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Rapid Letter

Augmentation of Sodium Butyrate-Induced Apoptosis by p38 MAP Kinase Inhibition in Rat Liver Epithelial Cells

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ABSTRACT

Sodium butyrate (NaBu) has an inhibitory effect on histone deacetylases (HDACs). The mitogen-activated protein (MAP) kinases, such as extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAP, kinase are known to be modulated during NaBu-induced apoptosis. In the present study, we showed that low concentrations of NaBu could induce apoptosis synergistically with the inhibition of p38 MAP kinase as proven by using specific p38 MAP kinase inhibitor and dominant negative p38 transfection in a ras-transformed rat liver epithelial cell line (WB-ras). There were no changes in HDAC1, suggesting that NaBu might be able to kill transformed cells bypassing the HDAC inhibitory effect. We further demonstrated that inhibition of p38 MAP kinase potentiated apoptotic cascades, including cleavage of poly(ADP-ribose) polymerase, caspase-3, and decrease in Bcl-2/Bax ratio even at a lower concentration of NaBu. Thus, p38 MAP kinase played inhibitory roles in NaBu-induced apoptosis, and simultaneous modulation of MAP kinases in NaBu treatment could increase the efficiency of the chemotherapeutic effect of NaBu. *Antioxid. Redox Signal.* 7, 1767–1772.

INTRODUCTION

SODIUM BUTYRATE (NaBu) is a sodium salt of a short-chain fatty acid, butyric acid, produced by luminal bacteria in colon during fermentation of dietary fiber (2). NaBu inhibits histone deacetylases (HDACs), which act as a transcriptional silencer in the modification of chromatin folding through catalyzing the transfer of an acetyl group from the acetyl coenzyme A to the ϵ-amino group of lysine residues in the histone tail, which in turn reduces accessibility of DNA-binding transcription factors, and is now regarded as potential therapeutics against cancer (9, 11, 14). Interestingly, NaBu has been used as an inducer of differentiation of transformed cell with no major side effects and, hence, is conceived to be relatively safe (8).

There is increasing evidence that HDAC inhibitors could contribute to cell differentiation and/or apoptosis through modulation of various protein kinases, including mitogen-activated protein (MAP) kinases and tyrosine kinases, as well as HDAC inhibition (12, 15). Moreover, activity of HDAC itself could be regulated by extracellular signal-regulated kinase 1/2 (ERK1/2), and its cellular localization was controlled by oncogenic ras, an upstream signaling molecule of ERK1/2 (16). Hence, it is possible that chemopreventive effects of NaBu would be modulated by a protein kinase pathway with or without involvement of HDAC proteins.

The p38 MAP kinase is a member of the MAP kinase family, and its role varies from proliferation to apoptosis (10). Rahmani *et al.* reported that the phosphorylation of p38 MAP kinase was transiently increased by NaBu until 30 min post treatment and reverted to its basal level in human leukemic cells (12). In addition, a specific p38 MAP kinase inhibitor failed to decrease apoptosis induced by NaBu, suggesting that the activation of p38 MAP kinase did not enhance apoptosis (12).

Multiple lines of evidence indicate that *ras* activation is involved in major carcinogenic processes. In addition, oncogenic

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mutations in ras lead to high transforming activity and occur in 30% of all human cancers (1). Recent reports showed that activation of Ras signaling pathways was also involved in induction of cyclooxygenase-2 (7), matrix metalloproteinase (6), and regulation of gap junctional intercellular communication (5). In a previous report in which we observed the activation of p38 MAP kinase during NaBu-induced apoptosis in ras-transformed rat liver epithelial cells (WB-ras), we proposed that the inhibition of p38 MAP kinase by SB202190 did not prevent, but enhanced apoptosis by NaBu-implicating that the p38 MAP kinase might act controversially in NaBu-induced apoptosis (4).

Thus, this study described the contribution of p38 MAP kinase to NaBu-induced apoptosis, focused on their potential synergy, and aimed to define the role of p38 MAP kinase in the regulation of the chemotherapeutic mechanisms involved.

MATERIALS AND METHODS

Chemicals

NaBu was purchased from Sigma (St. Louis, MO, U.S.A.). SB202190, a specific p38 MAP kinase inhibitor, were obtained from Calbiochem (San Diego, CA, U.S.A.).

Cell culture

Ras-transformed WB-F344 rat liver epithelial (WB-ras) cells, a kind gift of Dr. J. Trosko of Michigan State University (East Lansing, MI, U.S.A.) were cultured in D-media (formula no. 78-5470EF, GibcoBRL, Grand Island, NY, U.S.A.) containing 3 ml/L penicillin, streptomycin, and neomycin mixture (GibcoBRL) in the presence of 5% fetal bovine serum (GibcoBRL). Cells within 75-mm tissue culture plates were incubated in a 37°C humidified incubator containing 5% CO_2 and 95% air, and the medium was changed every other day.

Measurement of cell survival

The effects on cell proliferation were measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, based on the ability of live cells to convert tetrazolium salt into purple formazan. In brief, the cells were seeded in 24-well microplates and treated as indicated. At the end of incubation, 50 µl of MTT stock solution (5 mg/ml; Sigma) was added, and the plates were further incubated for 4 h at 37°C. The supernatant was removed, and 500 µl of dimethyl sulfoxide was added to solubilize formazan crystals. The absorbency was measured with an EL800 microplate reader (BIO-TEK Instrument, Winooski, VT, U.S.A.). All the measurements were performed in triplicate.

Transient transfection

Transient transfection of dominant negative (DN) p38 MAP kinase was performed using SuperFect Transfection Reagent (Qiagen, Valencia, CA, U.S.A.) following the manufacturer's instructions. The DN-p38 construct was kindly provided by Prof. J. Lu of the University of Minnesota (Austin, MN, U.S.A.). Efficiency of transfection was determined by western blot analysis using phospho-specific antibody for p38 and morphological observation.

Apoptosis assay

Apoptosis of WB-ras cells was analyzed by Hoechst 33258 (Sigma). Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, washed with PBS, and stained with Hoechst 33258 at 1 µg/ml concentration in PBS for 30 min. Stained cells were washed twice with PBS. The changes in nuclei were observed with a fluorescent microscope (Olympus, Melville, NY, U.S.A.) through a UV filter.

Western blot analysis

Western blot analyses were performed as previously described (3, 13). Equal amounts of whole protein lysates (20 µg/lane) were subjected to 10–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were subsequently incubated with the corresponding primary antibodies as indicated and were detected by the ECL system (Amersham Pharmacia Biotech U.K. Ltd., Buckinghamshire, U.K.) after recognition with the respective secondary antibodies conjugated with horseradish peroxidase.

RESULTS AND DISCUSSION

Modulation of MAP kinases during NaBu-induced apoptosis in WB-ras cells

In order to assess signal transduction during NaBu-induced apoptosis, it was necessary to find a concentration of NaBu that inhibited cell growth minimally without cytotoxic changes in the MTT assay. A 1 m*M* concentration of NaBu decreased proliferation of WB-ras cells ~40% after 48 h of treatment in a time-dependent fashion. NaBu induced apoptosis not in nontransformed WB cells, but specifically in WB-ras cells evidenced by morphological change in the nuclei (Fig. 1A) and significantly decreased the level of unprocessed poly(ADP-ribose) polymerase (PARP) up to 20% and cleavage of caspase-3 (Fig. 1B). The phosphorylation of ERK1/2 was also completely decreased in a time-dependent manner following NaBu treatment (Fig. 1C). As shown in Fig. 1C, the phosphorylation level of p38 MAP kinase was up-regulated significantly up to 350% of control by NaBu. There were no significant changes in the phosphorylation level of c-Jun N-terminal kinase (JNK).

Inhibition of p38 MAP kinase increases apoptosis inducibility of NaBu

Our previous report suggested that the inhibition of p38 MAP kinase did not prevent NaBu-induced apoptosis; rather it increased apoptotic cells synergistically with NaBu (4). Based on the hypothesis that the p38 MAP kinase activation may be a way to survive NaBu-induced apoptosis, we examined whether the p38 MAP kinase is protective against NaBu-induced apoptosis or not. The highest apoptotic nuclei induction reached was 50% of total nuclei in cells cotreated with the higher concentrations of NaBu and SB202190 (1 m*M* and 4 µ*M*, respectively), as assessed by Hoechst 33258 staining (Fig. 2). Simultaneous treatment with NaBu and SB202190

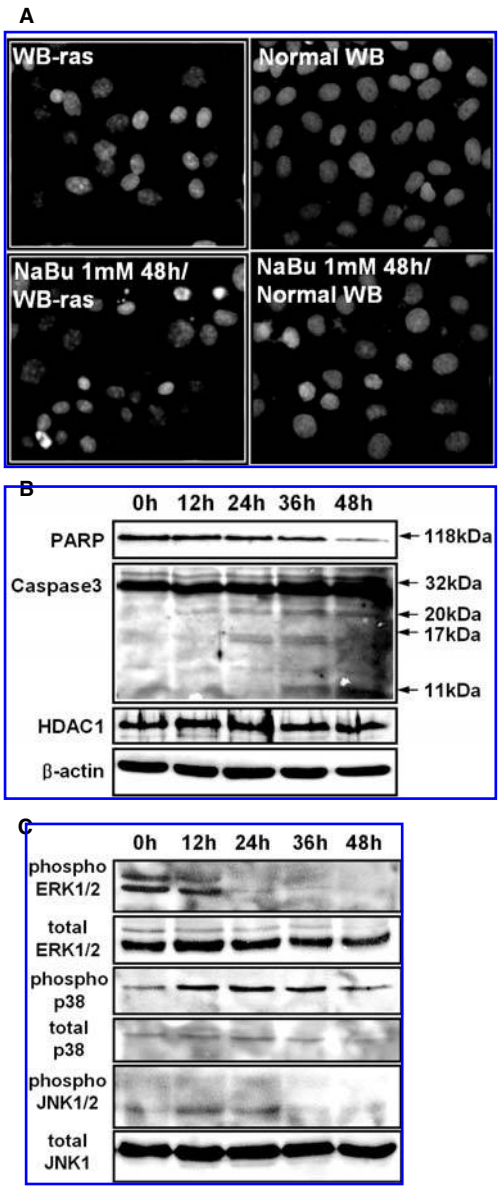

FIG. 1. Apoptotic nucleus induced by 1 m*M* concentration of NaBu in WB-ras cells. (**A**) Morphological change was observed after 48-h treatment of NaBu in WB-ras and nontransformed WB cell line. (**B**) Western blot observation of PARP and caspase-3. (**C**) NaBu-induced changes in MAP kinases. The cells were treated with 1 m*M* NaBu for the indicated periods.

obviously induced cleavage of PARP higher than 1,500% of control and a significant decrease in the Bcl-2/Bax ratio (Fig. 3).

Modulation of MAP kinases by NaBu and SB202190

Figure 4 shows that phosphorylation of ERK decreased by NaBu treatment, and further inhibition was found in NaBu and SB202190 cotreated cells. Phosphorylation of MAP kinase kinase 3/6 (MKK3/6) was obviously increased in SB202190-treated cells, as well as SB202190- and NaBu-simultaneously treated cells. In addition, the phosphorylation

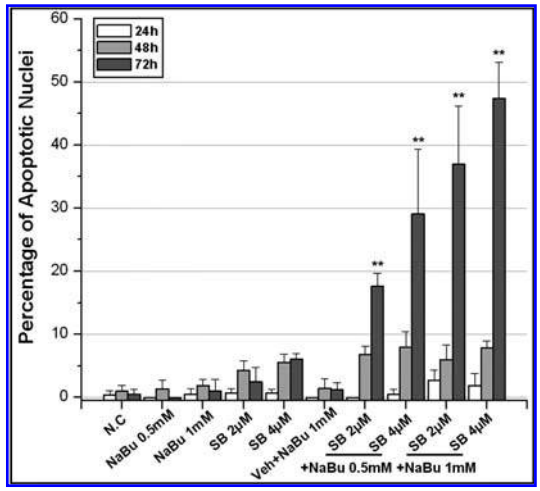

FIG. 2. Time- and dose-dependent induction of nucleus condensation by NaBu and SB202190 in WB-ras cell line. Each cell was treated as indicated for 72 h. $^{**}p < 0.01$, significantly different from SB treatment group. N.C, negative control.

level of p38 MAP kinase was significantly increased by 1 m*M* NaBu treatment for 72 h, and simultaneous treatment with SB202190 completely inactivated p38 MAP kinase ($p < 0.01$; data not shown). It was interesting that SB202190 also could inhibit ERK1/2 phosphorylation ($p < 0.01$), and that inactivation of p38 MAP kinase was further enhanced by NaBu and SB202190 cotreatment. Thus, p38 MAP kinase might be an upstream signal of ERK1/2, and the regulatory role of NaBu on p38 MAP kinase might be rather indirect via unknown upstream signal cascades.

Increased vulnerability to NaBu-induced apoptosis by p38 MAP kinase down-regulation

In order to elucidate how p38 MAP kinase contributes to NaBu-induced apoptosis, transfection of the DN-p38 MAP kinase construct was performed in WB-ras cells. The transfectant was more spindle-like in its shape than wild-type WB-ras cells (Fig. 5A). The phosphorylation level of p38 MAP kinase was decreased significantly ($p < 0.05$) after transfection of DN-p38 (Fig. 5B and C). The cell viability assay using MTT showed increased vulnerability of the transfectant against NaBu treatment (Fig. 5D). Furthermore, the transfectant was more sensitive to NaBu-induced nuclei condensation than wild-type WB-ras cells, as shown in Fig. 6A. Quantitative

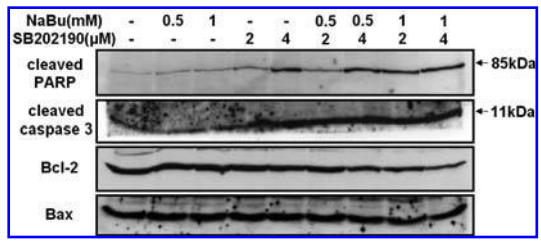

FIG. 3. Cleavage of PARP and caspase-3, as well as expression level of Bcl-2 and Bax, was evaluated using western blot. Each group was treated as indicated for 72 h.

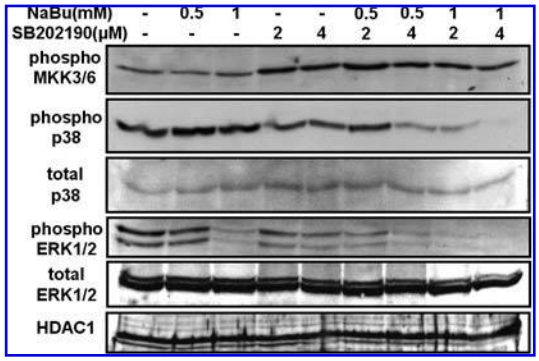

FIG. 4. Western blot analysis of protein kinases followed by NaBu and/or SB202190 treatment. Each group was treated as indicated for 72 h.

analysis revealed 10 times higher apoptotic cell number observed in the transfectant than in wild-type cells at 72 h (Fig. 6B).

Role of p38 MAP kinase down-regulation in NaBu-induced apoptosis

In order to verify the role of p38 MAP kinase down-regulation in NaBu-induced apoptosis, western blots of various apoptosis-responsive proteins were assessed. Cleavage of PARP, caspase-3, and Bcl-2 expression were observed with NaBu treatment in a time-dependent manner (Fig. 7A). There were no increases in Bax. Interestingly, simultaneous modulation of p38 MAP kinase with NaBu treatment also modulated the expression of apoptosis-related proteins (Fig. 7B). Densitometry results for each immunoblot also revealed significant increase in NaBu-induced apoptotic signals ($p < 0.01$ in groups that were treated with 1 m*M* concentration of NaBu for 72 h). Indeed the 0.5 m*M* concentration of NaBu was able to cleave PARP and caspase-3 in DN-p38 transfectant more strongly than the 1 m*M* NaBu in wild-type WB-ras cells.

Modulation of protein kinases during p38 down-regulation and NaBu-induced apoptosis

In order to examine the effects of NaBu-induced apoptosis simultaneously with p38 MAP kinase down-regulation on MAP kinases, western blot analyses were performed. The transfectants were less sensitive to p38 MAP kinase activation by NaBu than wild-type WB-ras cells (Fig. 8A). MKK3/6 was found to be significantly activated by 1 m*M* NaBu treatment for 72 h in wild-type WB-ras cells ($p < 0.01$). There was no significant change in MKK3/6 activity by NaBu treatment in transfectants, and its phosphorylation level was maintained highly, suggesting a negative feedback relationship between the two protein kinases. The p38 MAP kinase in DN-p38 transfectants was shown to be up-regulated by NaBu treatment in a time-dependent manner, whereas the degree of up-regulation was very slight compared with that in wild-type WB-ras cells (Fig. 8B). The phosphorylation level of MKK3/6 was not changed significantly by NaBu treatment in DN-p38 transfectant.

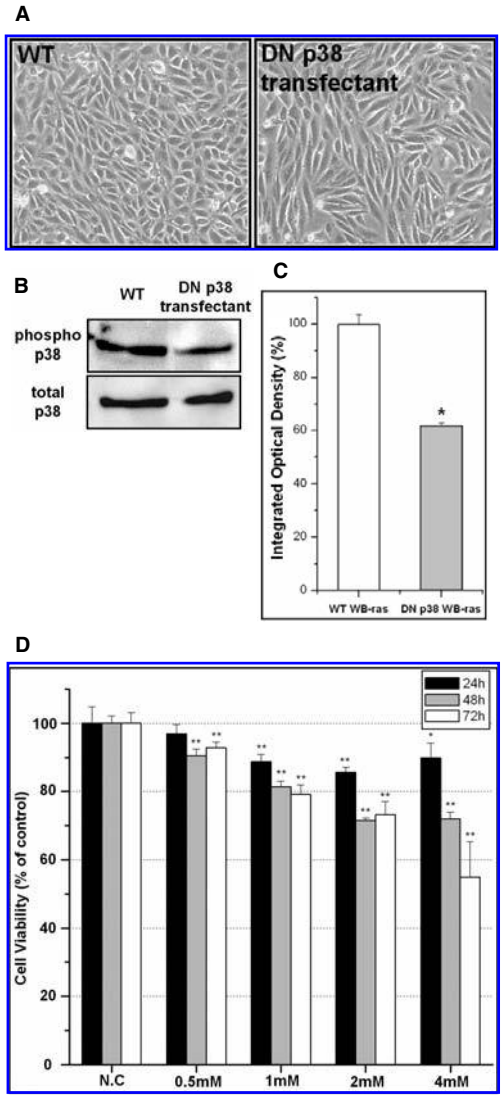

FIG. 5. Transient transfection of DN-p38 constructs into WB-ras cells (A, B, C) and cytotoxicity assay against NaBu treatment (D). (**A**) Morphological analyses were performed under ×200 inverted microscope. (**B**) The efficiency of transfection was determined using western blot analysis. (**C**) $*p < 0.05$, significantly different from wild type (WT). (**D**) Cytotoxicity assay of DN-p38 WB-ras on NaBu treatment. $*p < 0.05$, $**p < 0.01$, significantly different from negative control (N.C).

Molecular synergism between NaBu-induced apoptosis and inhibition of p38 MAP kinase in WB-ras cells

As shown in Fig. 9, the results of this study indicate that p38 MAP kinase is involved in preventing NaBu-induced apoptosis presumably by decreasing susceptibility of WB-ras cells to apoptosis. In addition, apoptosis induced by NaBu and p38 inhibition showed typical apoptotic changes. Thus, p38 inhibition could work synergistically with NaBu to induce apoptosis and, as a result, a lowered apoptotic threshold of NaBu.

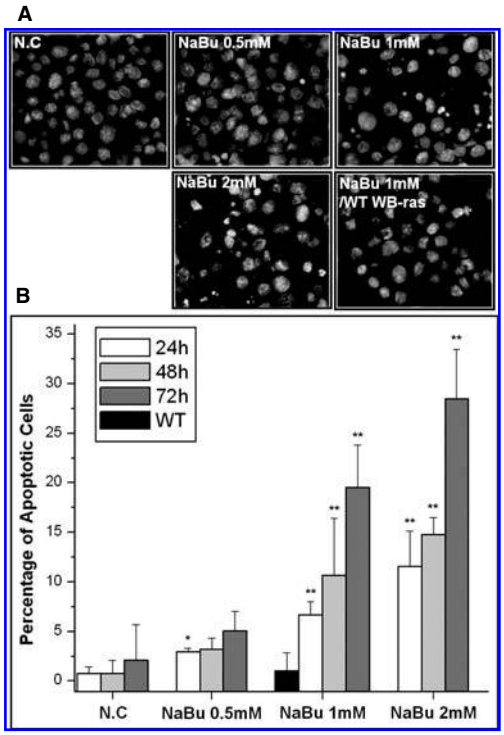

FIG. 6. Effects of DN-p38 transient transfection on NaBu-induced apoptosis in WB-ras cells. (**A**) Each cell was treated as indicated for 72 h. (**B**) Quantitative analysis of apoptotic nuclei. *$p < 0.05$, **$p < 0.01$, significantly different from negative control (N.C).

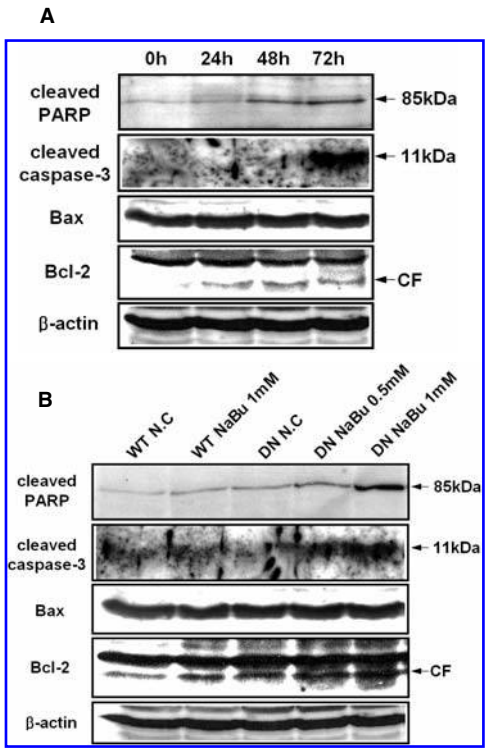

FIG. 7. Confirmation of apoptosis induced by NaBu in WB-ras cells using western blot analysis. (**A**) Time-dependent changes in apoptosis-related proteins of DN-p38 WB-ras cells. (**B**) Comparison of changes in apoptosis-related proteins reacted with NaBu between DN-p38 transfectant and wild-type (WT) WB-ras cells. CF, cleaved form.

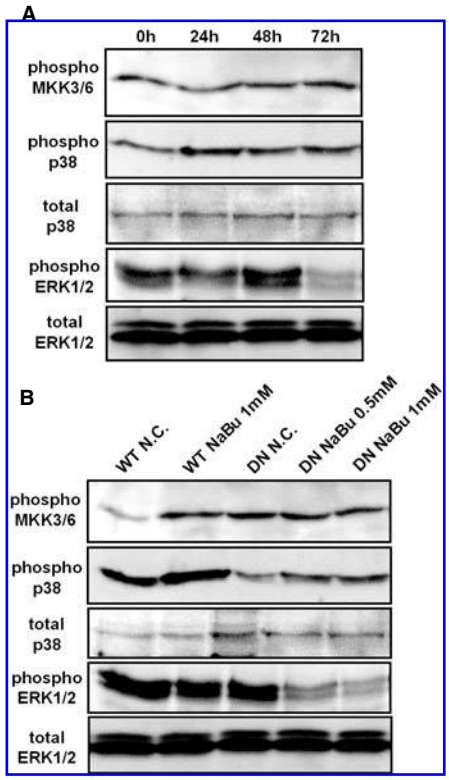

FIG. 8. Western blot analysis of protein kinases in DN-p38 transfectant and wild type WB-ras cells. (**A**) Time-dependent changes in protein kinases of DN-p38 WB-ras cells. (**B**) Comparison of changes in protein kinases between DN-p38 transfectant and wild-type (WT) WB-ras cells.

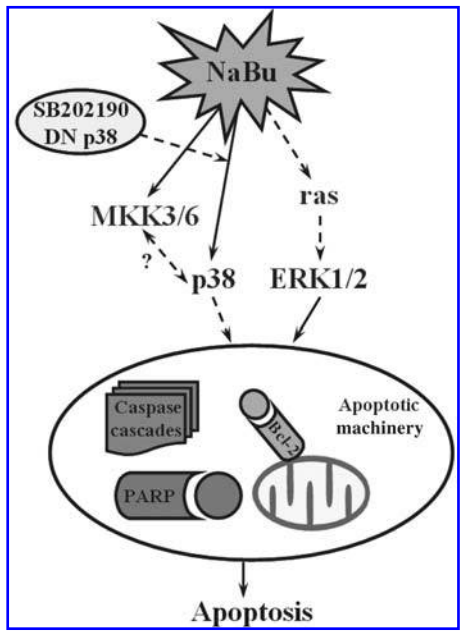

FIG. 9. The schematic diagram of the mechanism of NaBu-induced apoptosis in WB-ras cells, and the role of p38 MAP kinase.

In conclusion, apoptotic efficiency of NaBu was closely linked with p38 MAP kinase in WB-ras cells, and inhibition of p38 MAP kinase activity could increase this efficiency dramatically. Here, we suggest that approaches with concepts based on synergism between NaBu and p38 MAP kinase can lead to a higher chemopreventive effect with lower concentrations of NaBu, decreasing the possibility of adverse effect.

ABBREVIATIONS

DN, dominant negative; ERK, extracellular signal-regulated kinase; HDAC, histone deacetylase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MKK3/6, MAP kinase kinase 3/6; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NaBu, sodium butyrate; PARP, poly(ADP-ribose)polymerase; PBS, phosphate-buffered saline.

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