CURRENT THERAPEUTIC RESEARCH

VOLUME 71, NUMBER 3, JUNE 2010

Effects of a Histone Deacetylase Inhibitor, Sodium Butyrate, on 53-kDa Protein Expression and Sensitivity to Anticancer Drugs of Pancreatic Cancer Cells

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ABSTRACT

BACKGROUND: Several tumor-suppressor genes, such as 53-kDa protein (p53), are inactivated in some pancreatic cancers. The lack of a functional p53 has been proposed to be a component of resistance to chemotherapy, resulting in the inhibition of apoptosis. Therefore, reintroduction of wild-type p53 is a commonly used gene therapy strategy for the treatment of various types of cancer, including pancreatic cancer.

OBJECTIVE: The aim of this study was to examine the ability of the histone deacetylase inhibitor, sodium butyrate (NaB), to modulate the expression of p53.

METHODS: Five human pancreatic carcinoma cell lines (SW-1990, BxPC-3, PANC-1, MIA PaCa-2, JHP-1) were utilized. Two of the cell lines (SW-1990 and JHP-1) lacked p53 expression, as determined by Western blot analysis, and were investigated further. Expression of p53 was determined by densitometry of all bands present in the Western blot. Drug sensitivity was measured with a tetrazolium-based assay by exposing the cells to graded concentrations of NaB and/or anticancer drugs (cisplatin, fluorouracil, SN-38, and paclitaxel). Apoptosis was observed using gel electrophoresis.

RESULTS: In the SW-1990 and JHP-1 cell lines, use of 1 mM NaB was found to induce histone acetylation and p53 expression compared with those not treated with NaB ($P = 0.01$ and $P = 0.018$, respectively). Sensitivity to cisplatin ($P = 0.021$), fluorouracil ($P = 0.046$), and SN-38 ($P = 0.039$) was significantly enhanced by NaB treatment compared with nontreatment. However, sensitivity to paclitaxel was not significantly different between untreated and NaB-treated cells. A higher frequency of apoptosis was observed in NaB-treated cells compared with that of control cells.

CONCLUSION: This in vitro study found that NaB induced p53 expression in 2 pancreatic cancer cell lines (SW-1990 and JHP-1). Moreover, NaB acted on a biochemical modulator for antieuplastic therapy. Future research is necessary to assess the value of these findings. (*Curr Ther Res Clin Exp.* 2010;71:162–172) © 2010 Excerpta Medica Inc.

KEY WORDS: sodium butyrate, 53-kDa protein expression, human pancreatic carcinoma cell lines, in vitro.

Accepted for publication March 16, 2010.
doi:10.1016/j.curtheres.2010.06.002
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0011-393X/$ - see front matter

INTRODUCTION

The prognosis of patients with pancreatic cancer is poor (5-year overall survival rate is 2.5%)[1] since most cases are in an advanced stage at the time of diagnosis.[2] Early detection of pancreatic cancer using modern imaging techniques is required for curative resection.[3] Adjuvant chemotherapy is also important for improving the clinical outcome.[4] Because the effect of anticancer agents is limited, it is important to assess and develop new treatments.

53-kDa protein (p53) is a DNA-binding protein and a transcription factor that controls the expression of proteins involved in the cell cycle.[5,6] In response to DNA damage, p53 accumulates in the nucleus causing cells to undergo cell cycle arrest and DNA repair or apoptosis.[7] Inactivation of p53 can occur by several mechanisms, including direct genetic mutation, binding to viral oncoproteins or cellular factors, or alteration of its subcellular localization.[5,6]

The lack of functional p53 has been proposed as a component of resistance to chemotherapy, resulting in the inhibition of apoptosis.[8] Therefore, reintroduction of wild-type p53 is a commonly used gene therapeutic strategy for the treatment of various types of cancer, including pancreatic cancer.[9]

Pancreatic cancer represents 80% to 85% of all pancreatic malignancies and is one of the most lethal cancers (5-year survival rates after resection range from 10%–29%).[10–13] In addition to surgery, effective adjuvant therapy is necessary for treating pancreatic cancer. Recently, wild-type p53 was shown to induce apoptosis in a murine myeloid leukemic cell line[14] and a human colonic tumor cell line.[15] A therapeutic approach that induces overexpression of p53 in malignant pancreatic cells could potentially lead to reconstitution of their ability to undergo apoptosis. A critical determinant of the success of antineoplastic therapy is the ability of malignant cells to undergo apoptosis in response to DNA damage caused by radiation or cytotoxic agents. A deficit of p53, which is mutated in over 50% of pancreatic cancers, is thought to be a factor inhibiting apoptosis.[16]

Histone deacetylase (HDAC) inhibitors are a new class of antineoplastic agents that reactivate tumor suppressor genes, which results in growth inhibition, differentiation, and apoptosis of cancer cells.[17,18] HDAC inhibitors, including suberoylanilide hydroxamic acid, sodium butyrate (NaB), and trichostatin A, have been reported to induce apoptosis. NaB has been found to induce apoptosis in monocytic leukemia cells in mice.[19]

NaB, a 4-carbon fatty acid, is of particular interest because it has been reported to inhibit the proliferation of a number of cell types in vitro,[20] including colorectal tumor cells.[21,22] A previous study found that NaB is cytotoxic to colorectal cells; however, it is unclear whether this was due to the induction of terminal differentiation or to nonspecific toxicity.[23] Furthermore, we know that NaB induces several proteins but we do not know whether NaB affects p53 induction.[24–26] We used NaB, which is an HDAC inhibitor that induces an increase in the levels of membranous antigens and enzymic activities.[27–29] Other HDAC inhibitors are thought to act by promoting histone acetylation and, in turn, gene expression.

The p21 gene has been linked to p53 expression and inhibition of cell cycle progression.[30] The importance of p21 as a downstream mediator of tumor suppression

was enhanced when it was discovered that p21 was the prototype for a family of small cyclin-dependent kinase-inhibiting proteins.

The present study examined the effect of a very low amount (1 mM) of the HDAC inhibitor, NaB, on the expression of p53 protein in pancreatic cell lines. It also investigated whether low doses of NaB lead to increased sensitivity of pancreatic cancer cells to anticancer drugs. We also examined the status of p21 in cancer cells after NaB treatment.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Five human pancreatic cancer cell lines were used, SW-1990,[31] BxPC-3,[32] PANC-1,[33] MIA PaCa-2,[34] and JHP-1.[35] All cell lines were purchased from American Type Culture Collection, Manassas, Virginia. SW-1990 cells were maintained at 37°C in Leibovitz L-15 medium 10% fetal calf serum. PANC-1 cells were maintained at 37°C in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum. BxPC-3, MIA PaCa-2, and JHP-1 cells were maintained at 37°C in RPMI-1640 medium containing 10% fetal calf serum.

Protein Isolation and Western Blot Analysis

Cells were scraped into cell lysis buffer containing 10 mM Tris (pH 7.4), 150 mM sodium chloride (NaCl), 1% NP40, 1 mM EDTA (TNE), and 20 µg/mL aprotinin. Proteins (50 µg) were separated by 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electroblotted to a transfer membrane (Immobilon-P, Millipore, Billerica, Massachusetts). Nonspecific protein binding was blocked with 5% milk in TNE buffer (2 mM Tris [pH 7.4]; 2 mM NaCl; 1 mM EDTA; 0.15% polysorbate 20) for 1 hour. The membrane was incubated for 1 hour with a mouse monoclonal antibody to p53 and p21 (Santa Cruz Biotechnology, Inc., Santa Cruz, California) and a rabbit polyclonal antibody to acetylated histone H3 (Upstate Biotechnology, Lake Placid, New York), diluted to 1:1000 in TNE containing 5% milk. Since chromatin acetylation increases in cells treated with an HDAC inhibitor, it can be detected using an antibody against acetylated chromatin.[36] After washing, antimouse or antirabbit immunoglobulin horseradish-peroxidase–linked secondary antibody (Amersham Pharmacia Biotech Inc., Piscataway, New Jersey) was added for 1 hour. After washing, the membrane was developed in Western blotting detection reagents (ECL™, Amersham Pharmacia). The total value of the protein was determined by densitometry of all bands present in the Western blot.[37] The bands were quantified using a digital scanner and NIH Image software version 1.57 (Scion Corp., Frederick, Maryland). p53 expression was found in BxPC-3, PANC-1, and MIA PaCa-2 cell lines but not in the SW-1990 and JHP-1 cell lines (**Figure 1**). The latter 2 cell lines were examined further.

Growth Inhibition and Cell Viability

Cells (3×10^3) were produced in 180 µL of medium per well in 96-well plates in triplicate. Drug sensitivity was measured by exposing the cells to graded concentrations which were derived from previous studies[38–41] of NaB or anticancer drugs in a

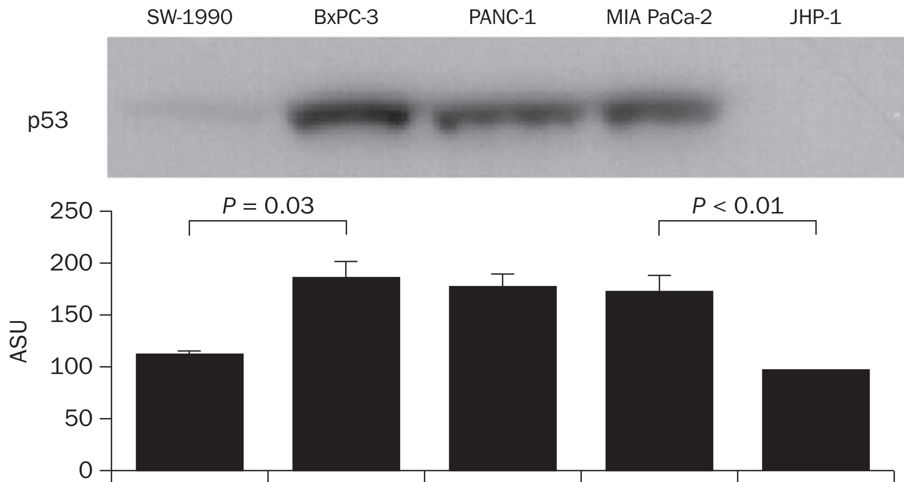


Figure 1. Western blot detection of 53-kDa protein (p53). Cell lysates from SW-1990, BxPC-3, PANC-1, MIA PaCa-2, and JHP-1 cells were examined by Western blotting with an antibody to p53. Data are the mean of 3 determinations. Error bars are 95% CIs. *P* values are 2-sided (Mann-Whitney *U* test). ASU = arbitrary subunit.

final volume of 200 µL. After 72 hours, viable cells were estimated with a colorimetric assay that measures the formazan reduction product of MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), which is produced by the mitochondrial activity of viable cells. The reduced product was dissolved in dimethyl sulfoxide and absorbance was measured with a plate-reader spectrophotometer (Bio Rad Benchmark Plus, Bio-Rad Laboratories, Inc., Hercules, California).

DNA Fragmentation Analysis

Nucleosomal DNA degradation was analyzed. SW-1990 cells (1×10^5) were seeded in 5-cm culture dishes and allowed to adhere overnight. On the next day, fresh medium was added with or without NaB (final concentration 2 mM). After 1 hour of incubation at 37°C in 5% carbon dioxide, the medium was aspirated, and fresh medium containing cisplatin (final concentration 0.01, 0.1, or 1 µM) was added. After 2 days of incubation, cells were harvested and lysed in a solution containing 100 mM NaCl, 10 mM Tris (pH 7.4), 25 mM EDTA, and 0.5% sodium dodecyl sulfate. After centrifugation, the supernatants were incubated at 65°C for 5 hours with 300 µg/mL proteinase K and then extracted with phenol chloroform. The aqueous layer was treated with 0.1 volume of 3 M sodium acetate, and the DNA was precipitated with 2.5 volumes of 95% ethanol. Following treatment with 100 µg/mL RNase A for 1 hour at 37°C, the sample was electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Statistical Analyses

Differences between groups were tested by ANOVA or Mann-Whitney *U* test. All *P* values are 2-sided. A 2-sided $P < 0.05$ was considered to be statistically significant. Statistical analyses were conducted using StatView version 4.0 (SAS Institute Inc., Cary, North Carolina).

RESULTS

The cytotoxicity of NaB to SW-1990 and JHP-1 cell lines was examined at various concentrations of NaB (0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 mM). MTT assays were performed after the 2 cell lines were exposed to NaB for 72 hours. NaB was not cytotoxic or, at most, minimally cytotoxic at 1.0 mM. At NaB concentrations >1.0 mM, cell growth was significantly inhibited (**Figure 2**). Therefore, a NaB concentration of 1.0 mM was used for all subsequent experiments.

When control cells and SW-1990 and JHP-1 cells treated with 1.0 mM NaB were examined by Western blot analysis, histone acetylation increased significantly in the SW-1990 and JHP-1 cells (**Figure 3A**). p53 and p21 expression in the 2 cell lines was detected by Western blotting after they were treated with 1 mM NaB for 1, 2, and 4 hours. p53 and p21 expression was also apparent after NaB treatment (**Figure 3B**).

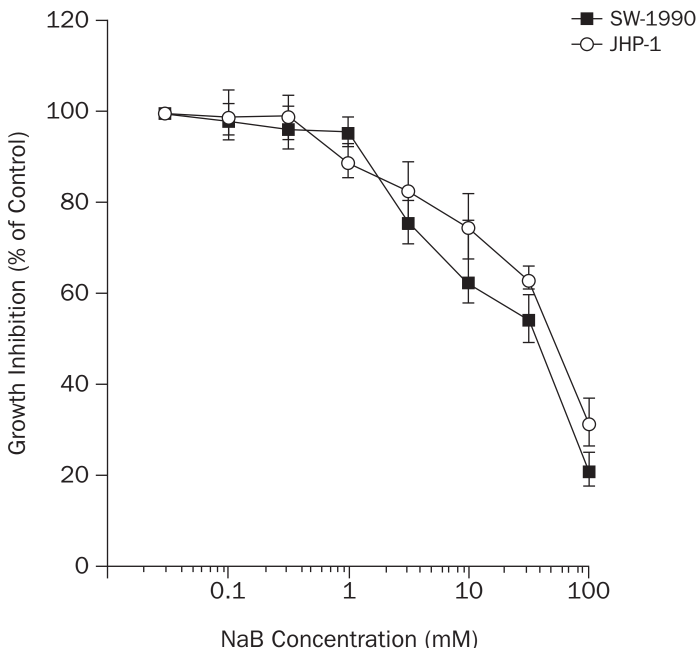


Figure 2. Cytotoxicity of sodium butyrate (NaB) to SW-1990 and JHP-1 cells. Each point represents the mean of triplicate analyses with SD.

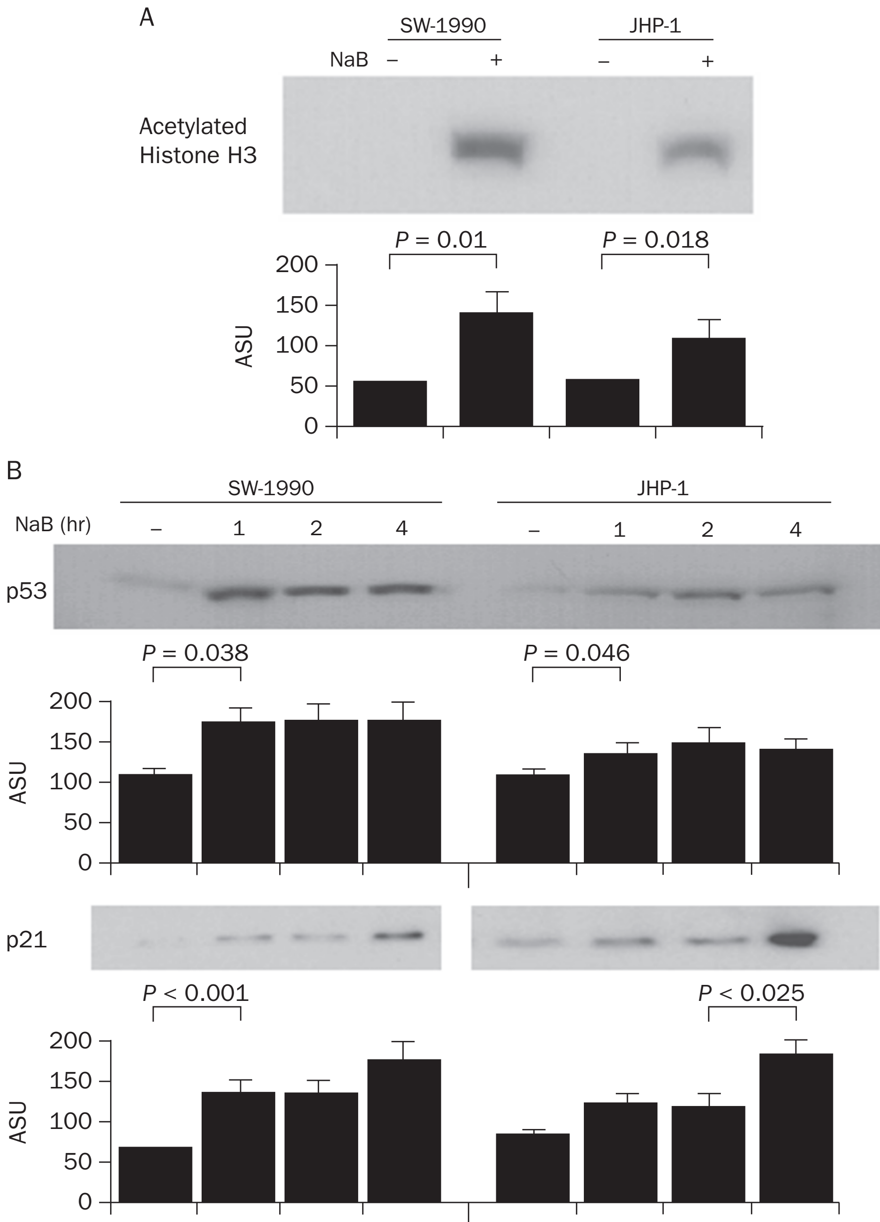


Figure 3. **(A) Two pancreatic cancer cell lines were treated with 1 mM sodium butyrate (NaB) for 24 hours and the expression of acetylated histone H3 was examined by Western blot analysis. (B) Western blot of 53-kDa protein (p53) and p21. SW-1990 and JHP-1 cells were treated with 1 mM NaB for the indicated times prior to harvesting protein. ASU = arbitrary subunit.**

MTT assays were used to investigate whether the induction of p53 increased the cytotoxic effect of an anticancer drug on the pancreatic cell lines. Following treatment with NaB, the 50% inhibitory concentration (IC_{50}) of cisplatin for SW-1990 cells was reduced to 0.012 µM from an IC_{50} of 0.02 µM for untreated cells. Similar results were obtained with fluorouracil and SN-38. However, sensitivity to paclitaxel was not increased significantly by NaB treatment (**Figure** 4).

When SW-1990 cells were exposed to 0.01, 0.1, and 1 µM cisplatin, apoptosis was observed in NaB-treated cells exposed to 0.1 µM cisplatin (**Figure 5**). On the other hand, untreated SW-1990 cells had a low frequency of apoptosis, and were not significantly affected by cisplatin, even at 0.1 µM.

DISCUSSION

In this study, p53 expression was not detected in 2 of 5 pancreatic cancer cell lines, SW-1990 and JHP-1. Functional p53 was induced in these cell lines by NaB treatment. Furthermore, growth inhibition tests with various anticancer agents showed that drug sensitivity was significantly increased in these cell lines by NaB treatment. These results suggest that enhancement of the effectiveness of anticancer drugs by p53 induction may be clinically useful for treating some cancers that have lost wild-type p53. Introduction of p53 was followed by induction of p21, suggesting the ability of p53 to respond to DNA damage and enhanced cisplatin sensitivity manifested as DNA degradation.

Interestingly, although overexpression of p53 in cells treated with NaB affected their sensitivity to cisplatin, fluorouracil, and SN-38, their sensitivity to paclitaxel did not increase.[42–45] In our DNA laddering study,[46] a high frequency of apoptosis was observed in the NaB-treated cells compared with that of control cells. Mandal and Kumar[47] reported that treating a human breast cancer cell line, MCF-7, with NaB induced apoptosis and growth inhibition (<2% without NaB; ~18% with NaB). This phenomenon is closely linked to the down-regulation of bcl-2 protein expression, and overexpression of bcl-2 in MCF-7 cells resulted in inhibition of apoptosis. Our results suggest that the same apoptosis pathway is present in pancreatic cell lines.

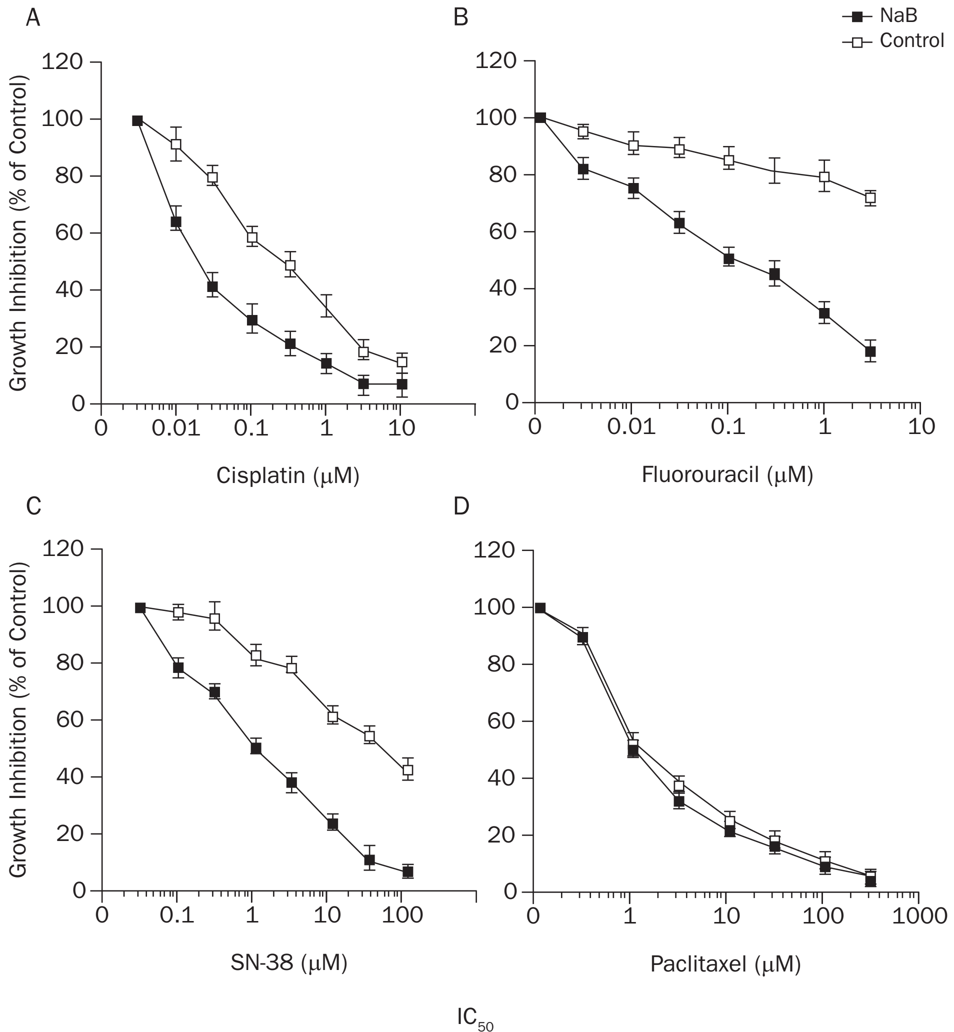
Future in vitro and experimental studies are needed to confirm these in vivo results.

CONCLUSIONS

This study found that NaB induced p53 expression in 2 pancreatic cancer cell lines (SW-1990 and JHP-1). Moreover, NaB acted on a biochemical modulator for antieuplastic therapy. These phenomena may be helpful for finding a new approach for treating pancreatic cancers lacking functional p53 expression.

ACKNOWLEDGMENTS

This study was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology, Tokyo, Japan. The authors have indicated that they have no other conflicts of interest regarding the content of this article.



Drug	IC$_{50}$ +NaB	IC$_{50}$ Control	*P*
Cisplatin	0.036 (0.029–0.044)	0.259 (0.183–0.333)	0.021
Fluorouracil	0.158 (0.146–0.169)	32.612 (31.619–33.600)	0.046
SN-38	1.144 (0.974–1.315)	65.417 (64.339–66.494)	0.039
Paclitaxel	1.257 (1.153–1.361)	1.247 (1.083–1.412)	0.981

Figure 4. Sensitivity of SW-1990 cell lines to anticancer drugs with or without sodium butyrate (NaB) pretreatment. After NaB treatment, cells were treated with various concentrations of cisplatin, fluorouracil, SN-38, and paclitaxel for 72 hours and cell viability was determined by MTT assay. Each point represents the mean of triplicate analyses with SD. IC$_{50}$ = 50% inhibitory concentration.

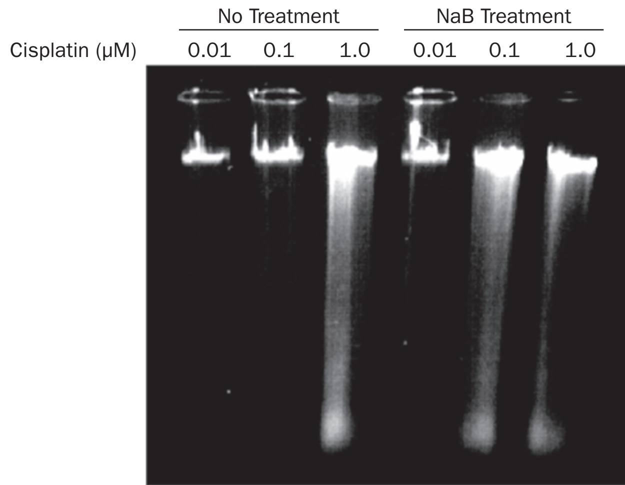


Figure 5. DNA ladder formation in SW-1990 cells treated with cisplatin in the presence or absence of sodium butyrate (NaB). The cells were lysed and extracted DNA was analyzed by conventional electrophoresis.

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