Effect of sodium butyrate on human breast cancer cell lines

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Abstract. We have investigated the effects exerted by sodium butyrate (NaBu) on the growth and cell cycle perturbations of four human breast cancer cell lines (MCF7, T47D, MDA-MB231 and BT20) with different steroid receptor profiles. Moreover, since one of the supposed mechanisms of action for NaBu activity involves the induction of apoptosis, we have studied the effects of NaBu on DNA fragmentation by agarose gel electrophoresis and flow cytometry. In all investigated cell lines, NaBu exerted a time- and dose-dependent inhibition of growth and caused a maximum inhibitory effect (85% to 90%) at the concentration of 2.5 mM. The inhibition was already evident after 3 days of treatment. The antiproliferative effect of NaBu was associated with a persistent block of cells in the G₂M phase. The block was associated with apoptosis only in oestrogen-receptor positive cell lines. The inhibiting effect of NaBu in hormone-dependent and independent cell lines and its ability to induce apoptosis through a cell cycle perturbation in hormone-dependent cell lines may have important implications in the treatment of human tumours including breast cancer.

Cancer can be regarded as a disruption of the equilibrium between proliferation and differentiation, the two processes at the basis of growth regulation. Compounds able to regulate these processes have been proposed as alternative treatment strategies. In this perspective, butyric acid, normally present in the human colon as a major product of bacterial fermentation of unabsorbed carbohydrate, could be a representative agent. In fact, experimental evidence has demonstrated that salts of butyric acid, particularly the sodium salt (NaBu), possess an antiproliferative activity and are potent inducers of cytodifferentiation in a wide panel of neoplastic cells (Leder & Leder 1975, Collins *et al.* 1978, Prasad 1980). It has been observed that NaBu is able to induce a series of cellular alterations, associated with the differentiation of a tumour-cell phenotype, which include an increase in cell volume, changes in cell morphology, substantial redevelopment of the cytoskeleton extracellular matrix and modulation of the expression of several membrane-associated antigens (Tsao *et al.* 1982, Tsao *et al.* 1983, Langdon *et al.* 1988).

Although the molecular mechanisms through which NaBu exerts its antiproliferative effect are not completely understood, experimental evidence suggests that it could act through the

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regulation of gene transcription and in some cases through the induction of apoptosis. NaBu should provoke the hyperacetylation of histones H3 and H4 and increase susceptibility of the associated DNA sequences to DNase I attack by blocking the activity of histone deacetylase (Boffa *et al.* 1978, Candido *et al.* 1978, Sealy & Chalkley 1978). This could result in an immediate down-regulation of genes associated with cell proliferation, an arrest of cells in the G_{0/1} phase of the cell cycle, and ultimately in a fragmentation of DNA. In fact, NaBu has been shown to cause, within a few minutes, a down-regulation of the expression of the nuclear proto-oncogene *c-myc*, an immediate-early gene directly related to cell growth and differentiation (Dang 1991), and an increase in the expression of transforming growth factor β 1 (Barnard & Warwick 1993), which is able to suppress *c-myc* expression and to arrest cell proliferation. Moreover, NaBu has been found recently (Mandal & Kumar 1996) to down-regulate the expression of the *bcl-2* gene, which is known to be related to apoptosis. Treatment with NaBu could constitute an attractive alternative to the consolidated approaches of chemotherapy or hormone therapy, which are not devoid of important side effects.

The aim of our study was to investigate the effects exerted by NaBu on the growth of four human breast cancer cell lines, characterized by a different steroid receptor status, as a model representative of the heterogeneity of cell populations in human tumours. The effect of NaBu was investigated on cell growth, cell cycle distribution and apoptosis by flow cytometric and gel electrophoresis approaches.

MATERIALS AND METHODS

Cell lines and culture conditions

Four human breast cancer cell lines were used in the experiments: MCF7 (Soule, Vasques & Long 1973) and T47D (Keydar, Chen & Karby 1979) are oestrogen receptor-positive (ER+), while MDA-MB231 (Cailleau, Young & Olive, 1974) and BT20 (Lasfargues & Ozzello 1958) are oestrogen receptor-negative (ER-) cells. In particular, BT20 is a cell line classified as ER- by ligand-binding analysis but which expresses an ER mRNA coding for a constitutively active, but not oestradiol-binding ER protein. Cell lines were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM/F12) (Sigma Chemical Co., St Louis, MO) supplemented with 2% fetal calf serum (FCS) (v/v) in T-75 cm² plastic flasks (Corning Industries, Corning, NY), maintained at 37°C in a 5% CO₂ humidified atmosphere and passaged weekly. At the beginning of the experiments, cell lines in exponential growth phase were removed from the flasks with 0.05% trypsin-0.02% EDTA solution.

Cell proliferation experiments in culture

Cells were seeded in a 12-well plate (50 000 cells/well) in DMEM/F12 medium supplemented with 2% FCS. The cells were allowed to attach for 24 h, and the seeding medium was removed and replaced by experimental medium. Cells were maintained for 9 days in DMEM/F12 medium plus 2% FCS supplemented with increasing concentrations (0.5, 1, 2.5, 5 and 7.5 mM) of sodium butyrate (Sigma). The medium was removed and replaced by a fresh one every 3 days. The antiproliferative effect was evaluated in terms of variation of DNA content, as determined by the Burton method (Burton 1956).

Flow cytometric determinations

Cell cycle perturbations and apoptotic cells were analysed 3, 6 and 9 days after NaBu treatment at the concentrations of 1 and 2.5 mM which represent the concentration closest to

IC₅₀ in all cell lines and that able to induce the maximum inhibitory effect, respectively. Cells were washed and harvested by trypsin, and samples of 1×10^6 were stained in a solution containing 50 µg/ml propidium iodide (PI), RNase (100 KU/ml) and 0.05% Nonidet P40 for 30 min at room temperature in the dark.

The fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, San José, CA) equipped with an argon laser at 488 nm wavelength excitation, and 610 nm filter for the PI fluorescence detection. The red (PI) fluorescence signal was collected in linear and logarithmic mode. Data were acquired and processed on LYSIS II software (Becton Dickinson), and a minimum of 10^4 cells was measured for each sample. The percentage of cells in the different cycle phases was evaluated on DNA linear plots by CellFit software according to the SOBR model (Becton Dickinson).

The apoptotic cells showed a diminished PI staining, due to DNA degradation, and were identified as a distinct 'sub-G₁' peak on DNA logarithmic plots.

Analysis of DNA fragmentation

The analysis of DNA fragmentation was performed according to Colotta *et al.* (Colotta *et al.* 1992). Cells were seeded in T-75 cm² plastic flasks (1×10^6 cells/flask) in DMEM/F12 medium supplemented with 2% FCS and, after a 24 h attachment, exposed for 3, 6 and 9 days to 1 and 2.5 mM NaBu. Following exposure to NaBu, the floating cells were collected and adherent cells were removed from the flasks with a trypsin-EDTA solution and collected. After centrifugation at $2000 \times g$ for 15 min at 4°C, the pellets were washed with PBS and centrifuged at $2000 \times g$ for 15 min at 4°C. The pellets were resuspended in 1 ml of lysis buffer (0.5% Triton X-100, 0.5 M EDTA, 1 M Tris-HCl, pH 8.0) and cell lysates were centrifuged at $8000 \times g$ for 20 min at 20°C to separate fragmented DNA (small fragments in the supernatant and large fragments in the pellet). Supernatants were collected and frozen at -20°C and pellets were incubated with 0.8 ml of ETS buffer (0.5 M EDTA, 1 M Tris-HCl, pH 8, 10% *N*-lauroylsarcosine) and 2 µl of proteinase-K (20 mg/ml) (Oncogene Science Inc., Uniondale, NY) overnight at 48°C using a shaker. To remove residual RNA, each sample was treated with 8 µl of RNAase A (10 mg/ml) (Oncogene Science) for 1 h at 37°C. Protein contamination was removed from samples by a phenol-chloroform (1:1) (Sigma) mixture and the obtained DNA was precipitated with two volumes of absolute ethanol (Sigma) overnight, then centrifuged at $8000 \times g$ for 5 min at 4°C. The pellets were resuspended with TES buffer (1 M Tris, 0.5 M EDTA, 10% SDS). DNA fragmentation was analysed by conventional agarose gel electrophoresis at 20 V on 0.8% agarose gel containing ethidium bromide. Lanes were loaded with a suitable volume of extract, and multiple DNA molecular weight (lambda BstE II digest, Boehringer Mannheim, Mannheim, Germany) reference preparations were run in parallel. DNA fragments were visualized under UV light and photographed with a Polaroid 667, ISO 3000/36°.

Statistical analysis

To evaluate the effect of NaBu on cell growth, the differences between means (derived from 6 replicates) were analysed by Student's *t*-test.

RESULTS

Effect of sodium butyrate on cell growth

As shown in Figure 1, NaBu exerted a dose-dependent antiproliferative activity in all cell lines starting from 3 days of treatment, except for T47D cells, which displayed a long lag

Figure 1. Effect of NaBu on growth in various human breast cancer cell lines. Experimental points are the mean ($\pm$ SD) of six replicates. CV was $<5\%$ SD. For details see 'Material and Methods'.

phase probably due to its longer doubling time. After 6 days, a significant inhibition (about 40%, $P < 0.001$) was observed at the concentration of 0.5 mM in MCF7 and at the concentration of 1 mM in all the other cell lines. After 9 days, an inhibition of about 40% was observed even at the lowest concentration in all cell lines. In particular, the IC_{50} was similar in MCF7, MDA-MB231 and T47D (0.5, 0.6 and 0.7 mM, respectively) and was more than two times higher (1.4 mM) in BT20 cells. The antiproliferative effect of NaBu was associated with morphological alterations in three of the four cell lines (data not shown).

Effect of sodium butyrate on the cell cycle

The perturbations induced by NaBu on the distribution of cells in the different cell cycle phases were analysed after 3, 6 and 9 days of treatment with 1 and 2.5 mM concentrations. A G_2M block, more or less evident as a function of NaBu concentration, was observed after 3 days in MCF7, T47D (Figure 2) and BT20, and after 6 days in MDA-MB231 (Figure 3). The G_2M block was accompanied by an increase in the $G_{0/1}$ -phase cell fraction in the two oestrogen-receptor-positive cell lines. At the longest observation time (9 days), the efflux of cells from $G_{0/1}$ to S phase had partially recovered, whereas the G_2M block persisted.

Effect of sodium butyrate on apoptosis

Apoptosis was determined in adherent and floating cells. No evidence of typical DNA cleavage was detected by the gel agarose approach in any adherent cell line. DNA fragmentation was observed in floating cells (Figure 4) from MCF7 after 3, 6 and 9 days at 1 mM and only at the third day after treatment with 2.5 mM; afterwards cytotoxic effect led to cell disruption. In T47D floating cells, DNA disruption was evident at both NaBu concentrations starting from 6 days. Flow cytometric analysis evidenced a 'sub- G_1 ' apoptotic peak (Figure 5) after 3 and 6 days of treatment with 2.5 mM NaBu in T47D but not in MCF7 floating cells.

Figure 2. Perturbations induced by NaBu on the distribution of MCF7 and T47D cells in different cell cycle phases after 3, 6 and 9 days of treatment. ■, control; ▲ 1 mM NaBu and ● 2.5 mM NaBu. Experimental points are the mean ($\pm$ SD) of three independent experiments. CV was $<5\%$ SD.

Figure 3. Perturbations induced by NaBu on the distribution of MDA-MB231 and BT20 cells in different cell cycle phases after 3, 6 and 9 days of treatment. ■, control; ▲ 1 mM NaBu and ● 2.5 mM NaBu. Experimental points are the mean ($\pm$ SD) of three independent experiments. CV was $<5\%$ SD.

Figure 4. Agarose gel electrophoresis in MCF7 and T47D floating cells after 3 (T3), 6 (T6) and 9 (T9) days of treatment. First lane for each time (1), control; second lane (2), 1 mM NaBu; third lane (3), 2.5 mM NaBu; fourth lane (M) for molecular weight standard (range 8.45–225 bp (base pair)).

(data not shown). DNA fragmentation was not detectable by gel agarose or by flow cytometric analysis in MDA-MB231 and BT20 cell lines (data not shown).

DISCUSSION

In the present study, we investigated the effect of NaBu in four human breast cancer cell lines characterized by a different steroid receptor profile. The drug exerted a dose- and time-dependent effect on the growth of all cell lines, thus indicating that a similar antiproliferative effect can be reached with low-dose treatment for a long period or with high-dose treatment for a short period.

Figure 5. DNA content frequency histograms of T47D cells, untreated (a,b) and treated with 2.5 mM NaBu for 3 days (c,e) and 6 days (d,f). Panels c and d represent adherent cells, while panels e and f represent floating cells. Note the position of apoptotic cells as a 'sub-G₁' peak in floating cells (e,f).

The antiproliferative effect of NaBu was associated with morphological alterations in three of the four cell lines. Although, in agreement to other authors (Stevens *et al.* 1984, Guilbaud *et al.* 1990, Planchon *et al.* 1991), we found an oestrogen receptor reduction in both ER+ cell lines (data not shown), we suppose that the down-regulation of steroid receptor content could be ascribable to a general block of protein synthesis, including oestrogen receptor production, rather than to a specific effect on oestrogen machinery.

The actual mechanism by which NaBu affects cellular proliferation remains unclear. Our findings indicate that, in asynchronous cells, NaBu induces significant cell cycle perturbations which result in a persistent G₂M block. Such a block is time- and dose-dependent and is preceded or accompanied by a block of G_{0/1}-S transition. Similar findings have been observed in several cell lines (Ronot, Hecquet & Larho 1986, D'Anna, Toboy & Gurley 1990, Staiano-Coico & Higgins 1990). Moreover, Fallon & Cox (1979) showed that HeLa cells treated with NaBu in early G_{0/1} or G₂M phases were arrested in G_{0/1} phase near the G_{0/1}-S boundary, whereas those exposed to the drug in the S phase were irreversibly blocked in G₂M.

DNA fragmentation was detected by gel electrophoresis in both ER+ cell lines but not in ER- cell lines, and a 'sub-G₁' peak by flow cytometry was detected only in T47D cells. DNA fragmentation, as detected by gel electrophoresis, was associated or consequent to a transient or permanent arrest of cells in G_{0/1} and G₂M phases, whereas a 'sub-G₁' apoptotic peak detected by flow cytometry was observed only after a persistent block in G_{0/1}. Failure to detect apoptosis by gel electrophoresis or flow cytometry in the two ER- cell lines supports the theory that a G₂M block does not necessarily result in apoptosis. However, an activation of the apoptotic process through mechanisms that do not produce DNA fragmentation cannot be excluded (Marini *et al.* 1996).

The interesting results obtained *in vitro* and the supposed lack of side effects due to the presence of NaBu in the normal mucosa of the colon have led to the clinical use of the drug in human malignancies. To our knowledge, NaBu has been used in two clinical studies on leukaemic patients (Novogrodsky *et al.* 1983, Miller *et al.* 1985). In both cases, only partial responses were observed probably due to the very short half-life (about 5 min) of the compound and its rapid clearance, which prevents an adequate concentration in the target cells. It has been demonstrated that intravenous infusion of NaBu at 500 mg/kg body weight per day results in peak blood levels (0.05 mM) (Miller *et al.* 1987) about 10 times lower than that required to induce *in vitro* differentiation of leukaemic cells (Calabresse *et al.* 1993).

Our results are consistent with the clinical findings reported for leukaemic patients. In fact, the growth of breast cancer cell lines was almost completely inhibited at the concentration of 1 mM NaBu. Therefore, high plasma concentrations are required to improve therapeutic clinical results. Such a goal could be reached by esterification of butyric acid with a consequent lower release of the functional group (Raphaelli *et al.* 1991, Pouillart *et al.* 1991). For example, an attempt has been made using tributyrin, a more stable analogue of butyrate found to be a potent inhibitor of cell proliferation even though less effective than sodium butyrate (Feng *et al.* 1996). Another suggestive approach is the encapsulation of the drug into liposomes to avoid drug degradation (Frangos *et al.* 1990, Storm, Wilm & Crommelin 1991, Owen *et al.* 1992).

In conclusion, the inhibiting effect of NaBu in hormone-dependent and independent cell lines and its ability to induce apoptosis through a cell cycle perturbation in hormone-dependent cell lines may have important implications for the treatment of human tumours including breast cancer. At this point, it is mandatory to elucidate mechanisms of NaBu activity by studying interactions on the expression of those molecules with an established or putative role in cell proliferation control (c-myc, cyclin, p53) and apoptosis (bcl-2, bax) in

breast epithelial cells as well as to develop new pharmaceutical approaches in order to improve NaBu stability. Our studies are actively moving in these two directions.

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