

Enhancement of Sodium Butyrate-induced Cell Death by Hyperthermia in HCT 116 Human Colorectal Cancer Cells

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Abstract. *Aim: The possible enhancing effect of the combined use of sodium butyrate (SB) and hyperthermia to kill HCT 116 cells was evaluated. Materials and Methods: HCT 116 cells were subjected to SB (1 mM) treatment followed by hyperthermia (44°C 60 min) and the effects on cell death, cell proliferation and the cell cycle were examined. Apoptosis-indicating protein expressions and intracellular superoxide formation were also analysed. Results: A marked reduction in the growth rate of the combined-treatment group was observed compared to those of the single-treatment groups. This involved the increased expression of p53 and p21, the alteration of the balance of anti- and proapoptotic Bcl-2 family proteins and enhanced superoxide formation. However, the death receptor pathway played no role. Conclusion: Hyperthermia synergistically promoted cell death induced by SB. Thus, the combined treatment led to mutual potentiation of the killing effects of each agent.*

Colorectal cancer is one of the three leading causes of cancer deaths worldwide (1). Most colorectal cancer cases are sporadic, with dietary risk factors implicated in their development. Despite curative surgery, patients still have a significant probability of disease relapse and cancer-related death. Much interest has been generated in the last few decades in adjuvant treatment that would eliminate microscopic disease, thus preventing recurrent diseases (2).

Hyperthermia is usually applied as an adjunct to an already established treatment modality, especially radiotherapy and chemotherapy, although the mechanisms leading to favorable clinical results of hyperthermia have not yet been identified (3). Synergy between heat and drugs may arise from multiple

events such as heat damage to ABC transporters (drug accumulation), intracellular drug detoxification pathways and repair of drug-induced DNA adducts (4). Many trials are being conducted to improve the outcome of cancer therapy and decrease the recurrence possibility. Tracing the underlying molecular mechanisms within the adopted strategy for cell death is a cornerstone for the success of such trials.

Sodium butyrate (SB) is a four-carbon fatty acid and a natural component of the colonic milieu derived from anaerobic microbial fermentation of diet-derived complex carbohydrates. In molecular terms, the action of butyrate is probably related to deacetylase inhibition, leading to hyperacetylation of chromatin components such as histones and nonhistone proteins, and alterations in gene expression. SB has been shown to induce apoptosis in a number of types of cancer cells (5). It has received much attention as a potential chemopreventive agent and for its protective action against early tumorigenic events in colorectal cancer (6). However, during the first clinical trials, limited efficacy was found (7).

In addition to its effectiveness as an anticancer agent by itself, SB was shown to stimulate apoptosis induced by heat shock under acidic conditions; but the possible mechanism remains unclear (8). As it is known, apoptosis induced by SB in human colorectal adenoma and carcinoma cell lines is caspase-dependent (9). Heat-induced apoptosis proceeds via a caspase pathway (10). Therefore, we propose that their combined treatment might have amplified effects on related pathways.

In this study, we applied the combined treatment to a human colon cancer cell line (HCT 116) and assessed whether hyperthermia affects butyrate-induced apoptosis in as much as butyrate sensitizes the cells to hyperthermia. We also tried to depict the interplaying apoptotic pathways involved in cell death.

Materials and Methods

Cell culture and treatments. Human colon carcinoma cells (HCT 116) (obtained from DS Pharma Biomedical Co., Ltd., Osaka, Japan) were grown in a modified McCoy's 5A medium (Gibco, #16600-082; Grand Island, New York, USA) containing 10% heat-inactivated fetal bovine serum. The cells were maintained in a

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humidified atmosphere containing 5% CO₂ at 37°C, and passaged using 0.25% trypsin three times weekly. All experiments were performed using logarithmically growing cells divided into 6 groups as follows: control (C), 44°C 30 min (H30), 44°C 60 min treatment (H60), sodium butyrate (SB), SB+H30, and SB+H60. Approximately 1×10⁶ cells were inoculated in 6-cm culture dishes containing culture medium (4 ml). SB (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to the SB-designated groups media at a final concentration of 1 mM.

On the second day of culture, the hyperthermia-designated groups were exposed to hyperthermia for the determined time by immersing the plastic culture dish in a water-bath (NTT-1200; Eyela, Tokyo, Japan) at 44.0±0.05°C. All analytical testing was carried out 24 h after hyperthermia, *i.e.* on the third day of the experiment, unless otherwise mentioned. The temperature of the solution inside the dish was monitored with a digital thermometer (#7563, Yokogawa, Tokyo, Japan) coupled with a thermocouple 0.8 mm in diameter during heating.

Survival curve studies. The trypan blue dye exclusion test was performed on all groups for 5 consecutive days starting from the first day of the experiment. Cell suspensions were mixed with equal amounts of 0.3% trypan blue solution (#T6146, Sigma-Aldrich, Inc. St. Louis, MO, USA) in PBS. After 5 min incubation at room temperature, the number of unstained (viable) cells and stained (non-viable) cells was counted using a Burker Turk hemocytometer. The percentage of dead cells was determined from the ratio of the number of stained cells to the total number of stained and unstained cells. The validity of this method has been demonstrated elsewhere (11).

Analysis of the cell cycle. Cell phase distribution was determined using flow cytometry. Cells (1-7×10⁶) were harvested and washed with PBS. The cells were fixed overnight in 10 ml of 70% (-20°C) ethanol at 4°C, washed with PBS and incubated for 60 min at 37°C with RNase (25 mg/ml) (Wako Pure Chemical Industries, Ltd.). Then they were treated with propidium iodide (50 µg/ml) (Wako Pure Chemical Industries, Ltd.), and incubated for 30 min at 4°C in the dark followed by flow cytometry (Epics XL; Beckman-Coulter, Miami, FL, USA). A minimum of 10,000 cells were collected in each run. The cells were selected by pulse-height (doublet elimination) analysis and only the integrated signals were collected to reject doublets. All histograms were evaluated by Multicycle software (version 3.0; Phoenix Flow Systems, San Diego, CA, USA). According to the instruction, we adopted the program of overlapped peak to calculate the proportions of different phases.

DNA fragmentation assay. The amount of DNA extracted from cells was assayed using the method of Sellins and Cohen with few modifications (12, 13). Briefly, approximately 3×10⁶ cells were lysed using 200 µl of lysis buffer (10 mM Tris, 1 mM EDTA, 0.2% Triton X-100; pH 7.5) and centrifuged at 13,000 ×g for 10 min. Subsequently, DNA from each sample in the supernatant and the pellet was precipitated in 12.5% trichloroacetic acid (TCA) at 4°C overnight and spectrophotometrically quantified using diphenylamine reagent after hydrolysis in 5% TCA at 90°C for 20 min. The percentage of fragmented DNA for each sample was calculated as the amount of DNA in the supernatant divided by the total DNA for that sample (supernatant plus pellet).

Measurements of caspase-3 and caspase-8 activities. FLICE/Caspase-3 and FLICE/Caspase-8 Colorimetric Protease Assay Kits (MBL, Nagoya, Japan) were used to measure the activities of caspase-3 and

caspase-8, respectively. The proteins were extracted from 1-2×10⁷ cells and prepared following the manufacturer's procedures. A total of 100 µg of extracted protein was reacted with Ac-DEVD-pNA substrate (caspase-3) or Ac-IETD-pNA substrate (caspase-8) at a final concentration of 200 µM with 50 µl of 10 mM dithiothreitol (DTT) for 60 min at 37°C in a final volume of 100 µl reaction buffer. The amount of the cleaved chromophore p-nitroanilide (pNA) was then quantified spectrophotometrically at 400 nm using a spectrophotometer (Beckman Instruments Inc., CA, USA).

Flow cytometry to determine mitochondrial transmembrane potential (MMP or Δψ). The cationic fluorophore, tetramethyl-rhodamine methyl ester (TMRM) (Molecular Probes, Eugene, OR, USA), accumulates electrophoretically in mitochondria in response to Δψ and is released upon the loss of Δψ. TMRM at concentrations as low as 10 nM has been shown to represent the Δψ loss in cells with long-lasting opening of permeability transition pores (14). Cells were harvested 0, 6, 12, 24 and 48 h after hyperthermia and stained with 10 nM TMRM for 15 min at 37°C in PBS containing 1% fetal bovine serum, followed by immediate flow cytometry (excitation at 488 nm; emission at 575 nm) with a flow cytometer (Epics XL, Beckman-Coulter, Miami, FL, USA). At least 10 000 cells were collected in each sample. Percentage of low MMP cells was obtained from cell counts falling into a 0.1~12 low window of the TMRM log scale.

Determination of superoxide (O₂^{•-}) levels by dihydroethidine (DHE). To measure the relative levels of intracellular oxidative stress caused by different treatment regimens, the dye DHE, which is easily oxidized by O₂^{•-} to red-fluorescent ethidium (Eth), was used. Cells were collected 0, 6, 12, 24 and 48 h after hyperthermia and incubated with 5 µM DHE (Molecular Probes) in PBS for 15 min at 37°C and immediately analyzed by flow cytometry (excitation at 488 nm; emission at 620 nm) with a flow cytometer (Beckman-Coulter) (15). At least 10 000 cells were collected in each sample. Percentage of cells in high window of the Eth log scale was controlled within 3% in control group. The scale determined in control group was applied to all other groups.

Western blot analysis. A total of 0.6-1×10⁷ cells per assay were collected, washed with cold PBS and lysed at a density of 10⁷ cells/100 µl of RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 (v/v), 1% sodium deoxycholate, 0.05% sodium dodecyl sulfate (SDS), 1 µg/ml of each of aprotinin, pepstatin, and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] for 20 min on ice. After brief sonication, lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The protein content in the supernatant was measured using a DC Protein Assay Kit (Bio-Rad Laboratories, Inc, Hercules, California, USA). Western blotting for p53, p21, Bcl-2, Bak, Bax, Bid, PCNA, procaspase-8 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), Bcl-xL, procaspase-3 (Cell Signaling Technology, Inc. Danvers, MA, USA) and anti-β-actin mAb (Sigma-Aldrich) was carried out (16). Using the respective secondary horseradish peroxidase (HRP) conjugated anti-rabbit and anti-mouse IgGs (Sigma-Aldrich), band signals were visualized on an X-ray film using an ECL system (GE Healthcare, UK).

For the preparation of cytosolic extracts, cells suspended in an ice-cold solution containing 20 mM HEPES (pH 7.5) were disrupted using a Dounce homogenizer. Samples were centrifuged at 1,500 ×g for 5 min at 4°C to remove nuclei and intact cells. The supernatant was centrifuged again at 105,000 ×g for 30 min at 4°C.

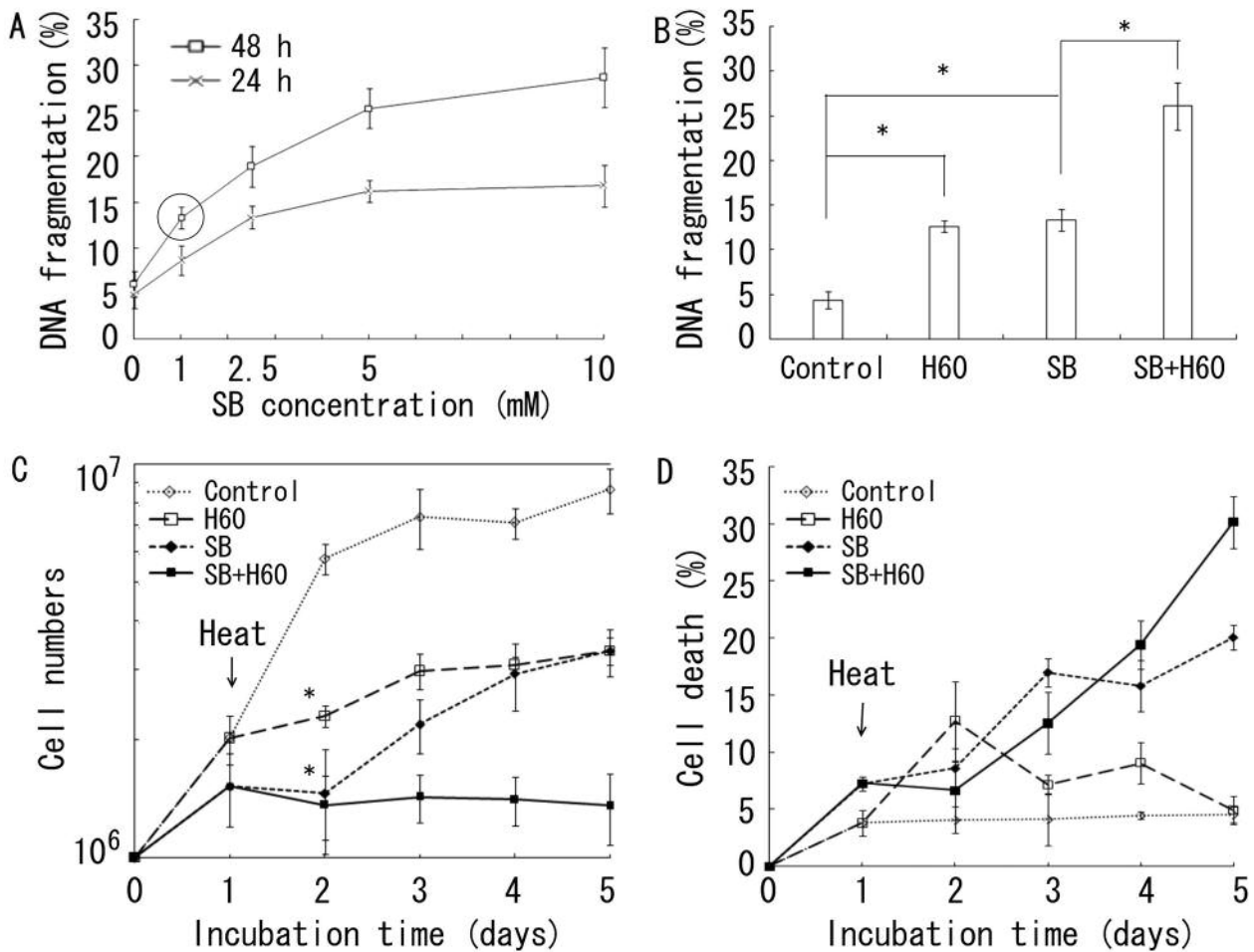


Figure 1. Effects of sodium butyrate (SB) treatment and hyperthermia on DNA fragmentation, cell growth and cell killing in HCT 116 cells. (A) Effects of SB at graded doses on DNA fragmentation. (B) Effects of hyperthermia (44°C 60 min), SB treatment (1 mM) and their combination. * $p < 0.05$, compared with appropriate control. (C) The relationships between cell numbers and incubation time (days). * $p < 0.05$, compared to control. (D) The relationships between cell death and incubation time (days). H60: 44°C 60 min treatment; SB+H60: combination.

The resulting supernatant was used as the soluble cytosolic fraction. Following measurement of the protein content, SDS-PAGE was carried out. Then Western blotting was performed to detect cytochrome *c* release to cytosol using anticypochrome *c* pAb (Santa Cruz Biotechnology Inc.) and anti- β -actin mAb (Sigma-Aldrich).

Statistical analysis. All experiments were conducted in triplicates unless otherwise specified. All results are presented as means \pm SD. Statistical analysis was carried out using a two-factor ANOVA test. p -values less than 0.05 were regarded as significant.

Results

DNA fragmentation analysis. The different treatment groups were subjected to DNA fragmentation analysis for evaluating the extent of apoptosis on the third day of experiment. First, the effects of SB on apoptosis were

examined: the percentage of DNA fragmentation was found to increase in a dose- and time-dependent manner. Since 1 mM SB exerted a relatively low toxicity (Figure 1 A), it was used for further experiments. Both groups subjected to hyperthermia or SB exhibited significant DNA fragmentation (12.6% \pm 0.6% and 13.2% \pm 1.2% respectively, $p < 0.05$) (Figure 1 A and B). The groups subjected to combined treatment exhibited a significant increase in the percentage of DNA fragmentation compared with the SB group (26.0% \pm 2.6% when heated for 1 h, $p < 0.05$), although there was no significant change when heated for 30 minutes (data not shown). Thus, the combined treatment of 1 mM SB and hyperthermia synergistically augmented the apoptotic effect of SB (two-factor ANOVA, $p < 0.05$), which might be dependent on the treatment time.

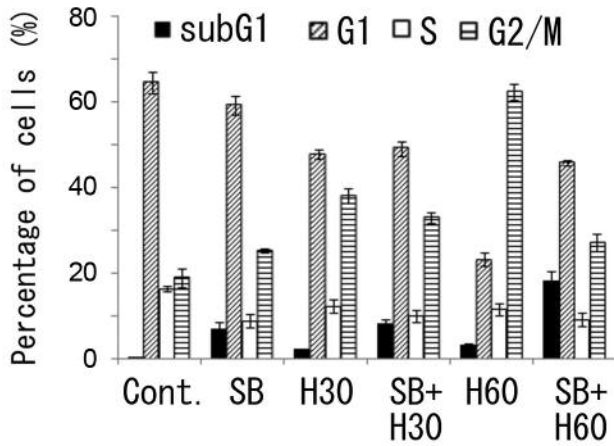


Figure 2. Cell cycle distribution after SB treatment (1 mM), hyperthermia (44°C 30 min or 60 min) or the combination in HCT 116 cells. Cell cycle distribution of cells after treatment with SB, hyperthermia and the combination at 48 h. Cont.: control; SB: sodium butyrate; H30: 44°C 30 min treatment; H60: 44°C 60 min treatment; SB+H30 and SB+H60: combination.

Analyses of cell growth and viability. Generally, growth curves can help identify the extent and the persistence of the lethal effects exerted by a therapeutic agent. The SB group showed a significant decrease (3.9-fold) in growth rates compared to the control group, as did the hyperthermia group over the 2 days following the heat treatment. The growth rate of the combination group exhibited the greatest decrease compared with the control, SB and heated groups (4.2-, 1.1- and 1.7-fold, respectively) with a similar modality of growth recovery to the heated group (Figure 1 C), while the pattern of cell death in combination group was similar to that in SB alone group (Figure 1 D). This augmented fall in survival rates bore evidence for a possible synergistic effect achieved by the combined therapy.

Cell cycle distribution. The cellular response to DNA damage involves checkpoint controls that delay cell cycle progression to provide time for either repairing of damaged DNA, or triggering of apoptosis. The analysis of the SB group showed notable subG1 and G2/M blockade characteristic of cell cycle arrest induced by SB, while the heated groups showed only high G2/M arrest. Interestingly, the combined treatment groups exhibited subG1 and G2/M block, similar to those of the SB group but different in proportion where there was an increase in the subG1 phase and more cells are accumulated in the G2/M phase (Figure 2).

Mitochondrial transmembrane potential (MMP or $\Delta\psi$) and superoxide formation patterns. To examine whether the mitochondrial pathway is related to the enhanced apoptosis on treatment with the combination of SB and heat, we

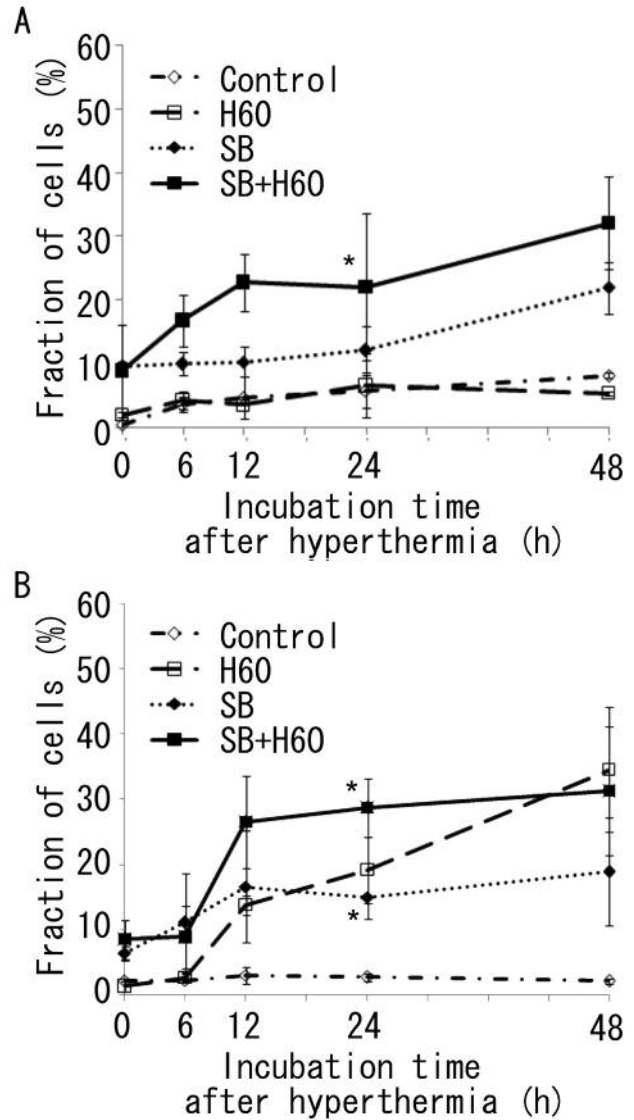


Figure 3. Flow cytometry of mitochondrial transmembrane potential (MMP) and superoxide generation in HCT 116 cells. (A) The relationship between fractions of cells showing low MMP detected using the fluorescence dye, TMRM, and incubation time (days). (B) The relationship between fractions of cells showing superoxide generation detected using a fluorescence dye, DHE, and incubation time (days). * $p < 0.05$, compared with control. H60: 44°C 60 min treatment; SB: sodium butyrate; SB+H60: combination.

measured the changes in MMP and reactive oxygen species (ROS) which have been shown to be responsible for outer mitochondrial membrane rupture (17). SB induced a notable loss of MMP [2.1-fold that of the control 24 h after heat treatment (where given), but not significant, $p > 0.05$] and a significant increase in superoxide production [5.6-fold that of the control 24 h after heat treatment (where given), $p < 0.05$] (Figure 3 A and B). On the other hand, hyperthermia led to

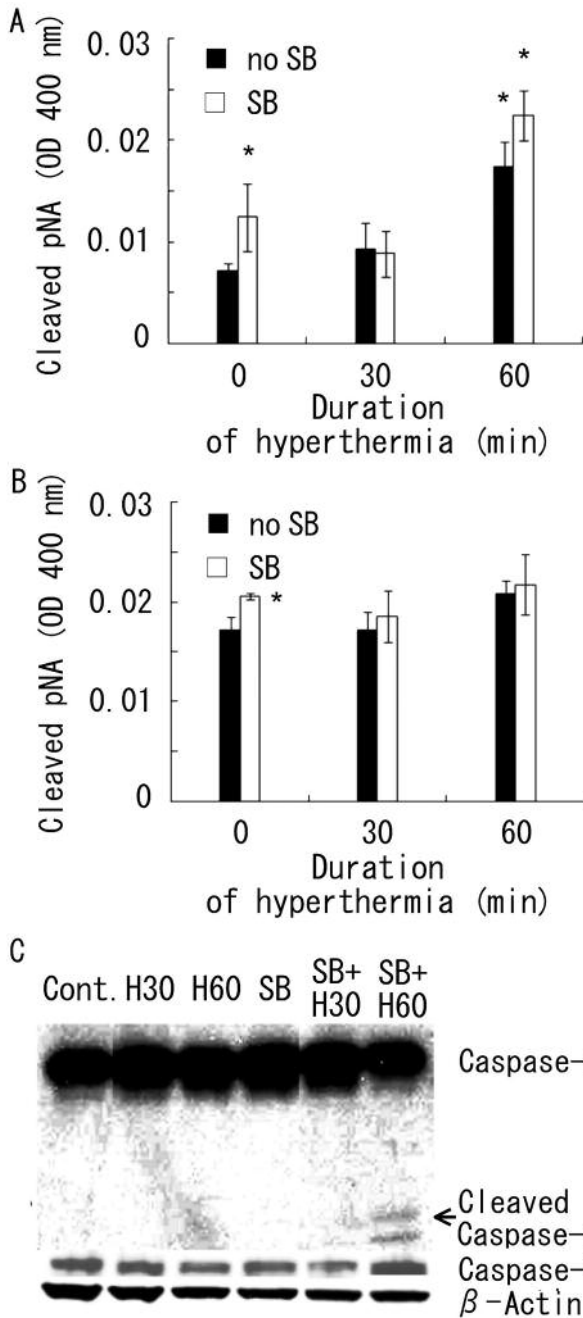


Figure 4. Enzyme activities and protein expressions of caspase-3 and caspase-8 in HCT 116 cells after hyperthermia with or without SB treatment. (A) Caspase-3 activity. (B) Caspase-8 activity. (C) Expressions of caspase-3 and caspase-8 detected by western blotting. Cont.: control; H30: 44°C 30 min treatment; H60: 44°C 60 min treatment; SB: sodium butyrate; SB+H30 and SB+H60: combination. * $p < 0.05$, compared with control.

no significant loss of MMP but did induce a gradual increase in superoxide production after heat treatment. The (SB + H60) group exhibited reduced MMP and increased

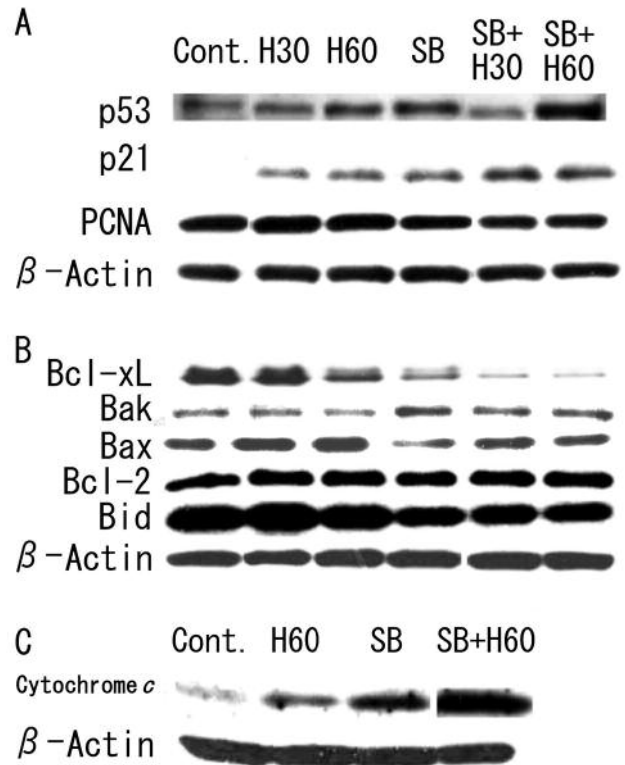


Figure 5. Western blotting analysis of protein expressions. HCT 116 cells were heated at 44°C for 30 min or 60 min following incubation with 1 mM SB for 24 h, and changes in protein expression levels were examined. (A) p53, p21 and PCNA. (B) Bcl-2 family proteins. (C) Cytochrome c. Cont.: control; H30: 44°C 30 min treatment; H60: 44°C 60 min treatment; SB: sodium butyrate; SB+H30 and SB+H60: combination.

superoxide production (3.8- and 10.6-fold that of the control 24 h after heat treatment respectively, $p < 0.05$) in a pattern similar to that induced by SB alone but to a greater extent. This similarity augments the hypothesis that hyperthermia increased the SB-induced apoptosis. These results also suggest that the enhancement of apoptosis involves the mitochondrial pathway under combined treatment.

Enzymatic activity of caspase-3 and caspase-8. To clarify whether the enhanced apoptosis is due to the caspase-dependent mitochondrial pathway, we studied the change of caspase-3 activity. The enzymatic activity of caspase-3 increased by 1.7-fold (SB) and by about 3.1-fold (SB + H60) compared to that of the control ($p < 0.05$), while hyperthermia of 60 min alone induced a significant 2.4-fold increase ($p < 0.05$) (Figure 4 A). To confirm these findings the protein expressions were also detected by Western blotting (Figure 4 C). The cleaved component was detected in the SB treated groups as well as the 60-minute heated

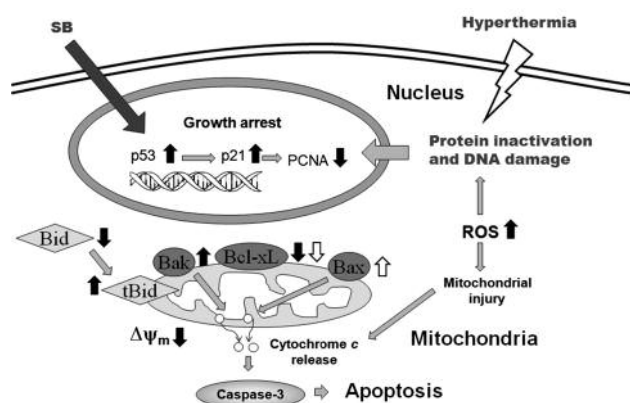


Figure 6. The mechanisms related to the enhancement of cell death by combined treatment with SB and hyperthermia. SB and hyperthermia regulate p53 and p21 proteins to arrest cell growth. SB alone changes the expression of Bcl-xL, Bak and Bid, whereas hyperthermia alone changes the Bax and Bcl-xL expression. Because caspase-3 cleavage and release of cytochrome c are increased, the enhanced cell death is associated with the stronger activation of the mitochondrial caspase pathway. Meanwhile, superoxide formation due to SB treatment or hyperthermia also contributes to the enhancement of apoptosis when both agents are combined. Arrows pointing up indicate stimulation while those pointing down indicate inhibition. The open arrows beside Bax and Bcl-xL are to emphasize the action of hyperthermia. SB: sodium butyrate; ROS: reactive oxygen species.

group. This suggests that caspase-3 could be partially responsible for the synergic effect in the combination groups.

To further demonstrate whether the activated pathway is intrinsic or extrinsic, the activity of caspase-8 was studied. Although the enhancement of activity was statistically significant, the increase was very slight when compared with that in the control (Figure 4 B). Furthermore, caspase-8 levels were practically the same among all the groups and no cleaved components were detected (Figure 4 C). Thus it seems that caspase-8 is not the primary activated caspase protein and that the extrinsic pathway has little effect on the enhanced apoptosis demonstrated by SB with heat treatment.

Expressions of apoptosis-related proteins. To investigate the mechanisms of cell death and cell growth arrest, we measured the expression of apoptosis-related proteins. Strikingly, the SB group exhibited large increases in p53 and p21 expression, while all heated groups showed a significant increase in p21. The (SB + H60) group expressed both proteins at a significantly greater level than the control (Figure 5 A). The PCNA protein expression was down-regulated with SB treatment.

Among the Bcl-2 family proteins, Bak expression increased and Bcl-xL and Bid expressions decreased with SB treatment, while Bax expression was enhanced by hyperthermia treatments. In addition, the expression of Bcl-

Table I. Western blotting analysis of protein expressions. HCT 116 cells were heated at 44°C for 60 min following incubation with 1 mM sodium butyrate (SB) for 24 h, and changes of protein expressions were examined.

Protein	SB	heat	SB + heat
P53	+	+	+
P21	+	+	+
PCNA	-	NC	-
Bcl-xL	-	-	-
Bak	+	NC	+
Bax	NC	+	+
Bcl-2	NC	NC	NC
Bid	-	NC	-
Cyto c	+	+	+

+ indicates increase, - decrease and NC no change compared to control cells.

xL was reduced to a greater extent in the combined treatment groups. However, no change in the expression of Bcl-2 was observed after any treatment (Figure 5 B). There was an overall increase in the production of apoptotic proteins with a concomitant decrease in the production of antiapoptotic ones in the SB and hyperthermia-treated groups (Table I).

In addition, cytochrome c was released to a significantly greater extent in the SB + H60 and SB groups, although it was also detected in the H60 group (Figure 5 C). This result was consistent with the loss of MMP.

Discussion

In this study, we have tried to determine if hyperthermia can potentiate SB-induced apoptosis in HCT 116 cells. As a result, we found that hyperthermia promoted synergistically cell death induced by SB. Moreover, the mechanism involved a pronounced activation of the mitochondrial apoptotic pathway with a specific alteration in the balance of anti- and proapoptotic Bcl-2 family proteins under the different treatments.

It is known that the effect of SB on mammalian cells in culture markedly varies depending upon the cell type (18). Here, we used HCT 116 cells, a colon cancer cell with wild-type p53 and exposed it to SB one day before hyperthermia and maintained it even after hyperthermia had been completed for the whole course of the experiment. The dose of SB used was 1 mM. This dose was shown to cause minimal DNA fragmentation and apoptosis (13.2 ± 1.2%) based on a dose- and time-dependent study. Under such conditions, we determined that a combination of SB treatment and hyperthermia could induce stronger growth inhibition and more cell death. We also found that there was a marked increase in the number of sub G1-phase cells in the combined treatment groups compared to that in the SB-

treated group. Furthermore, we found a synergistic effect of combined treatment on cell death compared with SB alone (26% to 13%, $p < 0.005$) in the DNA fragmentation assay. Therefore, we further studied why the combination treatment induced increased cell death.

Members of the Bcl-2 family proteins are central regulators of mitochondrial integrity and apoptotic cell death (19). Mammals possess an entire family of Bcl-2 proteins that includes proapoptotic as well as antiapoptotic members. The ratio of anti- to proapoptotic molecules such as Bcl-2/Bax constitutes a 'rheostat' that sets the threshold of susceptibility to apoptosis for the intrinsic pathway, which utilizes organelles such as the mitochondrion to amplify death signals. It is known that Bax or Bak is an essential gateway to mitochondrial dysfunction required for cell death in response to diverse stimuli (20). In our study, increased Bak expression was observed by SB treatment while Bax expression was enhanced by hyperthermia treatment, although Bax decreased in the SB-treated group. From our data, the increased expression of both proteins in the combined treatment probably contributes to the enhanced cell death observed. This is consistent with the cytochrome *c* release, loss of MMP, caspase-3 cleavage and DNA fragmentation.

It is worth mentioning that Bid expression was highly diminished in all SB-treated groups. Bid is cleaved and activated to tBid after activation, which induces the oligomerization of Bak and Bax, inducing cytochrome *c* efflux (21). In fact, the change in the expression of Bid may play a small role in the enhancement in our study. However, we cannot confirm the factor responsible for the activation of Bid, because one of the upstream proteins, caspase-8, is not activated efficiently. Thereby, the death receptor may not be the major target during the treatment.

In contrast, the antiapoptotic protein Bcl-xL was down-regulated in all SB-treated groups, more so in the combination groups, while Bcl-2 was consistently expressed in all groups. The antagonistic relations of Bcl-2 (22), Bcl-xL/Bax (23) and Bcl-xL/Bak (24) have been demonstrated. The impairment in the expressions of the antiapoptotic proteins suggests that the enhanced effect in the combination group is partially due to a change of the antagonism. While we found an attenuation of Bcl-xL in the group heated for 1 hour, no significant change was determined after 30 minutes treatment. The different expression levels might be one of the reasons for trivial effects when heated for 30 minutes. As for the slight increase of cell death after combined treatment with 30 minutes hyperthermia compared with that in SB group (data not shown), we propose that there must be inhibition of the caspase pathway which is not destroyed by the hyperthermia, considering the slight cleavage of caspase-3. More work is needed to clarify this. In addition, we also found the percentage of cells which lost MMP was as low as the control after hyperthermia treatment, while significant cell death and caspase-3 activity were shown.

This fact can be explained by the fact that the release of cytochrome *c* is not always accompanied by changes in MMP (25). The deeper relationship between those also needs more research. In summary, there was an overall increase in the production of apoptotic proteins with a concomitant decrease in antiapoptotic ones in the SB and hyperthermia-treated groups, so we can draw the conclusion that the enhanced cell death observed was achieved by the enhanced activation of the mitochondrial pathway by the prevailing effect of the proapoptotic Bcl-2 family members.

Taking account of the upstream factors of Bcl-2 family, p53 is one of the most important proteins. It can form complexes with the protective Bcl-xL and Bcl-2 proteins (26) and regulate Bax and Bak (27). In our study, SB induced a high level expression of p53 and greater expression was found in the combination group. This suggests that p53 expression is involved in the cell death effect in HCT 116 cells and is a basic factor in the effect of the combined treatment.

In this study, cell growth arrest was also observed and a significant change was noted in the combined treatment group. It is well known that p21 is necessary for the p53-mediated G1 (28) and G2 (29) arrests. Moreover antiproliferative effects require p21 gene expression (30). To investigate the mechanism for the enhancement, we examined the expression of p21 by western blotting. P21 expression was significantly elevated in both SB and hyperthermia groups and further increased in the combination treatment group, which is confirmed by the cell growth curve. Since p21 was shown to inhibit cell cycle progression by inhibiting the functions of cyclin/CDK complexes, and the PCNA function in both G1 and G2 arrests (31), we further detected the expressions of PCNA and cyclin D1. PCNA was down-regulated in the SB-treated group, but no change was observed in the hyperthermia group. As for cyclin D1, it increased in the SB-treated group (data not shown). Furthermore, in the cell cycle analysis, only G2/M block was found in all the groups. These results suggest that the growth arrest occurred in the G2-phase, not in the G1-phase, which explains the increase of cyclin D1 expression. As to the effector of the growth arrest, PCNA is at least a promoter. Further work is needed for clarification.

The enhancement of intracellular superoxide formation is closely related to mitochondrial dysfunction and cell apoptosis (32). In our study, a close association was clearly shown, that is, the decrease in MMP was associated with the increase of superoxide formation, except in the hyperthermia treatment. It may be that SB can inactivate some protection against oxidative damage to mitochondria. Alternately, this effect may be responsible for the enhanced cell death observed in the combination treatment group.

In conclusion, we have shown the presence of mutual potentiation exerted by both butyrate and hyperthermia adjuvant therapy. We also explored the interplay of molecular

pathways that culminate in cell death (Figure 6). With the combined treatment, the apoptotic mitochondrial pathway was enhanced significantly and found to be involved in the increased expression of p53 and p21, the release of cytochrome *c*, and the alteration in the balance of anti- and proapoptotic Bcl-2 family proteins. Note that enhanced superoxide formation was observed. However, the death receptor pathway was not found to play a role in the phenomenon observed here.

References

- Jemal A, Siegel R, Ward E, Murray T, Xu J and Thun MJ: Cancer statistics, 2007. *CA Cancer J Clin* 57: 43-66, 2007.
- Chau I and Cunningham D: Chemotherapy in colorectal cancer: new options and new challenges. *Br Med Bull* 64: 159-180, 2002.
- Hildebrandt B, Wust P, Ahlers O, Dieing A, Sreenivasa G, Kerner T, Felix R and Riess H: The cellular and molecular basis of hyperthermia. *Crit Rev Oncol Hematol* 43: 33-56, 2002.
- Kampinga HH: Cell biological effects of hyperthermia alone or combined with radiation or drugs: a short introduction to newcomers in the field. *Int J Hyperthermia* 22: 191-196, 2006.
- Chopin V, Toillon RA, Jouy N and Le Bourhis X: Sodium butyrate induces P53-independent, Fas-mediated apoptosis in MCF-7 human breast cancer cells. *Br J Pharmacol* 135: 79-86, 2002.
- Sengupta S, Muir JG and Gibson PR: Does butyrate protect from colorectal cancer? *J Gastroenterol Hepatol* 21: 209-218, 2006.
- Kelly WK, O'Connor OA and Marks PA: Histone deacetylase inhibitors: from target to clinical trials. *Expert Opin Investig Drugs* 11: 1695-1713, 2002.
- Narita N, Fujieda S, Tokuriki M, Takahashi N, Tsubuki H, Ohtsubo T and Matsumoto H: Inhibition of histone deacetylase 3 stimulates apoptosis induced by heat shock under acidic conditions in human maxillary cancer. *Oncogene* 24: 7346-7354, 2005.
- Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D and Paraskeva C: Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer* 55: 498-505, 1993.
- Yasumoto J, Kirita T, Takahashi A, Ohnishi K, Imai Y, Yuki K and Ohnishi T: Apoptosis-related gene expression after hyperthermia in human tongue squamous cell carcinoma cells harboring wild-type or mutated-type p53. *Cancer Lett* 204: 41-51, 2004.
- Patterson MK Jr: Measurement of growth and viability of cells in culture. *Methods Enzymol* 58: 141-152, 1979.
- Sellins KS and Cohen JJ: Gene induction by gamma-irradiation leads to DNA fragmentation in lymphocytes. *J Immunol* 139: 3199-3206, 1987.
- Hopcica KL, McCarey YL, Sylvester FC and Held KD: Radiation-induced apoptosis in HL60 cells: oxygen effect, relationship between apoptosis and loss of clonogenicity, and dependence of time to apoptosis on radiation dose. *Radiat Res* 145: 315-323, 1996.
- Petronilli V, Penzo D, Scorrano L, Bernardi P and Di Lisa F: The mitochondrial permeability transition, release of cytochrome *c* and cell death. Correlation with the duration of pore openings in situ. *J Biol Chem* 276: 12030-12034, 2001.
- Gorman AM, Heavey B, Creagh E, Cotter TG and Samali A: Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *FEBS Lett* 445: 98-102, 1999.
- Zhao QL, Kondo T, Noda A and Fujiwara Y: Mitochondrial and intracellular free-calcium regulation of radiation-induced apoptosis in human leukemic cells. *Int J Radiat Biol* 75: 493-504, 1999.
- Zhao QL, Fujiwara Y and Kondo T: Mechanism of cell death induction by nitroxide and hyperthermia. *Free Radic Biol Med* 40: 1131-1143, 2006.
- Prasad KN: Butyric acid: a small fatty acid with diverse biological functions. *Life Sci* 27: 1351-1358, 1980.
- Danial NN and Korsmeyer SJ: Cell death: critical control points. *Cell* 116: 205-219, 2004.
- Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB and Korsmeyer SJ: Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 292: 727-730, 2001.
- Yin XM: Bid, a BH3-only multi-functional molecule, is at the cross road of life and death. *Gene* 369: 7-19, 2006.
- Antonsson B, Conti F, Ciavatta A, Montessuit S, Lewis S, Martinou I, Bernasconi L, Bernard A, Mermod JJ, Mazzei G, Maundrell K, Gambale F, Sadoul R and Martinou JC: Inhibition of Bax channel-forming activity by Bcl-2. *Science* 277: 370-372, 1997.
- Yang E, Zha J, Jockel J, Boise LH, Thompson CB and Korsmeyer SJ: Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. *Cell* 80: 285-291, 1995.
- Sattler M, Liang H, Nettesheim D, Meadows RP, Harlan JE, Eberstadt M, Yoon HS, Shuker SB, Chang BS, Minn AJ, Thompson CB and Fesik SW: Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. *Science* 275: 983-986, 1997.
- Kluck RM, Bossy-Wetzel E, Green DR and Newmeyer DD: The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275: 1132-1136, 1997.
- Mihara M, Erster S, Zaika A, Petrenko O, Chittenden T, Pancoska P and Moll UM: p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* 11: 577-590, 2003.
- Pearson AS, Spitz FR, Swisher SG, Kataoka M, Sarkiss MG, Meyn RE, McDonnell TJ, Cristiano RJ and Roth JA: Up-regulation of the proapoptotic mediators Bax and Bak after adenovirus-mediated p53 gene transfer in lung cancer cells. *Clin Cancer Res* 6: 887-890, 2000.
- Waldman T, Kinzler KW and Vogelstein B: p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res* 55: 5187-5190, 1995.
- Bunz F, Dutriaux A, Lengauer C, Waldman T, Zhou S, Brown JP, Sedivy JM, Kinzler KW and Vogelstein B: Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* 282: 1497-1501, 1998.
- Hinnebusch BF, Meng S, Wu JT, Archer SY and Hodin RA: The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. *J Nutr* 132: 1012-1017, 2002.
- Cayrol C, Knibiehler M and Ducommun B: p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene* 16: 311-320, 1998.
- Chakraborti T, Das S, Mondal M, Roychoudhury S and Chakraborti S: Oxidant, mitochondria and calcium: an overview. *Cell Signal* 11: 77-85, 1999.

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