 substrates may lead to synergistic and more efficacious treatments. For instance, chlorambucil exerts its antitumor activity in variety of cancer cells, including lymphocytic leukemia, ovarian cancer, colorectal cancer and lung cancer (35,36). In the current study, the combination of NaB and chlorambucil exerted a synergistic effect in colorectal cancer and lung cancer. However, NaB could increase MDR1 expression, which may lead to drug resistance towards MDR1 substrates, such as steroids, vinca alkaloids and anthracyclines. The present study highlighted the importance of understanding the mechanism of drug interactions in order to achieve a more efficacious cytotoxic effect in cancer cells.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

BS contributed to the study design, data acquisition and analysis and drafted the manuscript. PF participated in the study design, data acquisition and revision of the manuscript. FFX, CPX, RJ, CHY, SQM, NW and AJW performed the statistical analysis. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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