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Isolation of a mitomycin-resistant human lung adenocarcinoma cell subline to investigate the modulation by sodium butyrate of cell growth and drug resistance

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We developed a mitomycin C (MMC)-resistant human lung adenorcarcinoma cell subline, SPC-A1/DM4, from cloned SPC-A1/D13 parent cells by 1 h exposures to escalating concentrations of the drug over 17 months. A 5.9-fold resistance to MMC and a 3.8-fold cross-resistance to cisplatin were present in resistant cells compared with parent cells. This phenotype was stable in the absence of drug exposure for at least 6 months. Sodium butyrate (NaBu), a widely used differentiating agent, was shown to inhibit cell proliferation in a dose-dependent manner, with the cytostatic concentration of 2 mM. This NaBu-induced growth inhibition was reversible. However, SPC-A1/DM4 cells, after recovery from the cytostasis induced by 2 days treatment with 2 mM NaBu, became 2-fold more sensitive to MMC than the cells not exposed to the agent. Meanwhile, the cisplatin response of these treated cells reached a level comparable to the parent cells. This modulation by NaBu of drug resistance could be retained for at least 1 month. Treatment with 2 mM NaBu for 2 days caused inhibition of DNA synthesis and accumulation of cells in the G_1 and G_2/M-phases of the cell cycle. Correlated with these were a marked increase of protein content in these cell subpopulations and an enhanced RNA synthesis. In addition, NaBu-treated cells acquired development of endoplasmic reticulum and accumulated lipid droplets. These morphological alterations were accompanied by a significant decrease in the ratio of nuclear to cytoplasmic areas. These findings suggest that NaBu is potentially useful in the treatment of drug-resistant non-small cell lung cancer. Information about the NaBu-induced phenotypic alterations may offer a clue to the understanding of its long-term effect on drug resistance.

Key words: Cisplatin, drug resistance, growth inhibition, human lung adenocarcinoma, mitomycin C, sodium butyrate.

Introduction

Non-small cell lung cancer (NSCLC) has long been recognized to be refractory to chemotherapy. The main cause of this seems to be the resistance of NSCLC cells to cytotoxic drugs.[1] Various trials to circumvent this drug resistance are in progress, but there is as yet no efficient method to control the proliferation of drug-resistant NSCLC cells.

Over the past decade, several lines of evidence suggested that the combination of a cytotoxic antitumor drug and a relatively non-toxic differentiating agent, each with distinct mechanisms of action and complementary biological effects, may represent a promising strategy to approach the problem of drug resistance.[2] Indeed, some differentiating agents were previously found to enhance the chemosensitivity of tumor cells or to modulate their drug resistance.[3–5] Sodium butyrate (NaBu) is a member of this group of agents. It has been shown to have diverse effects on cell proliferation, phenotype and metabolism in a variety of solid tumor cell lines *in vitro*, such as HeLa cells,[6] melanoma,[7] hepatoma,[8] colon carcinoma,[9] breast carcinoma,[10] neuroblastoma[11] and lung cancer (small cell carcinoma and adenocarcinoma),[12] to potentiate the anti-proliferative effect of some commonly-used antitumor drugs, including 5-fluorouracil, vincristine and cisplatin,[13,14] and to modulate the multidrug resistance by inhibiting P-glycoprotein phosphorylation.[15] These findings thus suggest the possibility that NaBu may be useful in the treatment of drug-resistant NSCLC.

To evaluate this possibility, we first isolated a mitomycin C (MMC)-resistant cell subline from a cloned human lung adenocarcinoma cell subline (SPC-A1 D13) by 1 h exposures *in vitro* to escalating concentrations of the drug. This established cell subline SPC-A1 DM4, which also exhibited a strong cross-resistance to cisplatin, was then used to examine the effect of NaBu on cell growth and drug resistance.

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Materials and methods

Chemicals

The following chemicals were used: RPMI 1640 medium (Gibco, Grand island, NY); new-born calf serum (SiJiQing Biotech, Hangzhow, PRC); L-glutamine, trypsin and RNase A (DongFeng Biochemical, Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, PRC; Sodium butyrate (NaBu, analytic grade), dimethyl sulfoxide (DMSO) and EDTA (Shanghai No. 1 Reagent Factory, Shanghai, PRC); MMC (Kyowa Hakko, Tokyo, Japan); cisplatin (Ebewe Arzneimittel, Australia); vincristine (VCR, Shanghai No. 12 Pharmaceutical Factory, Shanghai, PRC); MTT, propidium iodide (PI) and fluorescein isothiocyanate (FITC) (Sigma, St Louis, MO); [methyl-^{3}H] thymidine (41 mCi/mmol) and [5-^{3}H] uridine (24 mCi/mmol) (Shanghai Institute of Nuclear Energy, Chinese Academy of Sciences). NaBu, MMC and VCR were dissolved in 0.9% NaCl saline and diluted with medium immediately prior to each experiment. The stock solution of cisplatin was kept at 4°C and diluted as described for other drugs.

Cell lines and culture techniques

The human lung adenocarcinoma cell line SPC-A1 was established at this center from a metastatic pulmonary lesion of primary lung adenocarcinoma, as described elsewhere.[16] Cells were maintained as monolayer cultures in RPMI 1640 supplemented with 10% new-born calf serum, L-glutamine and antibiotics (referred to as growth medium), in a humidified incubator at 37°C, 5% CO_2 in air. For cloning, cells were detached with trypsin-EDTA and diluted to a density of 1 cell/ml. Aliquots (200 μl) of cell suspension were distributed into the wells of 96-well plates (Falcon). The monoclonality and cell growth were inspected under an inverted microscope. Twenty-one clones were obtained from 480 wells and one clone (D1,3) was selected for further studies and designated as SPC-A1/D13. Its resistant variant SPC-A1/DM4 was established by an *in vitro* stepwise selection procedure as detailed in Results. This resistant subline was used throughout the studies without any further exposures to the selecting drug.

Cytotoxicity assay

Cell survival following cytotoxic drug treatment was assessed by the MTT colorimetric method[17] with some modifications. Briefly, 2×10^4 cells in 0.9 ml of growth medium were put into the 24-well tissue culture plates (Falcon). After 24 h, the attached cells were exposed to drugs at various concentrations (in 0.1 ml of volume) for 1 h. The treated cells were washed once with growth medium and incubated in 2 ml of fresh growth medium for a further 96 h. At the final 4 h, the growth medium in each well was replaced with 1 ml of serum-free RPMI medium containing 0.35 mg/ml of MTT. Supernant fluids were aspirated and formazen crystals were dissolved in 3 ml of DMSO. The solution was then transfered into colorimetric tubes within 30 min and the absorbance was determined on a model 752c UV-VIS spectrophotometer (Shanghai No. 3 Analytical Instrument Factory, Shanghai, PRC) at a wavelength of 540 nm. The percentage of cell survival was calculated by dividing the absorbance of drug-treated cells by that of untreated control cells. The relative resistance was the ratio of IC_{50} value of resistant cells to that of parent cells. Each drug concentration was tested in triplicate.

Cell growth determined by cell number

Cells (1×10^5) in 9 ml of growth medium were put into a 60 mm diameter Petri dish (Falcon). After 24 h, NaBu (1 ml) was added to give the desired concentrations and the cultures were incubated for 2–4 days. On the indicated day, cells were harvested with trypsin–EDTA and washed once by centrifugation. The viable cells were counted with the trypan blue dye exclusion method. The mean number of viable cells from duplicate dishes was determined at each time-point tested. In some experiments, the NaBu-treated cultures were washed once with growth medium and incubated in fresh growth medium until a confluent monolayer was reached.

Flow cytometry

Differential staining of DNA and total cellular protein has been described elsewhere.[18] Cells were fixed by rapidly adding 4 ml of ice-cold 70% ethanol to a cell pellet, then rapidly pipetting 4 times. Fixed cells were held at 4°C at least overnight and stained as follows. Cells were washed once with phosphate buffered saline (PBS) (pH 8.0), resuspended in the same solution and chicken erythrocytes (CRBC) were added to the cell

suspension as an internal standard. Following treatment with 1000 units/ml of RNase A at 37°C for 30 min, the cells were stained with 0.05 μg/ml of FITC. After allowing 30 min at room temperature for staining protein, an equivalent volume of 40 μg/ml of PI solution was added to stain DNA. The resultant red (DNA) PI fluorescence and green (protein) FITC fluorescence for 1×10^4 cells were recorded by the FACScan flow cytometer (Becton-Dickinson, Mountain View, CA) within 10 min. The two parameter distributions and single parameter histograms for DNA and protein content were anlayzed by Lysys II software.

Uptake of radiolabeled precursors

Cells (1×10^5) were treated with or without 2 mM NaBu for 48 h as described above. At the final 24 h, cells were labeled with 1 μCi/ml of [methyl-^{3}H] thymidine or [5-^{3}H]uridine, respectively. The radioactive medium was aspirated and the labeled cells were rinsed three times with PBS. The cells were harvested with trypsin–EDTA, washed once by centrifugation and resuspended in PBS. Aliquots of 2×10^4 cells were transferred onto disks of glass fiber filter which were then treated with 10% TCA and 70% ethanol. The disks were dried and the retained radioactivity was determined in a Beckman scintillation counter. The rate of precursor incorporation was expressed as c.p.m./2×10^4 cells/24 h.

Morphological observations

For light microscopy, control and NaBu-treated cultures were observed by phase-contrast microscopy and photographed on Shanghai 35 mm panchromatic film with an Olympus model PM-6 camera attached to an Olympus phase-contrast microscope.

For measurement of nuclear and cytoplasmic areas, glass coverslides were put into dishes prior to each experiment. Cells growing on the slides were washed once with PBS, fixed for 30 min at room temperature in Corney's fixative and stained with hematoxylin-eosin. The nuclear and cytoplasmic areas of individual cells were measured by a VIDAS imaging analysis system (Opton, Germany). The ratio of nuclear to cytoplasmic areas (N:C ratio) was then calculated for each cell measured. A total of 200 cells were determined for each sample.

For transmission electron microscopy, cells were harvested with trypsin–EDTA, washed once by centrifugation and fixed in 2% glutaraldehyde at 4°C. The cells were stained with uranyl acetate, postfixed in osmium tetroxide, dehydrated in graded ethanol solutions and embedded in Epon (812). Thin sections were cut and examined under a Jeol 100c-transmission electron microscope.

Statistical analysis

Statistical significance was established by Student's *t*-test when necessary.

Results

Isolation of drug-resistant sublines

In a given malignant cell population, resistance to cytotoxic drugs may be developed by drug selection of pre-existing irresponsive subpopulations and/or induction by the drug of biochemical events resulting in alterations of drug transport, metabolism and drug–target interactions.[19,20] To assure that the drug resistance is induced from initially sensitive cells, the human lung adenocarcinoma cell line SPC-A1 was cloned by use of the limiting dilution method described for lymphocyte hybridomas.[21] Twenty-one clones were obtained, among which there were some differences in their growth rate and chemosensitivity to MMC (data not shown). One clone (D1,3) was then selected for further studies because of its higher growth rate and sensitivity to MMC and referred to as SPC-A1/D13. Its resistant variants were isolated by a stepwise selection procedure without mutagenic pretreatment, as outlined in Figure 1. Briefly, 1×10^5 cells in 9 ml of growth medium were seeded into a 25 cm^2 glass cultural flask. After 24 h, the attached cells were exposed to MMC for 1 h. The treated cells were then washed twice with saline and incubated in fresh drug-free growth medium until a confluent monolayer was reached, at which time the cells were passaged and the MMC treatment cycle was repeated. The drug concentration was doubled after at least six cycles of MMC exposure or when the survivors demonstrated an adaptive growth pattern.

The MMC response of the highest resistant subline, SPC-A1 DM4, and its parent, SPC-A1 D13, was tested by MTT assay (Figure 2). The IC_{50} values indicate that the SPC-A1 DM4 subline

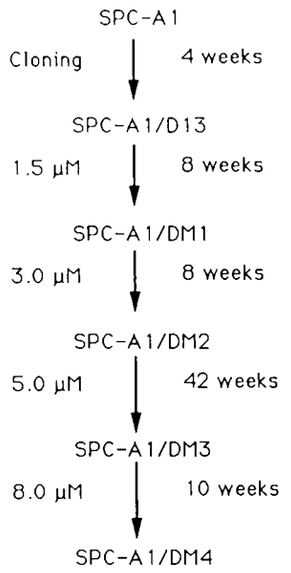

Figure 1. The stepwise selection procedure for isolating SPC-A1/DM sublines. SPC-A1 cells were first cloned by the limiting dilution method. The subline SPC-A1/D13 was treated by 1 h exposures to a fixed MMC concentration (left side of arrow) until the survivors demonstrated an adaptive growth pattern, as described in the text. The time period maintained at a selected MMC concentration is to the right of the arrow.

is approximately six times more resistant to MMC than its parent line (16.0 versus 2.7 μM).

Cross-resistance studies

Cross-resistance studies were performed using cisplatin (DDP) and VCR. As shown in Figure 2, MMC-resistant SPC-A1/DM4 cells have a strong cross-resistance to cisplatin, with a relative resistance of 3.8 (44.0 versus 11.6 μM). In contrast, no significant difference in the sensitivity to VCR was detectable at the IC_{50} level in both sublines. However, SPC-A1/DM4 cells exhibited a 2-fold increase in cell survival at higher concentrations (10 μM or more) when compared with SPC-A1/D13 cells, suggesting that the MMC-resistant cells still have a low level of cross-resistance to this drug.

Stability of the resistant phenotype

As shown in Figure 3, when SPC-A1/DM4 cells were maintained in continuous cultures for about 30 passages in MMC-free growth medium, their resistance to this drug remained relatively stable

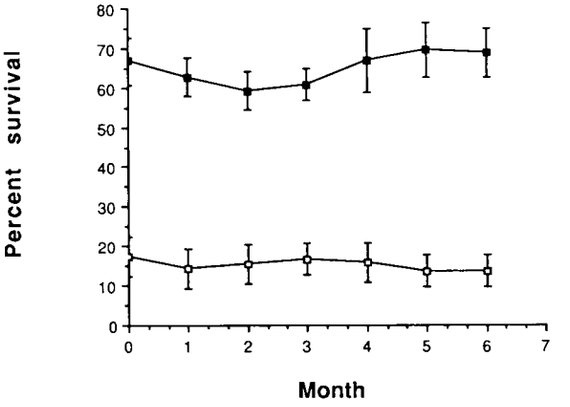

Figure 3. Stabiity of resistant phenotype. SPC-A1/DM4 (■) and SPC-A1/D13 (□) cells were maintained in the absence of drug exposure for 6 months. The cellular sensitivity to 10 μM MMC was determined by MTT assay at 1 month intervals. Each point represents the mean ± SD of three sample wells.

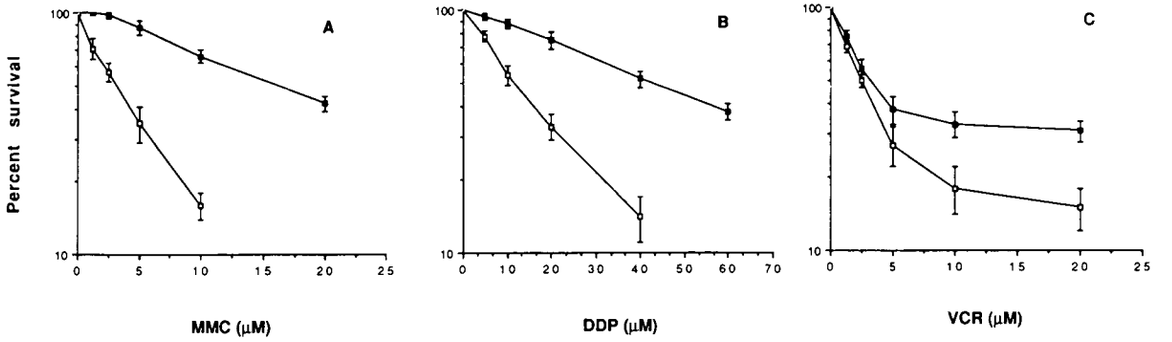

Figure 2. Survival curves for SPC-A1/DM4 (■) and SPC-A1/D13 (□) cells exposed to cytotoxic drugs. Cells were seeded into 24-well plates at 2 × 10^4 cells/well. At 24 h after seeding the attached cells were exposed to (A) MMC, (B) cisplatin and (C) VCR for 1 h. Cell survival was determined by MTT assay after 4 days of incubation. All values are the mean of three independent experiments, each performed in triplicate. Bars, SD.

during the entire 6 month testing period. Furthermore, their response to DDP was also assessed at 2 month intervals. However, no significant decline in the magnitude of cross-resistance was found (data not shown).

Effect of NaBu on cell growth

Exposure of SPC-A1/DM4 cells to various concentrations of NaBu for up to 4 days resulted in a dose-dependent decrease in cell growth (Figure 4a). Investigating the increase in cell number as a function of duration of exposure, it was found that continuous exposure to 2 mM NaBu prevented almost any increase in cell number, such that by 24 h there was a plateau in cell number of cultures exposed to that concentration of the agent (Figure 4b). The trypan blue exclusion assay revealed that the vast majority of treated cells were still viable at all time-points tested at 2 mM or less of NaBu, while a large proportion of dead cells occurred at 4 mM, suggesting a cytostatic nature of NaBu at low concentrations. However, this cytostasis was dependent on the continuous presence of the agent. As shown in Figure 4(b), if NaBu was removed from the cultures after 2 or 4 days, the treated cells appeared to return to log-phase growth immediately or after a lag of a few days, respectively.

Effect of NaBu on drug resistance

To explore the possible effect of NaBu on drug resistance, we examined the cytotoxicity of MMC and cisplatin on the NaBu-treated SPC-A1/DM4 cells. For these studies, the cells were first incubated with 2 mM NaBu for 2 days, then washed twice with saline and incubated in NaBu-free growth medium for a further 6 days. These survivors were used to test their response to MMC and cisplatin. The data presented in Figure 5 demonstrates that these cells, after recovery from the growth inhibition induced by NaBu, exhibited an approximately 2-fold increase in their sensitivity to MMC, while their response to cisplatin reached a level comparable to that of the parent cells. A similar, but lesser effect of NaBu on chemosensitivity was also found using SCP-A1/D13 cells as targets (data not shown).

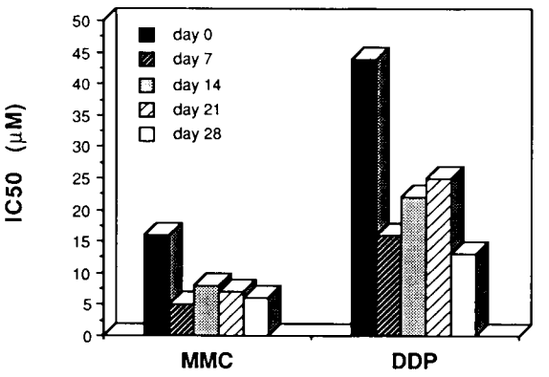

Figure 5. Effect of NaBu on drug resistance. SPC-A1/DM4 cells treated with 2 mM NaBu for 2 days were washed with saline. The treated cells were incubated and passaged in NaBu-free growth medium. Drug sensitivity was evaluated by MTT assay at 1 week intervals. The drug response of cells before NaBu treatment is represented as day 0.

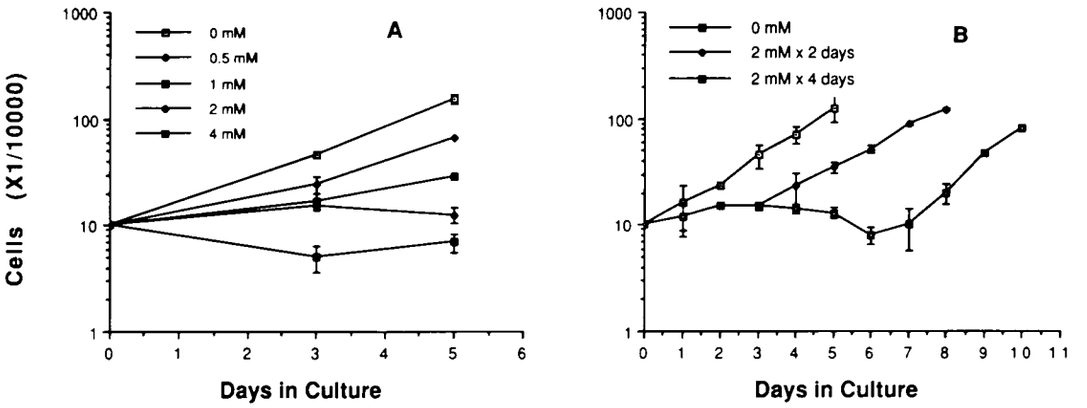

Figure 4. Effect of NaBu on cell proliferation. SPC-A1/DM4 cells were seeded on day 0. After 24 h, NaBu was added into the cultures. (A) Viable cells were counted on days 3 and 5 using the Trypan blue dye exclusion assay; (B) NaBu was replaced with fresh growth medium after 2 and 4 days treatment. Viable cells were determined every day. Each point represents the mean ± SD of duplicate cultures.

Strikingly, when these NaBu-treated SPC-A1/DM4 cells were further subcultured in the BaNu-free growth medium, they remained sensitive to the cytotoxicity of both drugs. As shown in Figure 5, this effect could be retained for at least 1 month.

Flow cytometric analysis

Flow cytometric studies were performed to evaluate the effect of NaBu on cell cycle progression and protein content. For this purpose, SPC-A1/DM4 cells treated with or without 2 mM NaBu for 2 days

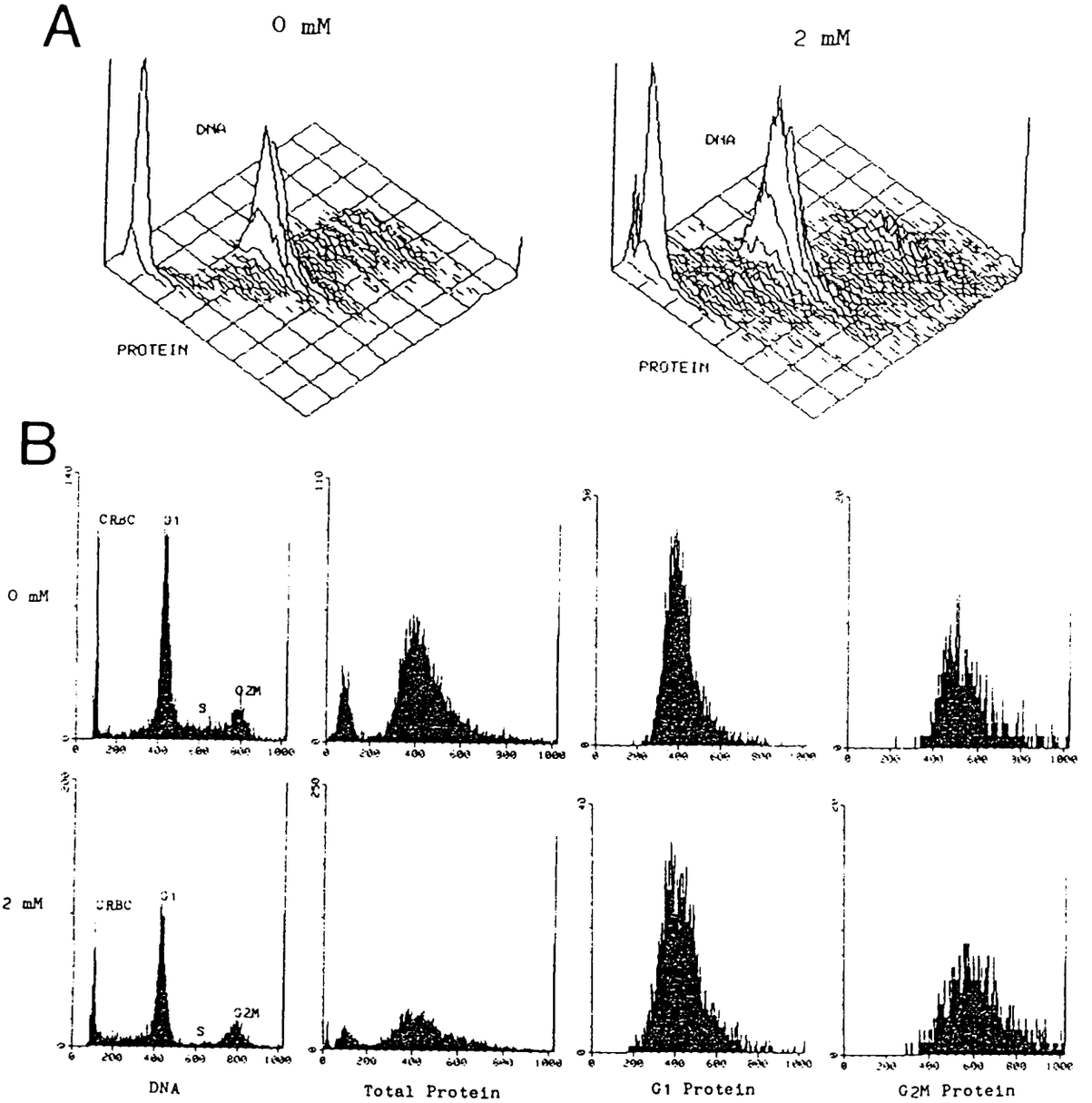

Figure 6. Effect of NaBu on the DNA and protein content. SPC-A1/DM4 cells were treated with 0 or 2 mM NaBu for 48 h and assayed by flow cytometry as described in Materials and methods. (A) Green (protein) FITC fluorescence was plotted versus red (DNA) PI fluorescence with cell frequency on the vertical axis; (B) the DNA and protein contents (channel number) were determined for the whole cell population and the subpopulations in the G_1 and G_2/M phases of the cell cycle, respectively. The percentage of cells in G1, S, and G_2/M phases of the cell cycle were 44.3, 36.6, and 19.2 in the control and 52.7, 4.4 and 42.8 in the treated group, respectively.

were stained simultaneously with FITC (to measure protein content) and PI (to measure DNA content), and the dual fluorescence was determined by flow cytometry. The two parameter distributions and single parameter histograms for DNA and protein are presented in Figure 6. Analysis of cell cycle, based on DNA content, indicated that a large proportion of cells has been arrested in the G_2/M and, to a lesser extent, G_1 phases of the cell cycle after 2 days exposure to 2 mM NaBu when compared with exponentially growing cells. This G_1–G_2/M arrest was accompanied by a significant decrease of cells in the S phase. However, there was a comparable level of DNA index between NaBu-treated and control cells. No significant difference existed in the DNA channel numbers of G_1 and G_2/M subpopulations between these two groups, suggesting that this treatment does not change the DNA content (Table 1). In contrast, the protein content in G_1 and G_2/M cells increased from the mean channel numbers of 408.8 and 537.7 in the control to 426.9 and 605.1 in the treated group, respectively. These differences in G_1 and G_2/M protein content between these two groups were statistically significant ($p < 0.01$) with reference to the cell number counted. Meanwhile, mean α_P, the ratio of mean protein to mean DNA content, which provides a quantitative measure of protein that can be normalized and therefore compared between different cell populations, also indicated an increased protein content in the NaBu-treated cells relative to the control (Table 1).

Table 1. Effect of NaBu on DNA and protein content in G_1 and G_2/M phase cells

NaBu (mM)	DI[a]	G_1 phase (channels)			G_2M phase (channels)		
		protein	DNA	α_P[b]	protein	DNA	α_P
0	4.2	408.8 (74.0)[c]	436.1 (29.9)	0.9	537.7 (97.8)	789.7 (35.8)	0.7
2	4.1	426.9[d] (86.4)	432.0 (28.3)	1.0	605.1[*] (102.1)	793.6 (35.9)	0.8

DNA and protein content in channels were calculated by the Lysys II software from the data presented in Figure 6.
[a] DNA index (use CRBC as standard).
[b] Ratio of mean protein to mean DNA channels.
[c] Standard deviation.
[d] $p < 0.01$.

Radiolabeled precursor incorporation

The radiolabeled precursor incorporation into DNA and RNA was determined in order to evaluate the effect of NaBu on biosynthesis of nucleic acids. The results listed in Table 2 demonstrate that 2 mM NaBu treatment for 2 days resulted in a marked inhibition of thymidine uptake in the SPC-A1/DM4 cells while uridine incorporation into RNA was significantly enhanced. These differences in DNA and RNA syntheses between the control and NaBu-treated cells were statistically significant ($p < 0.01$).

Table 2. Effect of NaBu on DNA and RNA synthesis

NaBu (mM)	Incorporation rate (c.p.m./2 × 10^4 cells/24 h)	
	[^{3}H]-thymidine	[^{3}H]-uridine
0	13353 ± 2273	1321 ± 291
2	4096 ± 480[a]	12715 ± 2617[a]

SPC-A1/DM4 cells (1×10^5) in 10 ml growth medium were exposed to 0 or 2 mM NaBu for 48 h. The tracer was added for the last 24 h of the assay. Results were expressed as mean ± SD of three separate experiments, each performed in duplicate.
[a] $p < 0.01$.

Morphological observation

Cell morphology was first examined at the level of light microscopy. As shown in Figure 7, exposure of SPC-A1/DM4 cells to various concentrations of NaBu for 2 days resulted in a dose-dependent increase in cell size. At 1–2 mM of NaBu, the treated cells became more irregular and many lipid droplets appeared in the cytoplasm. The nuclei of most of these cells also became easily observable when compared with the control. These alterations in morphology were further confirmed by transmission electron microscopy. At the ultrastructural level, the most obvious impression was a marked light-staining of the cells treated with 2 mM NaBu for 2 days. Preparation of the samples was repeated twice with comparable results, suggesting an alteration of components in the membrane. Moreover, as shown in Figure 7, NaBu-treated cells accumulated lipid droplets in the cytoplasm below the concave surface of the nucleus. Above the convex surface of the nucleus an area was observed with a well developed endoplasmic reticulum. These findings are in good concordance with the descriptions reported by Nordenberg *et al.* using melanoma cell cultures.[22]

Quantitative measurement with an imaging analysis system showed a significant enlargement of

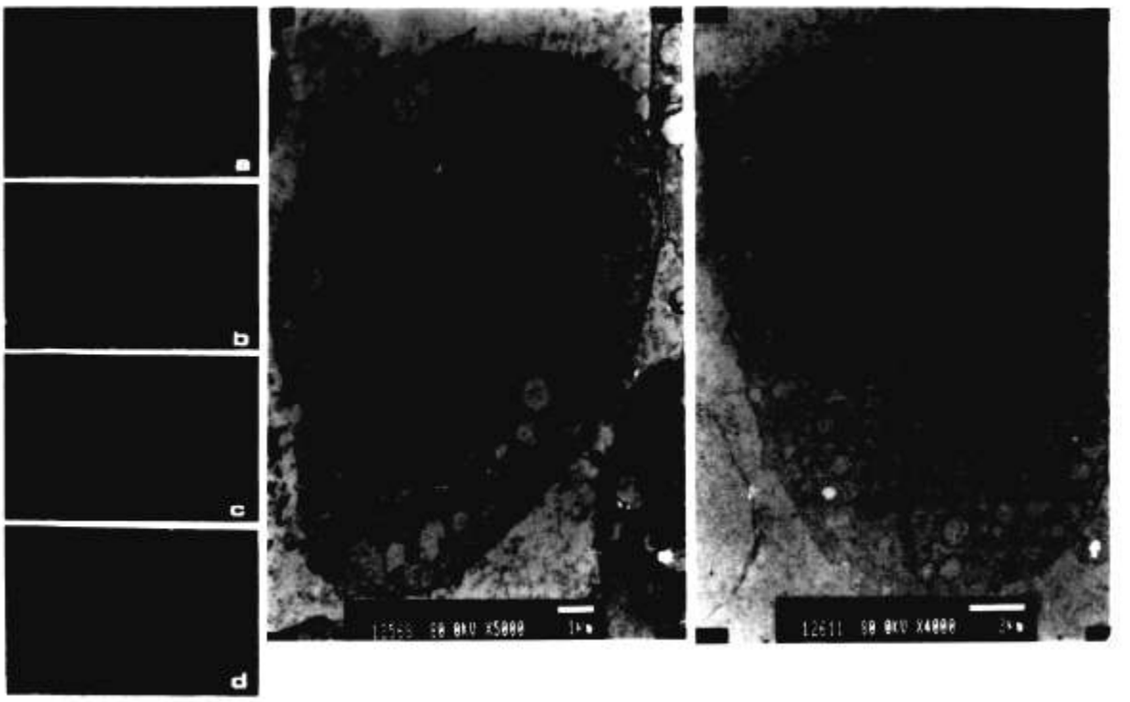

Figure 7. Morphology of SPC-A1/DM4 cells: 1×10^5 cells/dish were exposed to (a) 0, (b) 0.5, (c) 1.0 and (d) 2.0 mM of NaBu. Morphology was examined by phase-contrast microscopy after 2 days of treatment ($\times 200$); (e) and (f) represent the transmission electron micrographs of cells treated with 0 or 2.0 mM NaBu for 2 days, respectively.

Table 3. Effect of NaBu on the nuclear and cytoplasmic areas

NaBu (mM)	Areas (arbitrary units)		N:C ratio
	nuclear	cytoplasmic	
0	184.8 ± 80.0	718.5 ± 251.1	0.28 ± 0.15
0.5	150.7 ± 60.6	728.4 ± 269.1	0.23 ± 0.11[a]
1	275.9 ± 113.1	1249.0 ± 512.7	0.25 ± 0.14[b]
2	303.1 ± 158.4	1367.7 ± 509.4	0.24 ± 0.14[a]

SPC-A1/DM4 cells were exposed to NaBu for 48 h. The nuclear (N) and cytoplasmic (C) areas of each cell were measured by imaging analysis and the N:C ratio was then calculated individually. For each group, a total of 200 cells were determined. The results were expressed as mean ± SD.
[a] $p < 0.01$; [b] $p < 0.05$.

both nuclear and cytoplasmic areas in the cells exposed to 1–2 mM NaBu for 2 days (Table 3). However, as shown in Table 3, the ratio of nuclear to cytoplasmic areas (N:C ratio) was significantly decreased for all NaBu concentrations tested.

Discussion

MMC is one of the most consistently active single agents available for the treatment of NSCLC.[23] In combination with two other active drugs, cisplatin and vinca alkaloids, MMC can produce response rates greater than or equal to 50% in properly selected patients. However, clinical studies have raised an important problem. Although responding patients generally have improvement in both their performance status and survival, responses are frequently short-lived and more prominently, the maximum clinically apparent response occurs within the first three courses (for recent reviews, see refs 24 and 25). The exact reasons for this are at present unknown. A possible explanation is that a small proportion of tumor cells survives the combination chemotherapy and subsequently develops resistance to these active drugs. The results of this paper support this hypothesis. We reported herein that a cloned human lung adenocarcinoma cell subline SPC-A1/D13, after being induced to be resistant to MMC by a stepwise selection procedure *in vitro*, exhibited a strong cross-resistance to cisplatin and, to a lesser extent, to VCR (Figure 2). This drug resistance pattern of NSCLC cells has not been identified before to our knowledge.

The object of this study was to evaluate the effect of NaBu, a widely-used differentiating agent, on the cell growth and drug resistance of this established

cell subline. We show that NaBu inhibits cell proliferation in a dose-dependent manner. Concentrations of NaBu from 0.5 to 2 mM decrease cell growth without inducing cell death. A 4 mM concentration causes cell death which can be visible as early as 48 h after treatment. This antiproliferative effect is reversible (Figure 4). An important finding of the present study is that SPC-A1/DM4 cells, after recovery from the growth inhibition induced by 2 days treatment with 2 mM NaBu, become more sensitive to both MMC and cisplatin than the cells not exposed to the agent and this modulation of drug resistance by NaBu can be retained for at least 1 month (Figure 5). The mechanisms underlying this long-term effect remain poorly understood at present. However, our results indicate that this effect occurs 7 days after withdrawal of the agent which strongly suggests that this is not a result of a direct interaction between NaBu and cytotoxic drugs, but the result of some type of biochemical change induced by the agent.

The growth inhibition by NaBu is considered to be responsible for an enhanced gene expression in the sense that a high proportion of cells is shifted to the phase most amenable for transcription.[26,27] On a molecular level, the most evident targets of NaBu are the histone deacetylases which are inhibited uncompetitively. Blocking of deacetylating activities enables acetyltransferases to hyperacetylate the four core histones at distinct lysine residues within the N-terminal tails. Hyperacetylated nucleosomes in turn undergo conformational changes similar to those seen in active chromatin,[28,29] resulting in enhanced transcriptional activities and modulation of DNA higher-order structures during chromatin assembly.[30,31] In the present study, we also observed that NaBu treatment causes a marked inhibition of DNA synthesis (Table 2), an accumulation of cells in the G_2 M and, to a lesser extent, G_1 phases of the cell cycle (Figure 6), a corresponding increase of protein content in these subpopulations (Table 1), and an enhanced RNA synthesis rate (Table 2). These data support this model of action.

A key question is how the NaBu-induced metabolic alterations can affect the cellular response to MMC and cisplatin for such a long period of time. The effect of NaBu on cytotoxic drugs and irradiation has been investigated. Wasserman *et al.*[14] reported that co-administration of NaBu with cisplatin or adriamycin into human ovarian carcinoma cell cultures renders these initially irresponsive cells sensitive to cisplatin and enhances their response to adriamycin. However, the responsible mechanism(s) remain unclear. Leith *et al.*[32] found that NaBu potentiates the sensitivity of human colon carcinoma cells to X-ray by decreasing the intracellular content of glutathione, a main type of non-protein sulfhydryl in mammalian cells which has been shown to play a role in the development of resistance to MMC and cisplatin.[33,34] To evaluate this possible mechanism, we have determined the sulfhydryl content in crude cytoplasmic extract using Ellman's method.[35] We found that, in SPC-A1/D13 cells, the non-protein sulfhydryl content constitutes less than one fifth of the total content, and there are no differences in both non-protein and total sulfhydryl content between this subline and its resistant one, SPC-A1/DM4, suggesting that glutathione may not be a contributor to the drug resistance in this model (data not shown). Recently, Bates *et al.*[15] investigated the effect of NaBu on multidrug resistance using SW 620 human colon carcinoma cell line. They demonstrated that NaBu increases the intracellular drug accumulation by inhibiting P-glycoprotein phosphorylation. This is an important mechanism by which NaBu modulates drug resistance. However, this mechanism seems unlikely to act in our model for the following reasons: (i) cisplatin is not an appropriate substrate of P-glycoprotein, but NaBu can potentiate the cellular response to this drug (Figure 5); (ii) MMC and VCR are among the substrates of this drug efflux pump. However, the MMC-resistant SPC-A1/DM4 cells do not exhibit a strong cross-resistance to VCR (Figure 2) and (iii) it is difficult to imagine that the inhibition by NaBu of P-glycoprotein phosphorylation could be sustained for such a long duration when the inhibitor has been removed from the cell cultures. Thus it seems reasonable to presume that NaBu acts by other mechanisms by which the NaBu-induced molecular events could persist for a certain duration.

Sodium butyrate has been known to modify the action of certain enzymes involved in membrane synthesis and thereby might influence the transport of cytotoxic drugs. A sample of such an enzyme is glycerophosphate dehydrogenase. This enzyme was found to be enhanced in NaBu-treated melanoma cells[22] and fibroblasts,[36] and was supposed to be involved in lipid accumulation and adipose differentiation.[36] In addition, it has been reported that NaBu treatment of human and mouse melanoma cells induced a differentiation-associated enhancement of NADPH cytochrome *c* reductase, a marker enzyme of the endoplasmic reticulum.[22]

This enzyme is also a key enzyme for the one-electron reduction pathway of MMC activation.[37] In the present study, we observed that NaBu treatment results in some morphological alterations, including a marked lipid accumulation, the development of endoplasmic reticulum and a decreased N:C ratio (Table 3 and Figure 7). These findings suggest that the activity of the enzymes glycerophosphate dehydrogenase and NADPH cytochrome *c* reductase may also be induced in SPC-A1/DM4 cells. Although it is unclear whether this induction of enzymes reflects differentiated features in NSCLC cells, the decrease in the N:C ratio might represent a differentiated characteristic since poorly differentiated tumor cells tend to have a higher N:C ratio.

Recently, Sankaranarayanan *et al.*[38] reported that NaBu pretreatment of human lymphocytes resulted in an increased frequency of chromosomal aberrations when they were exposed to X-rays and/or bleomycin. This finding suggests that NaBu can inhibit the nuclear enzymes involved in repair of DNA damage. NaBu induced modification of nuclear enzymes was also described by Cosgrove and Cox.[6] They demonstrated that NaBu induced DNA hypermethylation in HeLa cells and, more importantly, this hypermethylation could be sustained for a relatively long period of time after withdrawal of the agent, a phenomenon similar to that observed in this study. Taken together, these findings, and many others, strongly suggest that NaBu can modify a variety of intra- and/or extra-nuclear enzyme activities. Some of these modifications can persist for a certain duration. These NaBu-inducible modifications of enzymes form the basis for its long-term effect on drug resistance. However, further investigations are needed before an unambiguous conclusion can be reached.

From a more practical point of view, however, our findings suggest that NaBu is potentially useful in the treatment of drug-resistant NSCLC, since it can exert antitumor effects both indirectly by modulating the chemosensitivity of drug-resistant tumor cells and directly by inhibiting the growth of tumor cells themselves. Considering the low toxicity of NaBu *in vivo*,[39] combined treatment of this agent with cytotoxic drugs might be feasible.

Conclusion

The object of this study was to evaluate the effect of NaBu on the cell growth and drug resistance of NSCLC cells. For this purpose, we first isolated a MMC-resistant human lung adenocarcinoma cell subline by a stepwise selection procedure *in vitro*. The established subline SPC-A1/DM4, which also exhibited a strong cross-resistance to cisplatin, was then used to examine this possibility. We show that NaBu inhibits cell proliferation in a dose-dependent manner with the cytostatic concentration of 2 mM. This growth inhibition is reversible. However, SPC-A1/DM4 cells, after recovery from NaBu-induced cytostasis, become more sensitive to both cytotoxic drugs than the cells not exposed to the agent and this modulation by NaBu on drug resistance can be retained for at least 1 month after withdrawal of the agent. Given the marked abilities of NaBu to inhibit cell growth and to modulate drug resistance in this cell subline, and given its low toxicity *in vivo*, combined therapy with this agent and cytotoxic antitumor drugs might be considered.

The mechanisms underlying this long-term effect of NaBu on drug resistance remain poorly understood at present. The results of this study suggest that NaBu inducible phenotypic alterations play a role in the modulation of drug resistance. However, further investigations are needed to characterize the associated molecular events.

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