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Antiproliferative effects of sodium butyrate in adriamycin-sensitive and -resistant human cancer cell lines

Dominique Fagot, Christine Buquet-Fagot, François Lallemand and Jan Mester
INSERM U55, 184, rue du Faubourg Saint-Antoine, 75012 Paris, France. Tel: (+33) 1 49284614; Fax: (+33) 1 43433234.

The proliferation of the MCF7 and MCF7-A (adriamycin-resistant) and KB-3-1 and KB-A (adriamycin-resistant) cell lines was arrested by sodium butyrate (NaBut) at 1 mM or higher concentrations. In the MCF7 and MCF7-A cell lines, an accumulation in the G_1 phase was observed, whereas the KB-3-1 and KB-A cell lines accumulated in both G_1 and G_2/M phases. The level of the mRNA coded by the 'early G_1' gene c-*myc* was high in all these cell lines, and was only transiently decreased by NaBut treatment. The 'late' mRNA coding for the proliferating cell nuclear antigen (PCNA) was also strongly expressed in all the cell lines studied; incubation with NaBut caused a decrease of the PCNA mRNA in the MCF7 and MCF7-A cells but not in the KB-3-1 and KB-A cells. The anti-oncoprotein p105RB was undetectable in the MCF7 and MCF7-A cells, while the KB-3-1 as well as KB-A cells contained a high level of this protein. Neither the content nor the apparent state of phosphorylation of the RB protein were affected by incubation (up to 48 h) with NaBut.

Key words: Butyrate, cell cycle, multidrug resistance, c-*myc*, PCNA, p105 RB.

Introduction

Sodium butyrate (NaBut) is well known for its growth-inhibitory and differentiation-inducing properties in cultured cells (reviewed by Prasad[1] and Kruh[2]). In exponentially growing cells, NaBut inhibits progression through G_1 phase of the cell cycle; however, this compound has been also shown to cause polyploidization in certain cell types: for instance, the rat 3Y1 cells released from the hydroxyurea block (G_1/S interphase) in the presence of NaBut will duplicate their DNA content but are unable to exit from G_2.[3] NaBut and derived compounds have a potential interest in cancer therapy, particularly in view of their low toxicity *in vivo*.

The mechanism of NaBut action remains obscure but may be related to the inhibition of protein desacetylases, leading to core histone hyperacetylation[4] and changes in the chromatin structure. In addition, NaBut inhibits phosphorylation of histones H1 and H2A.[5] An altered pattern of gene expression has been observed in cells incubated with NaBut; this may be a consequence of the overall changes in the chromatin structure but other (unknown) mechanisms are likely to be involved in the sequence-specific NaBut-mediated induction of gene expression. Both inhibition[6,7] and induction[8,9] of gene expression by NaBut have been reported and can be attributed to transcriptional processes. In particular, several groups have recently identified DNA sequence elements which can confer sensitivity to transcription induction by NaBut.[8–11] Post-transcriptional and indirect effects of NaBut on gene expression have also been described.[12]

In BALB/c mouse fibroblasts made to progress synchronously through G_1 phase by serum deprivation and re-stimulation, the addition of NaBut will rapidly block the cell cycle progression and accumulation of mRNAs characteristic of late G_1/S/G_2 phases such as *cdc2*[13] and PCNA ('proliferating cell nuclear antigen'; C Buquet-Fagot, unpublished data). However, NaBut does not interfere with either growth factor signaling itself, as it does not prevent the induction of early G_1-related gene expression.[13] Also, NaBut does not affect the incorporation of [^{3}H]thymidine into DNA in S phase cells (unpublished data). Interestingly, rat kidney fibroblasts stably transfected with the E1A gene display 'leakiness' in terms of NaBut inhibition of G_1 phase progression (T Joensuu and J Mester, manuscript in preparation); since the E1A gene product acts by sequestering the p105RB protein, this raises the possibility that the effect of butyrate may be linked to the modulation of the cell content or the state of phosphorylation of the p105RB protein.

In this work, we have studied the effects of NaBut in exponentially growing populations of human cancer cell lines expressing or not the *mdr1* gene.

Correspondence to J Mester

Materials and methods

Cell lines and culture

The human breast cancer cell line MCF7 and the derived subline adapted for growth in the presence of adriamycin (denoted 'MCF7-A' in this work[14]) were obtained from Dr F Calvo, St Louis Hospital, Paris. The human epidermoid cancer cell lines KB-3-1 and the subline KB-A (adriamycin-resistant[15]) were obtained from Dr S Chevillard, Institute Curie, Paris. These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS; Gibco-France, Eragny, France). Adriamycin (doxorubicin; Sigma, St Louis, MO; 1 μg/ml) was added to the MCF7-A and KB-A cells after passaging.

[^{3}H]Thymidine incorporation, cell cycle and cell growth studies.

For thymidine incorporation experiments, cells were seeded in 24-well plates (10–50×10^3/well); [^{3}H]thymidine (2 μCi/ml) incorporation was evaluated by 30 min pulses. For flow cytometry analysis, cells (1–2 × 10^6) were harvested from 100 mm Petri dishes by trypsin, fixed by ethanol (70%) and stored at 4 °C. For analysis, they were centrifuged, suspended in 0.1% Triton X-100, treated with RNAse (1 mg/ml) and propidium iodide (20 μg/ml) for 30 min at room temperature, and then analyzed at a flow rate of about 500 cells/s in Orthocytofluorograph 50H (Ortho Diagnostic Systems, Westwood, MN).[16] Cell growth rate was evaluated either by counting cells in a Coulter counter (Coultronics, Luton, UK), or by measuring the protein concentration (BioRad protein assay kit) in cells fixed by 5% trichloroacetic acid and solubilized in 0.1 N NaOH.

RNA analysis

Total cytoplasmic RNA was isolated from cells grown in 100 mm Petri dishes, denatured by formaldehyde, fractionated by agarose gel electrophoresis and transferred to nylon membranes.[17] The filters were then hybridized at 42°C in a solution containing 50% formamide, 0.45 M NaCl, 3 mM EDTA and 75 mM phosphate buffer, pH 7.4, with ^{32}P-labeled probes: *ClaI–EcoRI* fragment of the human c-*myc* gene,[18] PCNA cDNA[19] and rat α-tubulin cDNA.[20] The filters were washed at 45°C in 0.15 M NaCl and 0.15 mM sodium citrate, and exposed with XAR5 Kodak film and an intensifying screen (Eastman Kodak, Rochester, NY).

Detection of the p105RB protein

The cells were seeded as for RNA analysis. They were harvested in 0.5 ml of lysis buffer [50 mM Tris–HCl, pH 6.8, 120 mM NaCl, 0.5% Nonidet NP-40, 0.1 TIU aprotinin (Sigma)/ml] as described.[21] Aliquots containing 100 μg protein were denatured in the sample buffer (0.175 M mercaptoethanol, 1% SDS, 5% glycerol, 0.01% bromophenol blue, 40 mM Tris–HCl, pH 6.8) and fractionated by electrophoresis in 6% polyacrylamide gel. The portion of the gel covering the region between 90 and 130 kDa was transferred onto a nylon membrane and revealed by an anti-RB rabbit polyclonal IgG (Rb IC-15, directed against the COOH terminal 15 amino acids of the human RB protein; Santa Cruz, Santa Cruz, CA) followed by chemoluminiscence-labeled goat anti-rabbit peroxidase-coupled IgG (Amersham France, Les Ulis, France). For more details, see Chastre *et al.*[22]

Results

Cell growth

The effect of NaBut on [^{3}H]thymidine incorporation in the human epidermoid cell lines KB-3-1 and KB-A is illustrated in Figure 1(A). Both lines displayed about the same sensitivity to the drug, indicating that cells resistant to adriamycin do not display resistance to NaBut. The half-maximal inhibitory concentration (IC_{50}) was approximately 1 mM NaBut, both in terms of the labeled thymidine incorporation and cell density (data not shown). The [^{3}H]thymidine incorporation was inhibited by approximately 75% within 24 h of incubation with 3 mM NaBut (85% inhibition after 48 h).

Somewhat different data were obtained with the MCF7 and MCF7-A cell lines (Figure 1B): the original (adriamycin-sensitive) line MCF7 required approximately 2 mM NaBut for a 50% inhibition of [^{3}H]thymidine incorporation after 48 h of incubation, whereas for the adriamycin-resistant MCF-A cells, the IC_{50} of NaBut was approximately 0.7 mM. It should also be noted that the MCF7-A cells became rapidly (within 2 days) non-viable upon growth arrest, either by incubation with 3 mM or higher NaBut or with other inhibitors of G_1 progression such as lovastatin (data not shown).

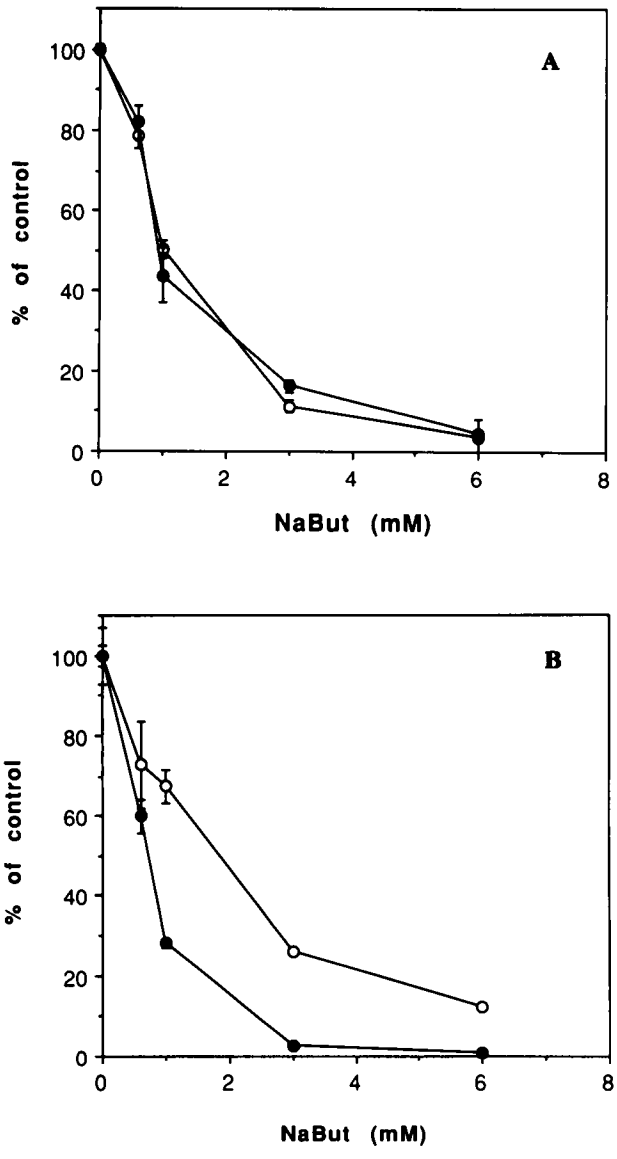


Figure 1. Effect of NaBut on [^{3}H]thymidine incorporation in (A) KB-3-1 and KB-A cells, and (B) MCF7 and MCF7-A cells. Cells were seeded at 50 000 per well in 24-well boxes. On the next day, NaBut was added at concentrations between 0 and 6 mM. After a further 48 h of culture, incorporation of [^{3}H]thymidine was measured by 30 min pulses. The data are presented as percentages of control (cells maintained without NaBut). MCF7 and KB-3-1 cells, ○; MCF7-A and KB-A cells, ●. Means of triplicates ± range are shown.

In contrast, the original MCF7 cells appeared to enter quiescence and were able to resume their growth even after 4 days of incubation with 3 mM NaBut. In the KB3-1- and KB-A cells, NaBut caused extensive cell death after 2 days or more. The cell death did not appear to result from apoptosis, either by morphological or by DNA fragmentation criteria (data not shown).

Cell cycle distribution

When the distribution of cells among the different phases of the cell cycle was studied, the responses to NaBut differed according to the cell line (Figure 2). A time-dependent increase of the cell fraction in the G_1 phase was observed in the MCF7 and MCF7-A cells, whereas in the KB-3-1 and KB-A cell lines the G2 phase fraction remained high and even increased with time. A transient increase in the G_2 phase population (maximum at approximately 7 h) was also noted in MCF7 and MCF7-A cells. In addition, a significant proportion of cells with DNA content corresponding to the S phase was frequently detected, in contrast with the strongly reduced [^{3}H]thymidine incorporation (*cf.* Figure 1). These observations indicate that the drug inhibited progression through G_1 as well as G_2 phases of the cell cycle and probably also DNA replication in S phase cells.

Gene expression

Earlier reports have suggested that the growth inhibitory effects of NaBut may result from a selective inhibition of the expression of cell cycle-related genes such as c-*myc*.[23] In the human cancer cell lines studied, the c-*myc* mRNA content decreased within 3 h following addition of NaBut to the culture medium (Figure 3), but this decrease was only transient and the c-*myc* mRNA returned to its initial level after 24–48 h.

The cell content of the mRNA coding for PCNA was evaluated as a marker of late $G_1/S/G_2$ (Figures 3B and 4). In the MCF7 and MCF7-A cells, a decrease in the PCNA mRNA level was observed during culture with NaBut. The decrease became detectable after a delay of approximately 3 h (Figure 4C); subsequently, the rate of disappearance of the PCNA mRNA was similar in cells incubated either with NaBut or with actinomycin D, suggesting that butyrate action was exerted by blocking the transcription of the PCNA gene. When transcription was blocked by actinomycin D, additional presence of NaBut did not lead to a faster elimination of the PCNA mRNA, indicating that butyrate does not affect the mRNA stability (Figure 4A and B). The expression of the PCNA gene appeared to require (a) short-lived cellular protein(s) as the presence of cycloheximide in the culture medium resulted in a decreased content of the PCNA mRNA. After incubation with both cycloheximide and NaBut the PCNA mRNA content was not significantly different

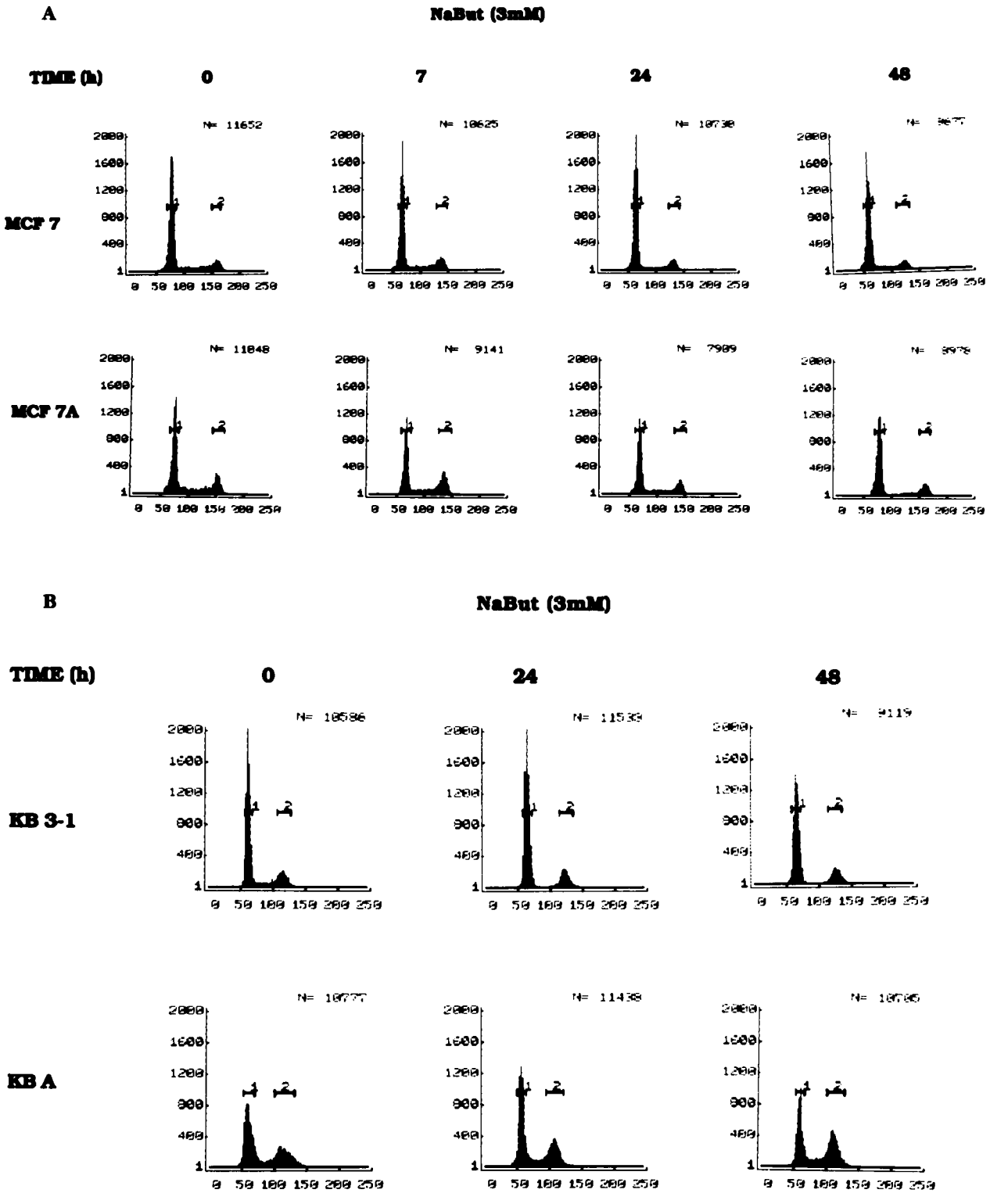


Figure 2. Effect of NaBut on cell cycle distribution in (A) KB-3-1 and KB-A cells, and (B) MCF7 and MCF7-A cells. Cells were seeded at 10^6 in 100 mm Petri dishes. On the next day, 3 mM NaBut was added and at the times indicated the cells were harvested for analysis by flow cytometry.

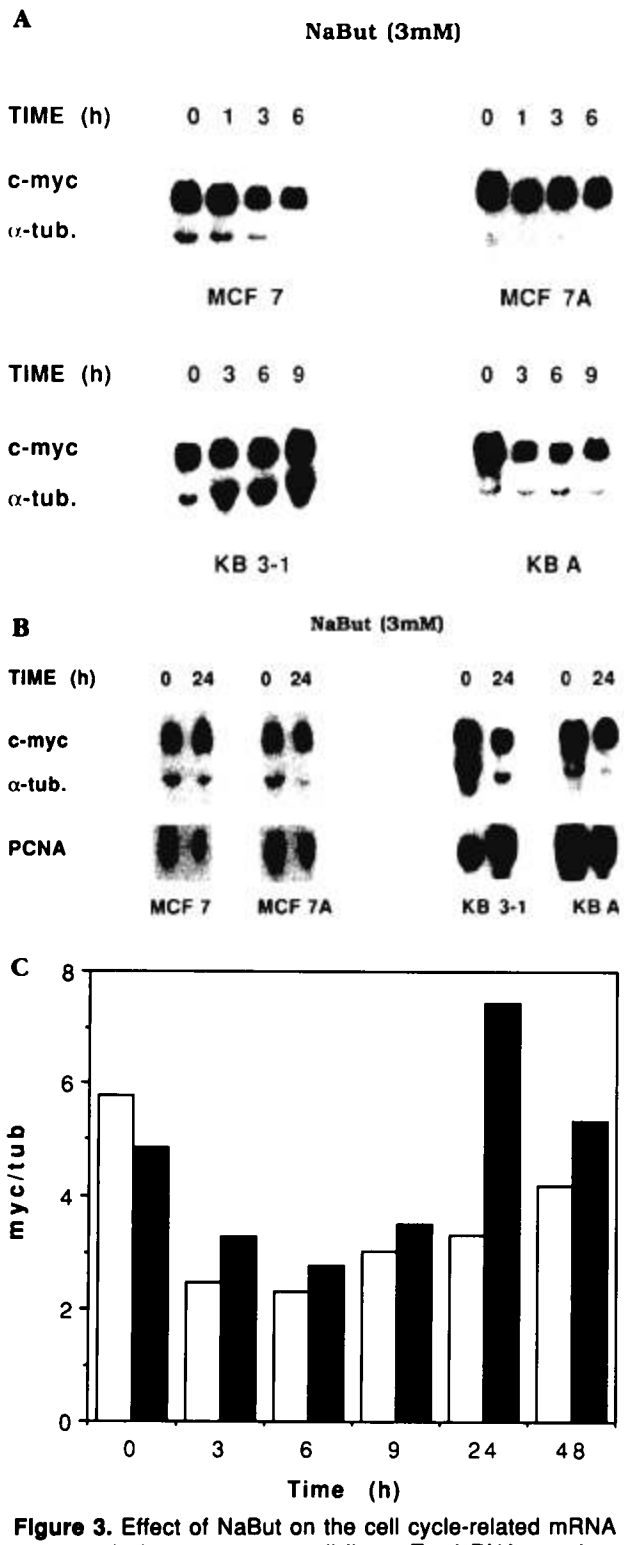


Figure 3. Effect of NaBut on the cell cycle-related mRNA contents in human cancer cell lines. Total RNA was isolated from cells incubated with NaBut (3 mM) for the different time periods (A and B) and analyzed by Northern blotting with the probes as indicated. (C) The ratio of the c-*myc* to α-tubulin signal for the KB-3-1 (□) and KB-A (■) cells was evaluated by densitometry.

from that detected in cells exposed to either drug alone.

In the KB-3-1 and KB-A cells, incubation with NaBut (for as long as 48 h) did not have a significant effect on the PCNA mRNA content (Figure 3).

p105RB protein

The MCF7 and MCF7-A cells did not express detectable amounts of the RB protein. In contrast, both KB-3-1 and KB-A cells gave a strong signal with the anti-RB antibody at the position of the slowly migrating band, previously identified as corresponding to the hyperphosphorylated form of p105RB.[24] After incubation with 3 mM NaBut (up to 48 h), approximately the same signal was detected, indicating that the drug had no effect on either the abundance of the RB protein or on its state of phosphorylation (Figure 5).

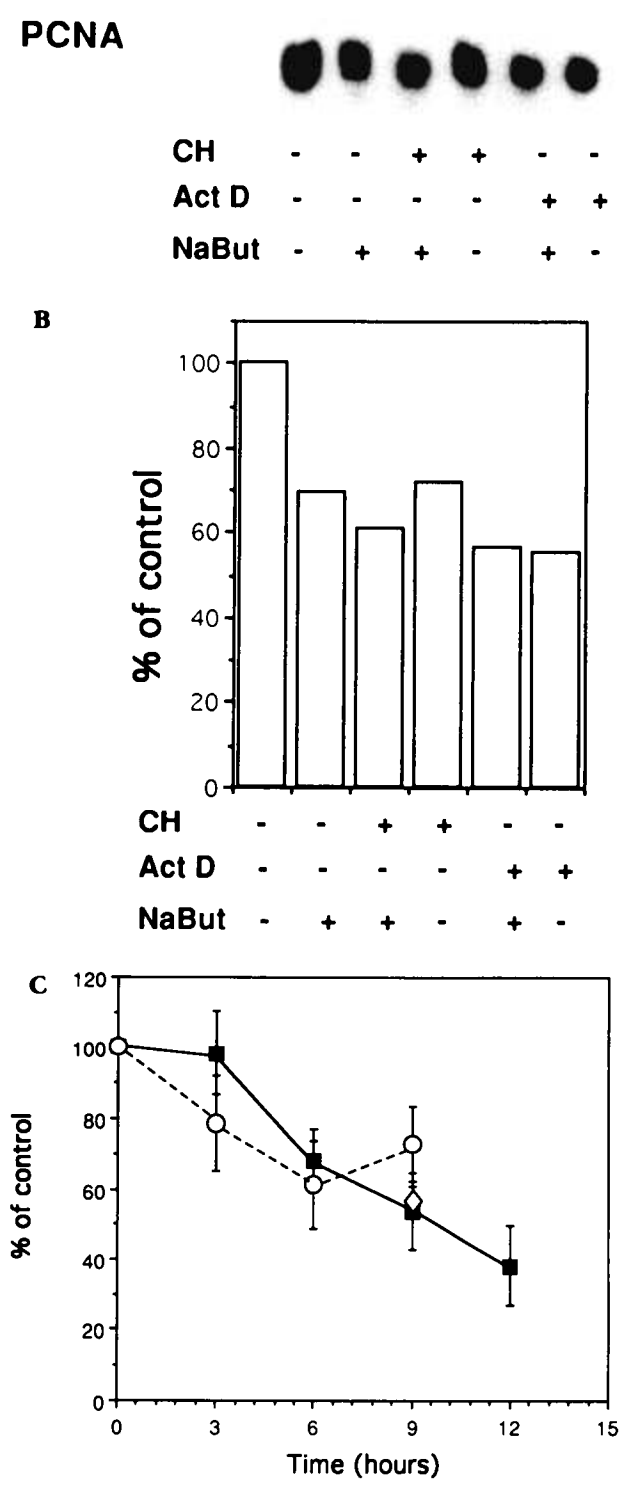
Discussion

It has been shown earlier that NaBut can arrest the G_1 progression in cells synchronized by serum deprivation and re-stimulation, and prevents the accumulation of *cdc2* mRNA characteristic of the late $G_1/S/G_2$ phase.[13] In this work, we have studied the effect of NaBut in exponentially growing human cancer cell lines. The following criteria were used: cell proliferation, DNA replication (incorporation of [^{3}H]thymidine), distribution among the phases of the cell cycle (flow cytometry), expression of genes characteristic of early and late phases of the cell cycle, and cell content of the hypo- and hyperphosphorylated forms of the RB protein.

Concentrations of 3 mM or higher of NaBut rapidly affect the pattern of abundance of cells with different DNA contents as revealed by flow cytometry analysis. We have frequently observed a transient accumulation in G_2 phase (maximum at approximately 7 h). At longer times (24–48 h), the cells accumulate either in the G_1 phase (MCF7 and MCF7-A cells) or in G_1 and G_2 phases (KB-3-1 and KB-A cells). We interpret this by proposing that the KB cells are partly resistant to the cell cycle inhibition by NaBut in the G_1 but not G_2 phase.

The question of the cellular target of NaBut action is of interest for a better understanding of the cell cycle regulatory processes. A reduction (due to post-transcriptional processes) of the expression of c-*myc*, one of the essential genes expressed early in the G_1 phase, has been observed for

instance in the colon carcinoma cell line Caco-2,[12] as well as in several other studies,[25,26] raising the possibility that the reduction in the c-*myc* mRNA may be the cause of the growth inhibition. Our data do not favor this hypothesis as the c-*myc* mRNA contents are only transiently affected by NaBut in all human cancer cell lines studied. It has been shown earlier that NaBut does not prevent the induction of the c-*myc* gene expression by serum in quiescent BALB/c mouse fibroblasts.[13] Of other 'immediate early' cell cycle-related genes, NaBut actually induces the expression of c-*fos*.[25] 'Late' genes such as PCNA appear to be better candidates to serve as a target of the growth inhibitory action of NaBut. In the MCF7-derived cells, the PCNA mRNA levels decreased during incubation with NaBut. The fact that the rates of elimination of the PCNA mRNA in cells incubated either with NaBut or with actinomycin D were the same (Figure 4B and C) indicated that NaBut may act at the gene transcription level. The question whether the effect of NaBut on the PCNA mRNA content requires protein synthesis (resulting for instance from the expression of butyrate-regulated genes) cannot be answered at this moment as the inhibition of protein synthesis is itself sufficient to induce a decrease in the PCNA mRNA content. It is probable that the expression of the PCNA gene depends on short-lived cellular proteins; a similar observation has



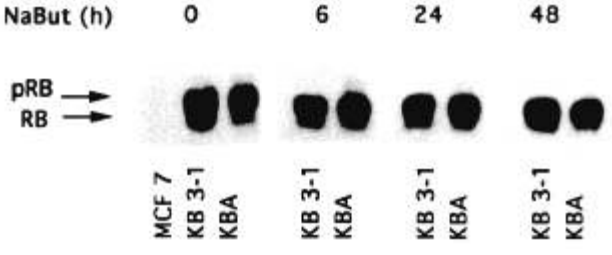


Figure 5. Detection of p105RB protein in NaBut-treated cells. The cells in 100 mm dishes were treated with 3 mM NaBut as indicated. They were lysed and the extracts analyzed by Western blotting as described in the Materials and methods section. The luminiscence was revealed by a 30 s exposure to a XAR5 Kodak film. The arrows indicate the migration of the hypophosphorylated (RB) and hyperphosphorylated (slower-migrating; pRB) forms of the RB protein.

Figure 4. Effects of NaBut, actinomycin D and cycloheximide on the cell contents of PCNA mRNA in human cancer cell lines. The cells were incubated with the different drugs as indicated. (A) Total RNA was isolated after 9 h of incubation and analyzed by Northern blotting with the PCNA probe. (B) The signal intensity of the experiment shown in (A) was evaluated by densitometry (arbitrary units). (C) The data (means ± range of two to three independent experiments) are plotted as percent of the signal in control (exponentially growing) cells versus time of incubation. Solid squares, cells treated with NaBut (3 mM); open circles, cells treated with actinomycin D (10 μg/ml); open diamonds, NaBut plus actinomycin D.

been made for the expression of another 'late' gene (*cdc2*) in mouse fibroblasts.[27] NaBut interfered also with the accumulation of the *cdc2* mRNA in fibroblasts (see Figure 7).[13] Since butyrate apparently can inhibit the G_1 progression also in its early stage,[13] it is possible that the function of a gene (or genes) coding for (the) short-lived protein factor(s) necessary for the 'late' gene expression may be affected by butyrate in the MCF7 cells.

In contrast, the PCNA mRNA levels were unaffected by NaBut in the KB-3-1 and KB-A cell lines. It is to be noted that these cells display a 'leaky' inhibition of G_1 phase progression by butyrate and accumulate partly in the G_2 phase. The PCNA gene product is the auxiliary protein of the DNA polymerase δ and it is indispensable for DNA replication in the S phase.[28] In addition, PCNA has been found to form multiprotein complexes involving D type cyclins (D1 and D3) and cell cycle-dependent kinases (*cdk 2, 4* and *5*), suggesting that it may have also non-DNA synthetic functions in the G_1 and/or S phases of the cell cycle.[29] The deregulation of the PCNA expression may be responsible for the partial resistance of the cell lines KB-A and KB-3-1 to NaBut in the G_1 phase.

In this context, it should be noted that the PCNA gene expression is apparently induced (directly or indirectly) by the E1A gene product in NRK-derived cell lines.[30] In addition, our recent unpublished data (T Joensuu and J Mester, manuscript in preparation) indicate that E1A-transformed normal rat fibroblasts are also 'leaky' to G_1 phase inhibition by NaBut. Since the E1A gene product is known to bind to the hypophosphorylated RB protein, we have considered the possibility that the action of butyrate could be transmitted via alterations in the cellular abundance of p105 RB, or in its state of phosphorylation. This is clearly not the case: the MCF7 cells are sensitive to NaBut in the G_1 phase although they lack p105 RB, whereas in the KB-3-1 cells the p105 RB is abundant, predominantly hyperphosphorylated and insensitive to butyrate. It remains possible that the G_1 progression block by NaBut could be a consequence of an inhibition of the expression or function of transcription regulatory gene(s) yet to be identified and whose products are required for the expression of late genes such as PCNA. Alternatively, the cessation of the PCNA gene expression may be a consequence of inhibition by NaBut of a process (unknown) necessary for G_1 progression. The resistance to the effect of NaBut in the G_1 phase may result from alterations of the gene expression pattern (implicated for instance in the oncogenic transformation which produced the KB-3-1 cells). Such cells, when cultured in the presence of NaBut, will accumulate (partly or predominantly) in the G_2 phase and continue to express the PCNA gene. (It has been shown that the PCNA mRNA accumulates in the G_1 phase and remains abundant throughout the late stages of the cell cycle.[19]) The inhibition of the G_2 phase progression by NaBut remains to be explored.

The effects of NaBut observed in cultured cells have incited several groups to suggest its use as a therapeutic drug, both for the treatment of malignancies and of genetic disorders linked to cell differentiation. The handicap of the extremely short half-life in the plasma (5 min or less[31]) can be conceivably overcome by the use of chemical conjugates of butyric acid.[32,33] In an animal model (mouse), plasma concentrations between 4 and 8 mM have been produced for a short period of time.[31] Clinical trials in humans have so far not detected any significant toxicity of butyrate; however, the plasma concentrations were not in the milimolar range required for *in vitro* effects. It is all the more remarkable that a butyric acid derivative, arginine butyrate, has proved to be efficient in the treatment of patients with β-thalassemia and sickle cell anemia.[34] Our results indicate that NaBut, besides arresting cell proliferation, causes extensive death in exponentially growing cell populations; the cytotoxic effect is particularly pronounced in those cell lines which have lost their differentiated character (e.g. the MCF7-A cells which are estrogen-independent, unlike the original MCF-7 line). We believe that further laboratory and clinical studies of butyric acid and its derivatives as potential anticancer drugs are justified, in particular in view of the efficacy of NaBut in multidrug-resistant cell lines (see Note).

Conclusion

NaBut blocks the proliferation of human cancer cell lines MCF7 and KB-3-1, as well as the sublines MCF7mdr1 and KB-A adapted to growth in the presence of doxorubicin. The growth inhibition is situated at several points of the cell cycle. All these cell lines displayed a high level of expression of the cell cycle-related genes c-*myc* (early) and PCNA (late). Butyrate causes a transient decrease in the cell contents of c-*myc* mRNA in all these cell lines. There were differences between the two types of cell lines: in the MCF7 and MCF7mdr1 cells, we observed an accumulation predominantly in the G_1 phase, absence of expression of the p105RB

protein and decrease in the PCNA mRNA content during incubation with butyrate, whereas in the KB-3-1 and KB-A cells, there was a high level of the p105RB protein, during incubation with butyrate these cells accumulated in both G_1 and G_2/M phases and their contents of the PCNA mRNA were unaffected by the drug. The mechanism of butyrate action may involve the activity of transcription factors required for both G_1 and G_2 phase progression. The activity of this compound in multidrug-resistant cells justifies further attention to its potential in therapy of cancer.

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Note

During the preparation of this manuscript, Qiang *et al.* have reported describing the growth inhibition of NaBut in mitomycin-resistant human lung adenocarcinoma.[35] These cells arrest in both G_1 and G_2/M phases of the cell cycle, in agreement with our data.

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