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Abbreviations: ACA, 1'-Acetoxychavicol acetate; NaB, Sodium butyrate; ROS, reactive oxygen species; AMPK, AMP-activated protein kinase; EATC, Ehrlich ascites tumor cells; HDAC, histone deacetylase; CI, combination index; DCFH-DA, 2',7'-dichloroihydrofluorescein diacetate;

Abstract

It has been suggested that the combined effect of natural products may improve the effect of treatment against the proliferation of cancer cells. In this study, we evaluated the combination of 1'-acetoxychavicol acetate (ACA), obtained from *Alpinia galangal*, and sodium butyrate, a major short chain fatty acid, on the growth of HepG2 human hepatocellular carcinoma cells and found that treatment had a synergistic inhibitory effect. The number of HepG2 cells was synergistically decreased via apoptosis induction when cells were treated with both ACA and sodium butyrate. In ACA- and sodium butyrate-treated cells, intracellular reactive oxygen species (ROS) levels and NADPH oxidase activities were increased significantly. The decrease in cell number after combined treatment of ACA and sodium butyrate was diminished when cells were pretreated with catalase. These results suggest that an increase in intracellular ROS levels is involved in cancer cell death. AMP-activated protein kinase (AMPK), a cellular energy sensor, plays an essential role in controlling processes related to tumor development. In ACA- and sodium butyrate-treated cells, AMPK phosphorylation was induced significantly, and this induction improved when cells were pretreated with catalase. These results suggest that the increase in intracellular ROS is involved in the increase of AMPK phosphorylation. In normal hepatocyte cells, treatment with ACA and sodium butyrate did not decrease cell numbers or increase ROS levels. In conclusion, combined treatment with ACA and sodium butyrate synergistically induced apoptotic cell death via an increase in intracellular ROS and phosphorylation of AMPK. Our findings may provide new insight into the development of novel combination therapies against hepatocellular carcinoma.

Key Words: 1'-Acetoxychavicol acetate (ACA), Sodium butyrate (NaB), Synergistic effect, reactive oxygen species (ROS), AMP-activated protein kinase (AMPK), HepG2 cell.

1. Introduction

Hepatocellular carcinoma represents the fifth most common cancer and the third most common cause of death from cancer [1, 2]. The incidence of this cancer is particularly high in Eastern Asian and African regions [3] but is rapidly rising in other parts of the world, such as the United States [4]. Unfortunately, hepatocellular carcinoma tumors are highly resistant to currently available chemotherapeutic agents. The clinical outcome of hepatocellular carcinoma treatment remains unsatisfactory. Therefore, new effective and well-tolerated therapy strategies are urgently needed.

Natural products are excellent sources for developing new medications for disease treatment. Recently, the low side effects of natural products in anti-cancer treatments have been recognized, and more than 60% of drugs used to treat cancer are of natural origin [5]. Therefore, it is important to study the anti-cancer effect of natural products.

1'-Acetoxychavicol acetate (ACA) naturally occurs in the rhizomes and seeds of Zingiberaceae plants such as *Languas galangal* and *Alpinia galangal*. Southeast Asia residents traditionally ingest ACA when using plants as a spice or medicine in everyday life. ACA exhibits chemopreventive effects on chemically induced tumors in mouse skin as well as on rat oral, colonic, esophageal, and pancreatic tumors [6-10]. In addition to these in vivo observations, ACA exerts antitumor activity by inducing apoptosis in various tumor cells such as Ehrlich ascites tumor cells (EATCs) [11], rat and human hepatocellular carcinoma cells [12], human colon cancer cells [13], and human myeloid leukemia cells [14]. In a previous study, we showed that ACA induced apoptosis in EATCs by decreasing intracellular polyamines and inducing caspase-3 activity [11].

Dietary fiber fermentation by colonic bacterial flora produces the short-chain fatty acids acetate, propionate, and butyrate. These acids, which occur in millimolar amounts, are rapidly absorbed in the colon and provide an important energy supply for the colorectal epithelium [15]. Sodium butyrate has multiple effects on tumor cells cultured in vitro, including, most notably, inhibition of cell proliferation and induction of apoptosis [16, 17], as well as initiating the differentiation of

various carcinoma cells [18-20]. Butyrate also alters the transcription of several genes related to tumor growth and invasiveness [20-22] and suppresses the growth of tumors implanted in nude mice [23-25]. NaB is an inhibitor of histone deacetylase (HDAC), which is a class of proteins that can inhibit malignant cell growth in vitro and in vivo, bring about the reversion of oncogene-transformed cell morphology, induce apoptosis, and enhance cell differentiation.

Reactive oxygen species (ROS) are the most important mediators of oxidative stress and produced in aerobic organisms during normal metabolism [26-28]. Hydrogen oxide (H_2O_2) is most important ROS and induces oxygen radicals that cause oxidative stress to cells [27]

H_2O_2 is known to regulate intracellular signal transduction pathways and to induce apoptosis in a variety of cell types [29]. Catalase is a major scavenger of H_2O_2 and we hypothesized that H_2O_2 may be involved in the decrease in the number of HepG2 cells after combined treatment of ACA and sodium butyrate, and thus, we examined the effects of catalase against ACA- and sodium butyrate-induced death pathway including phosphorylation of AMP-activated protein kinase (AMPK).

AMPK is expressed in all eukaryotic cells and is a critical enzyme that plays an essential role in cellular energy homeostasis as well as controlling processes related to tumor development, including cell cycle progression, cell proliferation, protein synthesis, and survival. It has been reported that activation of AMPK induces apoptotic cell death [30]. Therefore, AMPK has received intense attention as an anti-cancer target in recent years [31]. Recently, we found that ACA induced a dose-dependent activation of AMPK in 3T3-L1 cells [32] and IEC-6 cells [33].

The aim of our study was to elucidate the synergistic interaction of ACA and NaB on cell viability in the human hepatocellular carcinoma cell line HepG2 and to examine the mechanisms of the anti-cancer effect of combined ACA and NaB treatment.

2. Materials and Methods

2.1 Materials

Racemic ACA was prepared according to a previously described method [34]. Sodium butyrate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). FBS was purchased from Nichirei Biosciences, Inc. (Tokyo, Japan).

Anti-phospho-AMPK (pAMPK) and anti-AMPK antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-p53 and anti-p-extracellular signal-regulated kinases (ERK)1/2 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

All other chemicals used in this study were special grade commercial products.

2.2 Cell culture

HepG2 cells or HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a humidified incubator containing 5% CO_2 at 37°C for 3-4 days and were then washed and cultured again in fresh medium in plastic dishes overnight.

Rat hepatocytes were isolated by collagenase perfusion from 10-week-old male Wistar rats anesthetized with diethyl ester. The isolated hepatocytes were plated in plastic dishes at a density of 2.5×10^5 cells/ml in 2 ml Williams' Medium E supplemented with 10% FBS and cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C overnight.

2.3 Neutral red assay

Cell number was determined with the neutral red uptake assay, which is based on lysosomal uptake of neutral red [35]. Following specific incubations with test agents, neutral red solution (0.25 mg/ml) was added to the cell cultures at a final concentration of 50 μg/ml neutral red. After incubation at 37°C for 2 h, cells were rinsed twice with a mixture of 1% (v/v) formaldehyde and 1% (v/v) calcium chloride in distilled water. Subsequently, 1 ml of destaining buffer consisting of 1% (v/v) acetic acid and 50% (v/v) ethanol in distilled water was added to the cells, and the culture

plates were destained for 30 min. Lysosomal uptake of neutral red was determined spectrophotometrically at 540 nm [36, 37]. Cell number was expressed as (A_{540}-treated cells/A_{540} of appropriate control) ×100% after correction for background absorbance.

2.4 Trypan blue assay

HT-29 cell viability was detected by the Trypan Blue assay. Cells were incubated with ACA and/or NaB for 24 hours. After incubation, cells were added an equal volume of 0.4% Trypan Blue reagent, and total cell number was counted under the microscope.

2.5 Determination of combination index

The interaction between ACA and NaB was quantified by determining the combination index (CI), which was calculated according to the medium-effect principle [38, 39]. The equation for the isobologram is $CI=(D)_1/(Dx)_1+(D)_2/(Dx)_2$, where $(Dx)_1$ and $(Dx)_2$ indicate the individual doses of ACA and NaB required to inhibit a given level of cell growth, and $(D)_1$ and $(D)_2$ are the doses of ACA and NaB necessary to produce the same level of inhibition in combination. The combined effects of ACA and NaB can be indicated as follows: CI<1, synergism; CI=1, additive effect; and CI>1, antagonism.

2.6 Propidium iodide staining assay

Cells were stained with the fluorescent DNA-binding dye propidium iodide (PI). Cells were washed twice with calcium-free phosphate-buffered saline (PBS(-)) and fixed with 70% ethanol at 4°C for 30 min. Fixed cells were washed with PBS(-), and then treated with RNase A(1 mg/ml) at 37°C for 30 min. After washing with PBS(-), cells were stained with PI (5 μg/ml) at 4°C for 1 h. Chromatin structures were examined using a SFX100 Bio Imaging Navigator (Olympus, Japan).

2.7 Quantification of apoptosis by ELISA

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis in HepG2 cells. Briefly, after treatments, the cytoplasmic histone/DNA fragments from cells were extracted and bound to immobilized anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the samples was determined by using Wallac 1420 ARVOsx multi label counter at 405 nm.

2.8 DCFH-DA assay

A relatively specific probe for hydrogen peroxide, namely 2',7'-dichloroihydrofluorescein diacetate (DCFH-DA), was used to analyze intracellular ROS formation. Cells were incubated with 2.4 mM DCFH-DA (5 µl) during the final 30 min of treatment. Cells were washed twice with PBS(-). Fluorescence intensity was detected with a SFX100 Bio Imaging Navigator (Olympus, Japan). Quantification of the intensity of ROS expression was analyzed using a computer with Win ROOF Ver. 6.2 (Mitami Corporation, Japan).

2.9 NADPH oxidase activity assay

Cells treated with ACA and/or NaB were washed twice in PBS(-), scraped, and then suspended in ice-cold buffer containing 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 100 mM sucrose. The cells were disrupted by three cycles of freezing and thawing. The samples were centrifuged at 800×g at 4°C for 5 min to remove unbroken cells and nuclei. The supernatants were then centrifuged at 100,000×g for 30 min to separate the membrane fractions, which were used in the enzyme assay [40]. Generation of O^{2-} in the membrane fraction was measured by lucigenin chemiluminescence using 100 µM NADPH, 50 µM lucigenin, and the cell membrane proteins. After 5 min incubation at 37°C, chemiluminescence was measured using a

Wallac 1420 ARVOsx multilabel counter (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA). Protein concentrations were determined by the Bradford method.

2.10 Catalase activity assay

Cells treated with ACA and/or NaB were washed twice in PBS(-) and then scraped and suspended in ice-cold buffer containing 5 mM EDTA, 0.25% sodium cholate, and 0.01% digitonin. The cells were disrupted and centrifuged. Then the supernatants were used for the enzyme assay. The reaction solution (1M Tris-HCl, 5 mM EDTA, and 10 mM H_2O_2) was added, and the absorbance was determined at 240 nm for 2 min. Protein concentrations were determined by the Bradford method.

2.11 Glutathione peroxidase (GPx) activity assay

Cells treated with ACA and/or NaB were washed twice in PBS(-) and then scraped and suspended in ice-cold buffer containing 5 mM EDTA, 0.25% sodium cholate, and 0.01% digitonin. The cells were disrupted and centrifuged. Then the supernatants were used for the enzyme assay. The reaction solution (1M Tris-HCl, 5 mM EDTA, 100 mM GSH, 10 U/ml GR, 2 mM NADPH, and 7 mM t-BuOOH) was added, and the absorbance was determined at 340 nm for 5 min. Protein concentrations were determined by the Bradford method.

2.12 Western blot analysis

Cells were washed twice in PBS(-) and then dissolved in lysis buffer (150 mM NaCl, 50 mM Tris [pH 7.2], 1 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM sodium vanadate, 1 mM NaF, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 10 μg/ml pepstatin A, and 100 μg/ml phenylmethylsulfonyl fluoride). The solutions were then centrifuged at 2000×g for 20 min at 4℃. The supernatant was collected, and protein concentration was determined by the Bradford method.

Equal amounts of protein were fractionated on 10% SDS-PAGE gels and transferred to 0.45-μm PVDF membranes (Hybond, Amersham Pharmacia Biotech). After blocking overnight in 0.1% Tween-20 and 5% non-fat dry milk in PBS, the blots were incubated with anti-AMPK (1:1000 dilution), anti-pAMPK (1:1000 dilution), anti-p53 (1:200 dilution) or anti-pERK (1:200 dilution) antibodies for 1 h at room temperature. The blots were also incubated with anti-β-actin (1:1000 dilution) or β-tubulin (1:200 dilution) antibodies as a control. After washing, the membrane was incubated with 1:500 diluted biotinylated mouse or rabbit IgG for 1 h at room temperature. The membrane was washed several times and incubated with 1:500 diluted horseradish peroxidase-coupled streptavidin for 1 h at room temperature. After several washing steps, the color reaction was developed with DAB. Densitometry analysis of the protein bands was then performed with Scion Image software.

2.13 Statistical analysis

The data are expressed as the mean±standard deviation. The significance of differences in assay values was evaluated with ANOVA followed by Tukey's multiple tests. A value of $p<0.05$ was assumed to indicate statistical significance.

3. Results

3.1 Effect of ACA and NaB on HepG2 cell numbers

HepG2 cells were treated with ACA (10, 12.5, or 15 μM) and/or NaB (1 or 2 mM) for 24 h, and cell number was measured by a neutral red assay. Cell numbers were decreased after ACA or NaB treatment (Figure 1A). In addition, HepG2 cell numbers were synergistically decreased when treated with both ACA and NaB.

Analysis of the enhanced efficacy obtained by combining 10, 12.5, or 15 μM ACA and 2 mM NaB indicates synergism, as depicted in the isobologram: data points are positioned below the line

of additive effects (Figure 1B). A combination of 12.5 μM ACA and 2 mM NaB showed the strongest synergy. However, data points for 10, 12.5, or 15 μM ACA and 1 mM NaB are positioned above the line. Therefore, we performed all of the following experiments with 12.5 μM ACA and 2 mM NaB.

3.2 Effect of ACA and NaB on apoptosis of HepG2 cells

To investigate whether ACA and NaB induced apoptotic cell death, PI staining was performed. When HepG2 cells were treated with ACA and/or NaB for 24 h, the number of apoptotic cells increased. In addition, the rate of apoptosis increased significantly in cells treated with both ACA and NaB (Figure 2A). To further confirm whether ACA and NaB induced apoptotic cell death, we examined Histone DNA ELIZA assay using the Cell Apoptosis ELIZA Detection kit. As shown in Figure 2B, DNA fragmentation was induced significantly in the cells treated with both ACA and NaB.

3.3 Effect of ACA and NaB on intracellular ROS levels in HepG2 cells

Excessive intracellular ROS levels are known to cause apoptotic cell death. Therefore, HepG2 cells were treated with ACA and/or NaB for 30 min or 2 h and the intracellular ROS level was measured using a DCFH-DA assay. In ACA- or NaB-treated cells, intracellular ROS increased slightly. However, intracellular ROS levels increased significantly in cells treated with both ACA and NaB (Figure 3A and 3B).

3.4 Effect of catalase on cell number in ACA- and NaB-treated HepG2 cells

To clarify the relationship between apoptotic cell death and intracellular ROS levels, cells were exposed to catalase for 5 min prior to the addition of ACA and NaB. After 24 h, cell numbers were measured by a neutral red assay. The decrease in cell number after the combined treatment of ACA

and NaB was reduced following catalase pretreatment (Figure 3C). These results suggest that the increase in intercellular ROS levels may be involved in the decrease in cell number.

3.5 Effect of ACA and NaB on NADPH oxidase activity and oxide scavenging enzymes activities in HepG2 Cells

NADPH oxidase is known to generate ROS. HepG2 cells were treated with ACA and/or NaB for 2 h, and NADPH oxidase activity was measured. In ACA- or NaB-treated cells, NADPH oxidase activity increased. In cells treated with both ACA and NaB, NADPH oxidase activity in the cells increased significantly (Figure 4A).

Intracellular ROS levels are regulated by their formation and removal by scavenging. Catalase activity is decreased in apoptotic cell death [41, 42]. Therefore, we examined the effect of ACA and NaB combined treatment on catalase and glutathione peroxidase (GPx) activity. Catalase and GPx activities were not affected to combined treatment of ACA and NaB (Figure 4B and 4C).

3.6 Effect of ACA and NaB on AMPK phosphorylation in HepG2 cells

We focused on AMPK phosphorylation to elucidate whether ACA and NaB are involved in AMPK activation. HepG2 cells were treated with ACA and/or NaB for 2 h, and AMPK phosphorylation in these cells was evaluated by Western blot analysis. As shown in Figure 5A, ACA and NaB significantly induced the phosphorylation of AMPK.

The p53 tumor suppressor plays a major role in the cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead to either DNA repair or apoptosis. As shown in Figure 6A, p53 increased slightly in ACA- or NaB-treated cells. However, The p53 level increased significantly in cells treated with both ACA and NaB.

ERK regulate numerous cellular processes and lead to different consequences. Although activation of ERK is believed to be related to cell survival, there is growing evidence demonstrating

that activation of ERK (pERK) is strongly associated with apoptosis in a variety of cancers [43, 44], including a human hepatocellular carcinoma [45]. As shown in Figure 6B, ACA and NaB significantly induced the activation of ERK.

3.7 Effect of catalase on AMPK phosphorylation in ACA and/or sodium butyrate-treated HepG2 cells

To understand the relationship between AMPK phosphorylation and increased intracellular ROS levels, HepG2 cells were exposed to catalase for 5 min before the addition of ACA and NaB. After 2 h, phosphorylated AMPK levels were evaluated by Western blot. AMPK phosphorylation in ACA- and NaB-treated cells improved after pretreatment with catalase (Figure 5B). This finding suggests that the increase of intracellular ROS may be involved in the increase of AMPK phosphorylation.

3.8 Effect of ACA and NaB on the number of HT-29 cells or hepatocytes

To determine whether apoptotic cell death induced by the combined treatment of ACA and NaB is specific to HepG2 cells, we examined the effect of ACA and NaB on HT-29 cells, human colon adenocarcinoma cells and normal hepatocytes. As shown in Figure 7A, HT-29 cell numbers were synergistically decreased when treated with both ACA and NaB. Hepatocytes were treated with 12.5 μM ACA and/or 2 mM NaB for 24 h, at which point the number of cells was measured by neutral red. In ACA- and/or NaB-treated cells, the numbers did not decrease (Figure 7B), suggesting that the effects of ACA and NaB are specific to cancer cells.

3.9 Effect of ACA and NaB on intracellular ROS levels of hepatocytes

In HepG2 cells, an excessive accumulation of intracellular ROS causes apoptotic cell death. Therefore, we investigated whether the increase of intracellular ROS levels induced by the

combined treatment of ACA and NaB is specific to cancer cells. Hepatocytes were treated with ACA and/or NaB for 2 h, and then intracellular ROS levels in the cells were measured using a DCFH-DA assay. In ACA- and/or NaB-treated cells, intracellular ROS did not increase (Figure 7C).

4. Discussion

Natural products are excellent sources for the development of new medications for disease treatment. One of the main approaches in cancer therapy is to utilize a combination of chemotherapeutic agents with the objective of improving efficacy. In this study, we examined the effect of combined ACA and NaB treatment on HepG2 cell viability. ACA naturally occurs in the rhizomes and seeds of *Languas galangal* and *Alpinia galangal*. NaB is one of the short-chain fatty acids produced from dietary fiber by colonic bacterial flora. Our results indicate that the combined treatment of ACA and NaB synergistically induced cell death in HepG2 cells but had no cytotoxicity toward normal hepatocytes.

An increase in intracellular ROS levels causes apoptotic cell death [42, 43]. The present study demonstrates that upregulation of ROS by the combined treatment of ACA and NaB contributes to the induction of cell death in HepG2 cells. Cell numbers after the combined treatment of ACA and NaB recovered following catalase pretreatment.

Oxidative stress has been widely implicated in both apoptotic and non-apoptotic cell death [46-48]. The formation of ROS could be classified into two general categories: ROS derived from mitochondrial oxygen consumption or ROS that are mitochondrial independent. Recent studies, however, have also implicated ROS generated by a specialized plasma membrane oxidase, NADPH oxidase, in the apoptosis of cancer cells. In our experiments, ROS levels increased synergistically after combined treatment with ACA and NaB, but these levels were suppressed with DPI, an inhibitor of NADPH oxidase. Rotenone, a mitochondrial oxidase inhibitor, and allopurinol, a xanthine oxidase inhibitor, had no appreciable effect on ROS production (data not shown).

Furthermore, we found that NADPH oxidase in HepG2 cells increased synergistically after combined treatment with ACA and NaB. These results suggest that NADPH oxidase plays an important role in the production of ROS in HepG2 cells after combined treatment with ACA and NaB.

NADPH oxidase consists of multiple subunits, including p47phox, p67phox, p22, and Rac. p47phox is an important subunit; translocation of this subunit from the cytosol to the membrane is an essential event for activation of NADPH oxidase [49]. Phosphorylation of p47phox is induced by protein kinase C (PKC) and is necessary to activate the p47phox subunit [50]. Recently, it has also been reported that the effect of NaB on cell differentiation is significantly attenuated in the presence of PKC inhibitors [51]. These results suggest that NaB is involved in the activation of NADPH oxidase via phosphorylation of the p47phox subunit.

AMPK is a cellular energy sensor that inhibits ATP consumption and stimulates ATP production under energy-depleted conditions. AMPK also plays an essential role in controlling processes related to tumor development, including cell cycle progression, cell proliferation, and cell death. Activated AMPK plays two major functions, metabolic and non-metabolic [31]. Recently, we found that ACA induced a dose-dependent activation of AMPK in 3T3-L1 cells and IEC-6 cells [32, 33].

AMPK is a critical enzyme expressed in all eukaryotic cells and plays an essential role in cellular energy homeostasis [30]. Recently, Park *et al*. showed that magnolol, a hydroxylated biphenyl compound present in *Magnolia officinali*s, is a potent cell growth inhibitor and induces apoptosis via AMPK activation in HCT-116 human colon cancer cells. Inhibition of growth and expression of apoptotic proteins (p53, Bax, and Bcl-2) was associated with AMPK activation [52]. Recently, it has been reported that an increase in intracellular ROS levels induces activation of AMPK [53]. In our results, combined ACA and NaB treatment induced AMPK phosphorylation and increased intracellular ROS levels in HepG2 cells. After catalase treatment, AMPK activation was suppressed, suggesting that the increase in intracellular ROS levels may induce AMPK

phosphorylation.

NaB is an HDAC inhibitor, and HDAC inhibitors have shown synergistic and additive anti-tumor effects with a wide range of chemotherapeutic drugs [54]. Stiborova et al. have reviewed suitable therapeutic candidates for such combined treatments, including (i) other epigenetic modifiers such as inhibitors of DNA methyl transferase or histone demethylases, (ii) compounds mediating the formation of ROS, (iii) drugs inhibiting tumor growth by microtubule stabilization, (iv) proteasome inhibitors, (v) agents that cause DNA damage, and (vi) radiotherapy [53].

Our results also showed that ACA and NaB treatment did not affect normal hepatocyte numbers. Furthermore, we previously reported that ACA and NaB synergistically increased phase II enzyme activities in intestinal epithelial cells (IEC 6) without cell death [33]. Together, these results suggest that the effect of ACA and NaB on cell numbers is specific to cancer cells.

In conclusion, our current findings are consistent with the hypothesis that combination treatments can sensitize cancer cells more effectively than individual treatments. We reported that combination treatment of ACA and NaB synergistically induced apoptotic cell death via an increase in intercellular ROS and an increase in pAMPK levels. However, the detailed molecular pathway involved in this mechanism needs to be further investigated.

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Figure legends

Figure 1. Effect of ACA and NaB on HepG2 cell number.

(A) Effect of ACA and NaB on HepG2 cell number. HepG2 cells were incubated with various concentrations of ACA and/or NaB for 24 h. Cell viability was measured by the Neutral Red assay described in the Materials and Methods section. The results represent the mean ±SD from four experiments (* $p < 0.05$ compared to control cells). (B) The fraction-effect versus combination index (FA-CI) curve. Isobologram analysis and combination index analysis of cell death induction in HepG2 cells treated with a combination of ACA and NaB. A combination index of 1.0 reflects additive effects, whereas values greater than or less than 1.0 indicate antagonism and synergy, respectively. ●: 10 μM ACA plus 1 mM NaB, ▲: 12.5 μM ACA plus 1 mM NaB, ■: 15 μM ACA plus 1 mM NaB, ○: 10 μM ACA plus 2 mM NaB, △: 12.5 μM ACA plus 2 mM NaB, □: 15 μM ACA plus 2 mM NaB.

Figure 2. Effect of ACA and NaB on HepG2 cell apoptosis.

HepG2 cells were incubated with 12.5 μM ACA and/or 2 mM NaB for 24 h. (A) PI staining was performed as described in the Materials and Methods section. (a) Control; (b) 12.5 μM ACA; (c) 2 mM NaB; (d) 12.5 μM ACA plus 2 mM NaB. Apoptotic cell (arrow) is seen in (b) and (d). (B) Quantification of apoptosis by measurement of histone-complexed DNA. The Cell Apoptosis ELISA Detection Kit was used to detect apoptosis in HepG2 cells. The results represent the mean ±SD from three experiments (* $p < 0.05$ compared to control cells).

Figure 3. Effect of ACA and NaB on intracellular ROS levels in HepG2 cells.

(A) Photograph of Intracellular ROS expression in HepG2 cells. HepG2 cells were treated with 12.5 μM ACA and/or 2 mM NaB for 30 min (a-d) or 2 h (e-h). The intracellular ROS levels

were measured by the DCFH-DA assay, as described in the Materials and Methods section. (a) Control; (b) 12.5 μM ACA; (c) 2 mM NaB; (d) 12.5 μM ACA plus 2 mM NaB; (e) Control; (f) 12.5 μM ACA; (g) 2 mM NaB; (h) 12.5 μM ACA plus 2 mM NaB. (B) Quantification of the intensity of ROS expression was analyzed using a computer with Win ROOF Ver. 6.2. (C) Effect of catalase on cell number in ACA- and NaB-treated HepG2 cells. HepG2 cells were incubated with 12.5 μM ACA and/or 2 mM NaB for 24 h. Catalase (100 units/ml) was added 5 min before treatment with ACA and/or NaB. Cell viability was measured by the Neutral Red assay, as described in the Materials and Methods section. The results represent the mean±SD from four experiments (* $p < 0.05$ compared to control cells).

Figure 4. Effect of ACA and NaB on NADPH oxidase activity and oxide scavenging enzymes activities in HepG2 cells.

HepG2 cells were incubated with 12.5 μM ACA and/or 2 mM NaB for 2 h. (A) NADPH oxidase activity, (B) catalase activity and (C) GPx activity were measured as described in the Materials and Methods section. The results represent the mean ±SD from four experiments (* $p < 0.05$ compared to control cells).

Figure 5. Effect of ACA and NaB on AMPK phosphorylation in HepG2 cells.

(A) Effect of ACA and NaB on AMPK phosphorylation in HepG2 cells. HepG2 cells were incubated with 12.5 μM ACA and/or 2 mM NaB for 2 h. (B) Effect of catalase on AMPK in ACA- and/or sodium butyrate-treated HepG2 cells. HepG2 cells were incubated with 12.5 μM ACA and/or 2 mM NaB for 2 h. Catalase (100 units/ml) was added 5 min before treatment with ACA and/or NaB. Cell lysis and Western blot analysis were performed as described in the Materials and Methods section. The results represent the mean ±SD from four experiments (* $p < 0.05$ compared to control cells).

Figure 6. Effect of ACA and NaB on (A) p53 and (B) ERK phosphorylation in HepG2 cells

HepG2 cells were incubated with 12.5 μM ACA and/or 2 mM NaB for 2 h. Cell lysis and Western blot analysis were performed as described in the Materials and Methods section. The results shown represent the results from three separate experiments.

Figure 7. Effect of ACA and NaB on the number of (A) HT-29 cells or (B) hepatocytes

HT-29 cells or hepatocytes were incubated with 12.5 μM ACA and/or 2 mM NaB for 24 h. Cell viability was measured by the trypan blue assay or the Neutral Red assay, as described in the Materials and Methods section. The results represent the mean ±SD from four experiments (* $p < 0.05$ compared to control cells). (C) Effect of ACA and NaB on intracellular ROS levels of hepatocytes. Hepatocytes were treated with or without 12.5 μM ACA and/or NaB for 2 h. The intracellular ROS levels were measured by the DCFH-DA assay, as described in the Materials and Methods section. (a) Control; (b) 12.5 μM ACA; (c) 2 mM NaB; (d) 12.5 μM ACA plus 2 mM NaB.

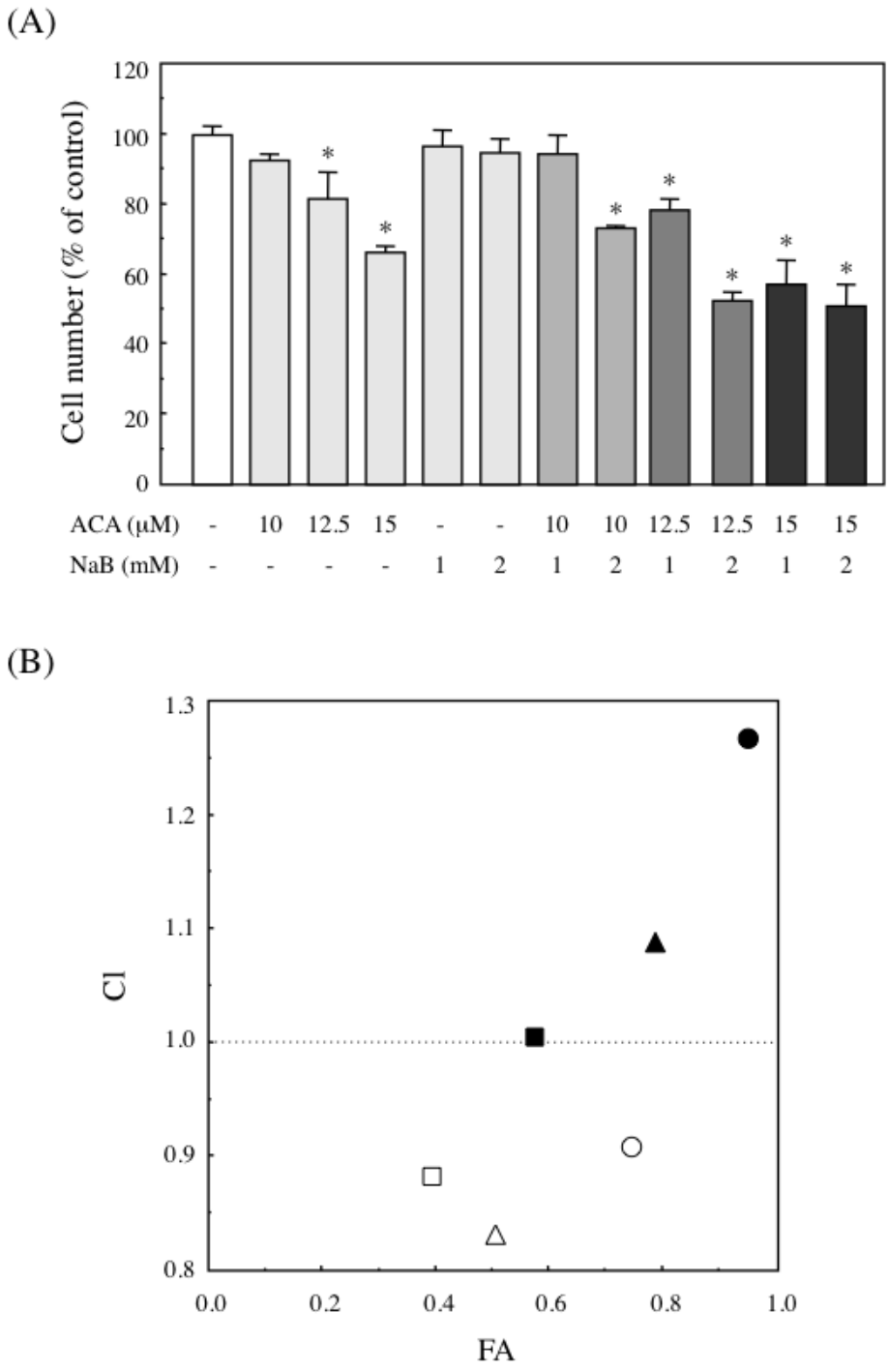
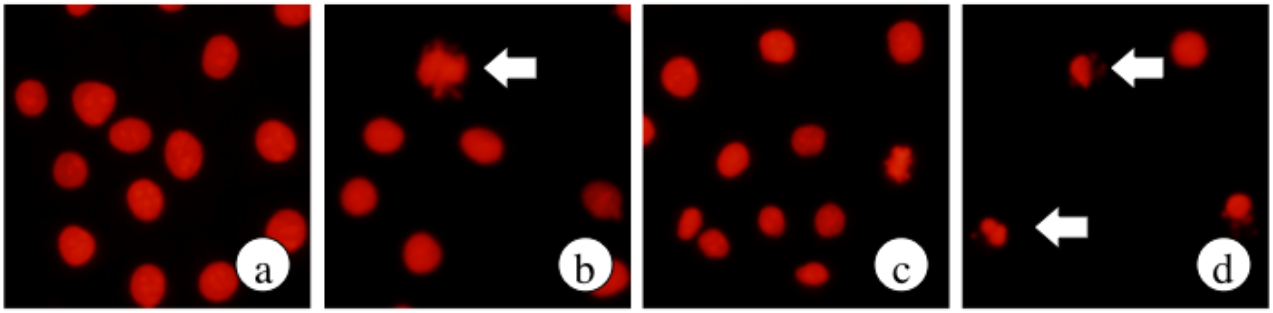

(A)
Cell number (% of control)
120
100
80
60
40
20
0
ACA (μM) - 10 12.5 15 - - 10 10 12.5 12.5 15 15
NaB (mM) - - - - 1 2 1 2 1 2 1 2
(B)
Cl
1.3
1.2
1.1
1.0
0.9
0.8
0.0
0.2
0.4
0.6
0.8
1.0
FA

Figure 1

(A)


a
b
c
d

(B)

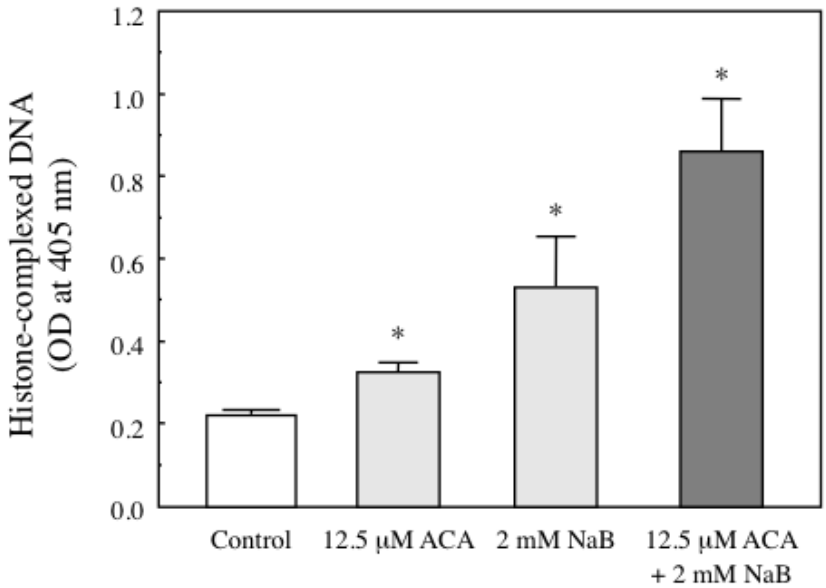
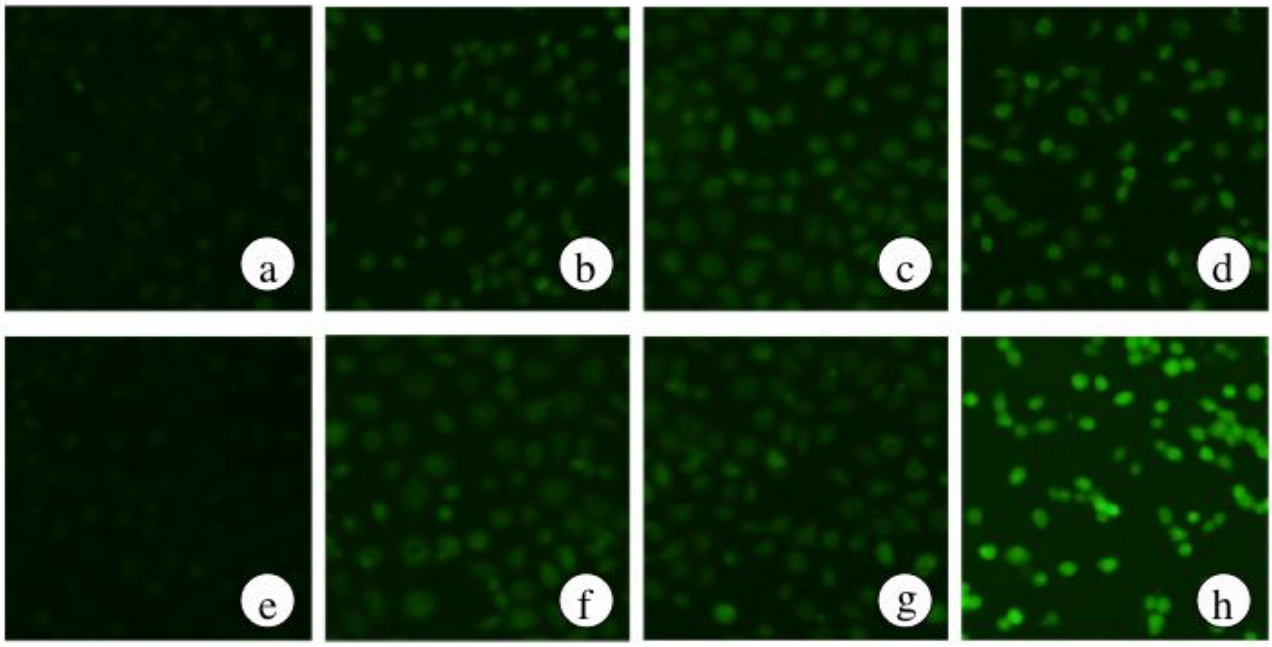

Histone-complexed DNA
(OD at 405 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB

Figure 2

(A)


a
b
c
d
e
f
g
h

(B)

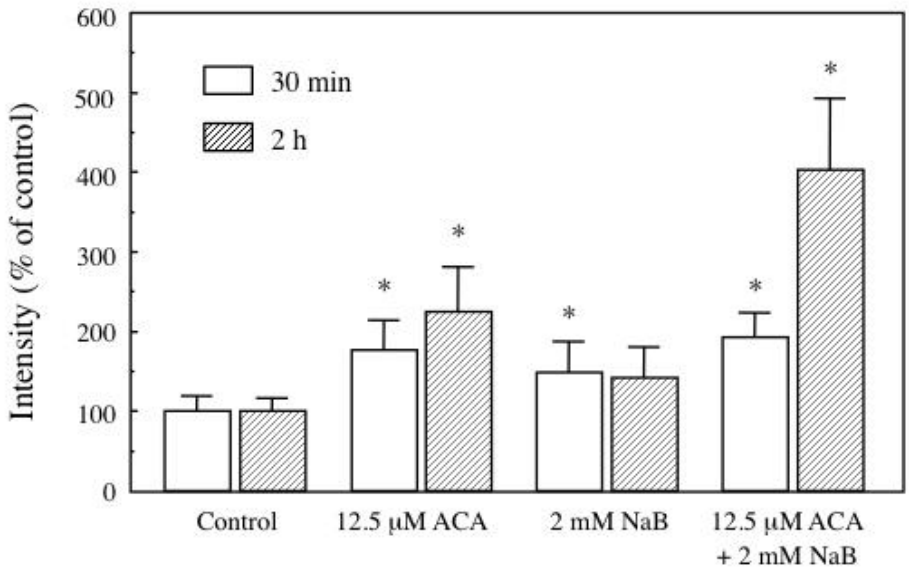

Intensity (% of control)
600
500
400
300
200
100
0
30 min
2 h
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB

Figure 3

(C)

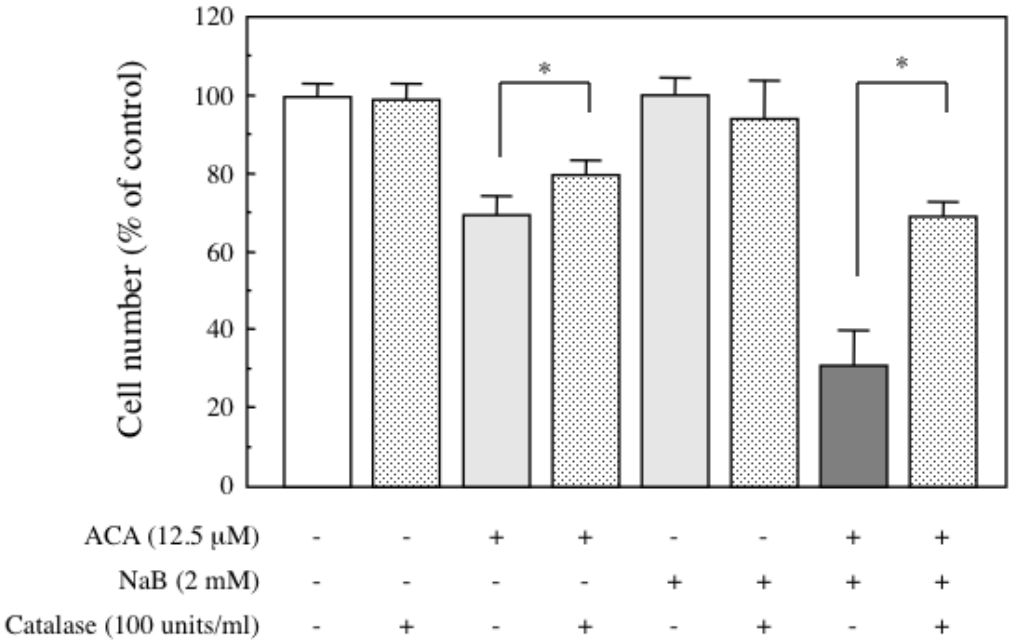

Cell number (% of control)
120
100
80
60
40
20
0
*
*
ACA (12.5 μM) - - + + - - + +
NaB (2 mM) - - - - + + + +
Catalase (100 units/ml) - + - + - + - +

Figure 3

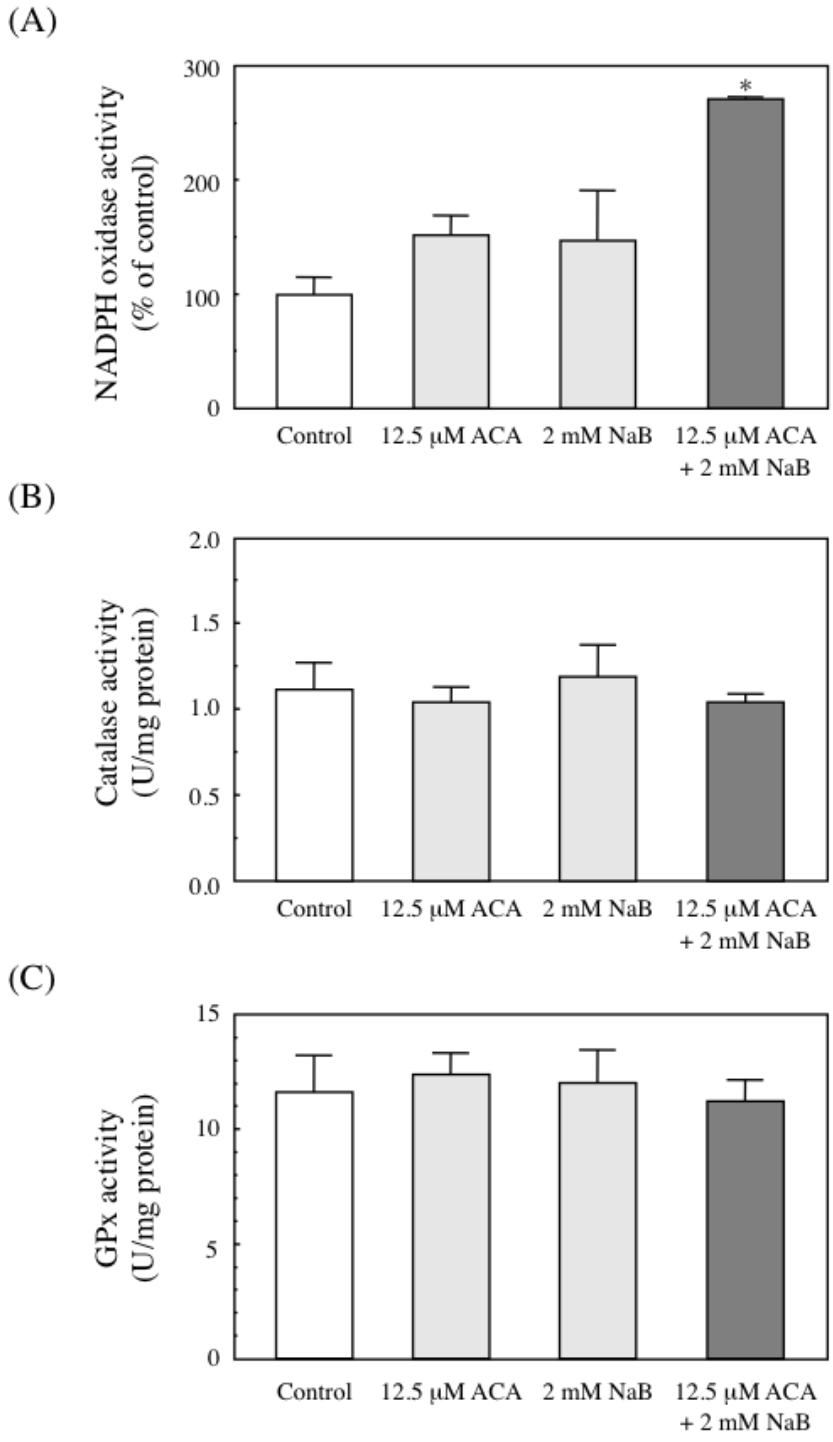

(A)
NADPH oxidase activity
(% of control)
300
200
100
0
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB
(B)
Catalase activity
(U/mg protein)
2.0
1.5
1.0
0.5
0.0
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB
(C)
GPx activity
(U/mg protein)
15
10
5
0
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB

Figure 4

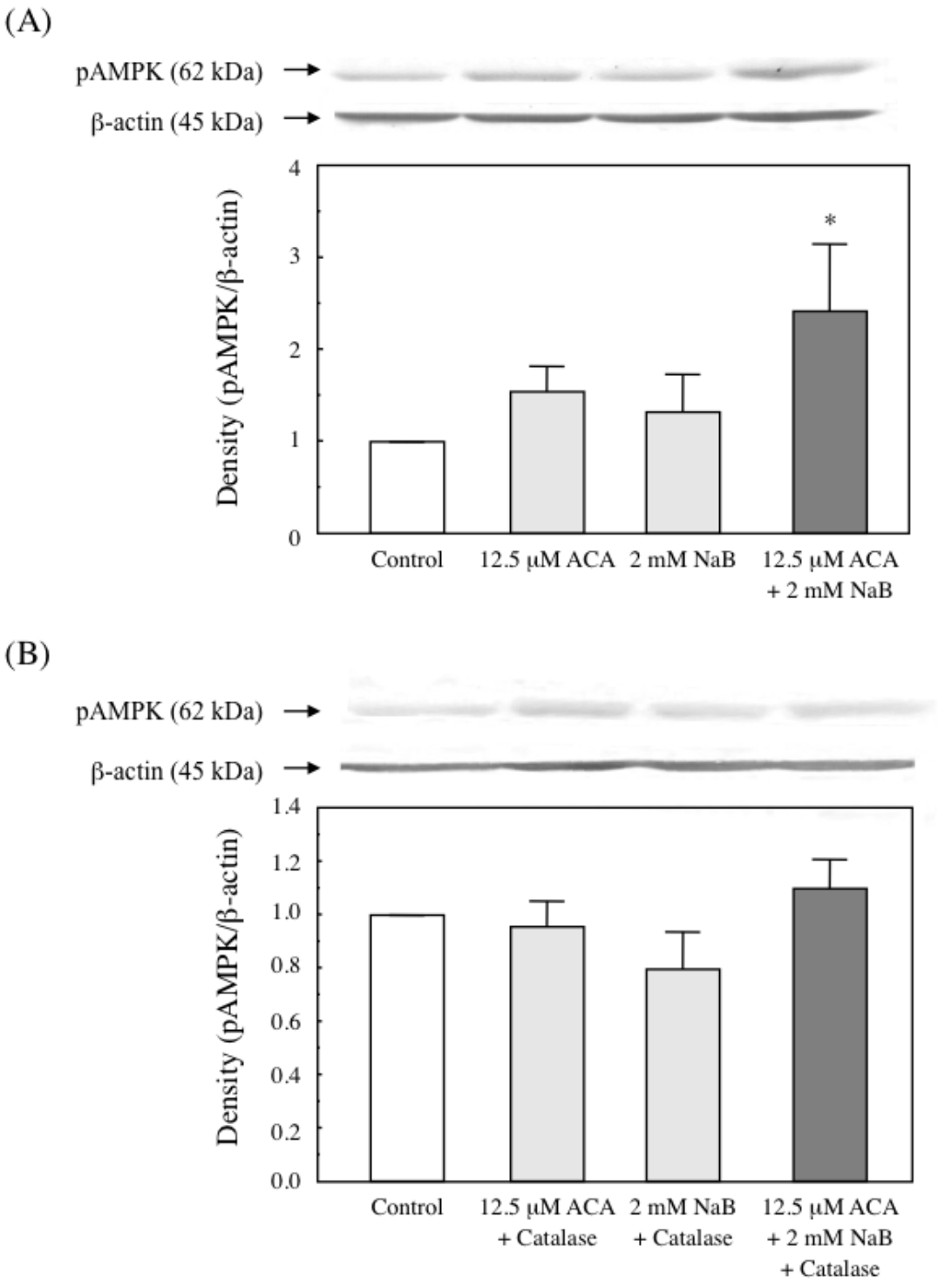

(A)
pAMPK (62 kDa)
β-actin (45 kDa)
Density (pAMPK/β-actin)
0
1
2
3
4
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA + 2 mM NaB
(B)
pAMPK (62 kDa)
β-actin (45 kDa)
Density (pAMPK/β-actin)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Control
12.5 μM ACA + Catalase
2 mM NaB + Catalase
12.5 μM ACA + 2 mM NaB + Catalase

Figure 5

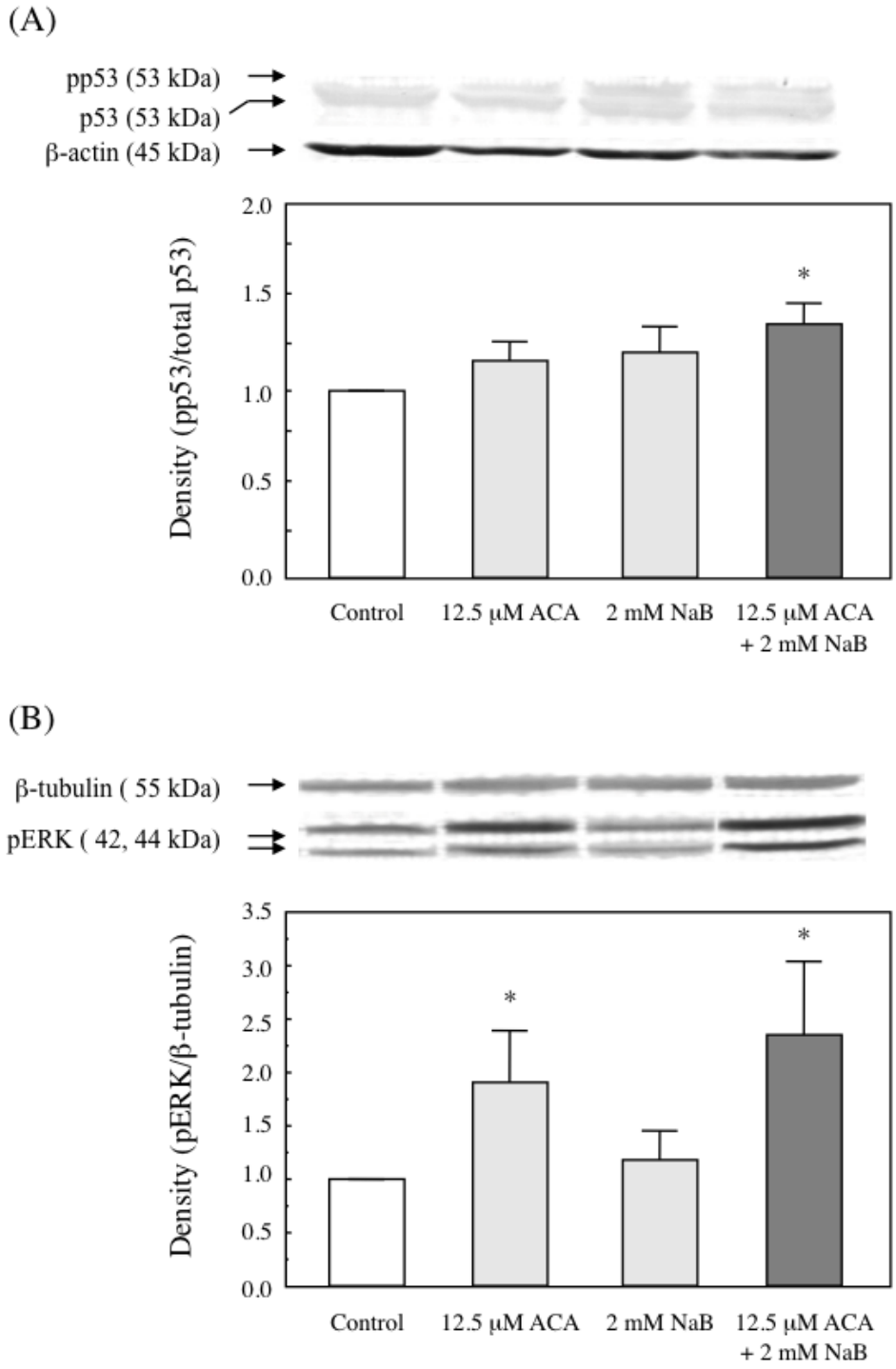
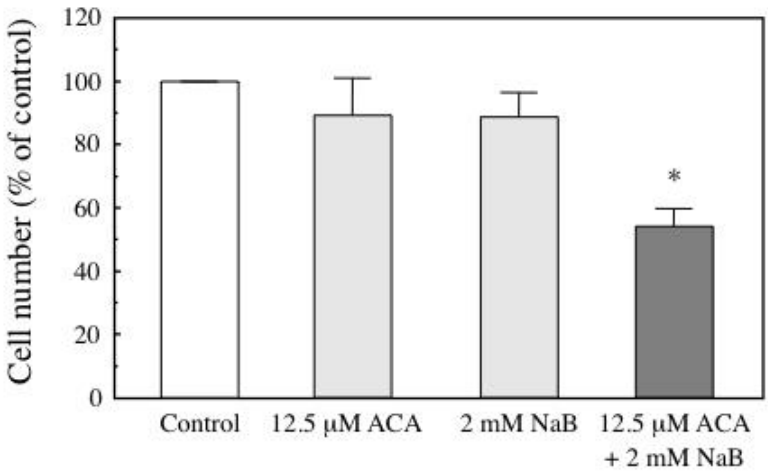
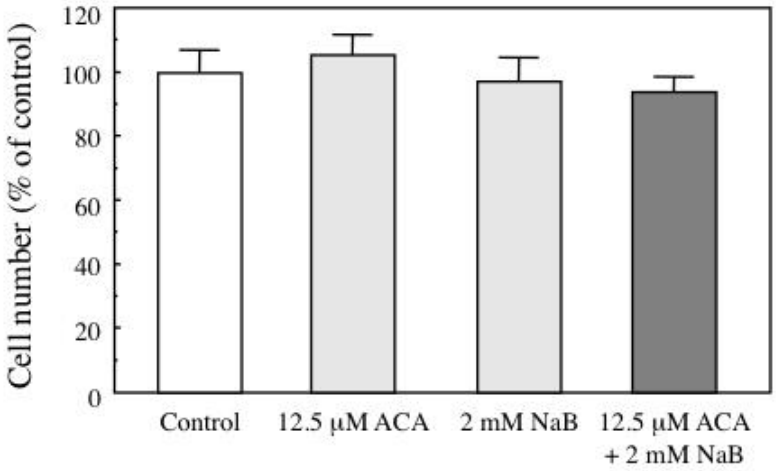

(A)
pp53 (53 kDa)
p53 (53 kDa)
β-actin (45 kDa)
Density (pp53/total p53)
2.0
1.5
1.0
0.5
0.0
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA + 2 mM NaB
(B)
β-tubulin (55 kDa)
pERK (42, 44 kDa)
Density (pERK/β-tubulin)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA + 2 mM NaB

Figure 6

(A)


Cell number (% of control)
120
100
80
60
40
20
0
*
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB

(B)


Cell number (% of control)
120
100
80
60
40
20
0
Control
12.5 μM ACA
2 mM NaB
12.5 μM ACA
+ 2 mM NaB

(C)

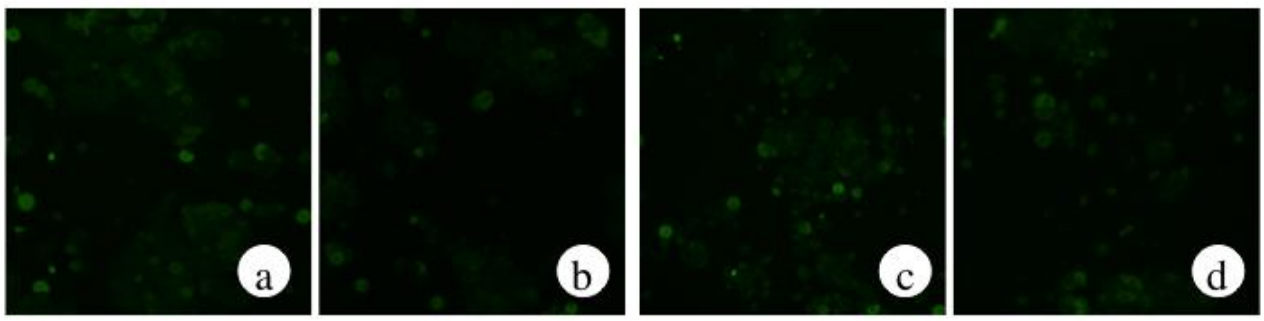

a
b
c
d

Figure 7