Cholesteryl butyrate solid lipid nanoparticles as a butyric acid pro-drug: effects on cell proliferation, cell-cycle distribution and c-*myc* expression in human leukemic cells

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Cholesteryl butyrate solid lipid nanoparticles (chol-but SLN) have been proposed as a pro-drug to deliver butyric acid. We compared the effects on cell growth, cell-cycle distribution and c-*myc* expression of chol-but SLN and sodium butyrate (Na-but) in the human leukemic cell lines Jurkat, U937 and HL-60. In all the cell lines 0.5 and 1.0 mM chol-but SLN provoked a complete block of cell growth. Cell-cycle analysis demonstrated in Jurkat cells that 0.25 mM chol-but SLN caused a pronounced increase of G_2/M cells and a decrease of G_0/G_1 cells, whereas in U937 and HL-60 cells chol-but SLN led to a dose-dependent increase of G_0/G_1 cells, with a decrease of G_2/M cells. In Jurkat and HL-60 cells 0.5 mM chol-but SLN induced a significant increase of sub-G_0/G_1 apoptotic cells. Cell growth and cell-cycle distribution were unaffected by the same concentrations of Na-but. A concentration of 0.25 mM chol-but SLN was able to cause a rapid and transient down-regulation of c-*myc* expression in all the cell lines, whereas 1 mM Na-but caused a slight reduction of c-*myc* expression only in U937 cells. The results show how chol-but SLN affects the proliferation pattern of both myeloid and lymphoid cells to an extent greater than the natural butyrate. *Anti-Cancer Drugs* 15:525–536 © 2004 Lippincott Williams & Wilkins.

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Introduction

Butyric acid, a short-chain fatty acid produced in the mammalian gastrointestinal tract from the bacterial metabolism of ingested soluble fibers, plays a considerable physiologic role in the integrity and function of the colon epithelium. Sodium butyrate (Na-but) causes a variety of morphological and functional cell modifications, which include differentiation induction, blockade at specific stages of the cell cycle and stimulation or inhibition of specific gene expression [1]. It inhibits cancer cell proliferation and induces pathways of cell maturation in a wide panel of neoplastic cell lines (human colorectal, breast, gastric, lung, brain, pancreatic cancers) [2–5]. Although the molecular mechanisms involved in the effects produced by this compound are not yet completely understood, it has been suggested that gene transcription regulation and, in some cases, apoptosis induction may play a major role [6,7]. Several studies suggest that butyric acid modulates the expression of oncogenes like c-*myc*, c-*fos* and c-*ras* [8,9], and induces hyperacetylation of histone by inhibiting histone deacetylases, a finding consistent with butyrate stimulatory effects on gene expression [10].

Na-but-inhibited cell growth is associated with multiple cellular pathways, including induction of cyclin-dependent kinase inhibitor proteins, p21 and p27, and in the case of apoptosis it induces the expression of the pro-apoptotic proteins, Bax, Bak and Bik [11]. Furthermore, activation of these cellular pathways appears to be independent of the tumor suppressor protein, p53. In human myeloma cell lines Na-but causes growth arrest and apoptosis associated with increased expression of p21 and Bax, and with the decrease of IL-6 receptor expression [12]. The efficacy of more stable mannose butyrate esters in inhibiting cell proliferation and in inducing programmed cell death has been analyzed in primary human acute myeloid and chronic lymphocytic leukemia [13,14].

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Treatment with butyric acid may represent an attractive alternative to the conventional chemotherapeutic approach. However, the infrequent application is not due to side-effects or general toxicity, but to the extremely short half-life of butyric salts derivatives, which impairs any long-lasting effect *in vivo* [15–17]. Furthermore the concentrations of butyrate produced in patients given continuous i.v. infusion of sodium butyrate or arginine butyrate are lower than the concentrations required to produce a pharmacodynamic effect *in vitro* [18]. There have been several attempts to overcome the rapid degradation of butyric acid *in vivo* by the synthesis of a monosaccharide stable pro-drug or with the use of butyric acid analogs like tributyrine and isobutyramide, an orally bioavailable active butyrate analog [19].

Among the approaches recently proposed for delivery of long-circulating, slow-release and target-specific antiproliferative compounds, solid lipid nanoparticles (SLN) present several chemical, pharmacological and clinical advantages [20,21]. Cholesteryl butyrate (chol-but), the ester of two natural molecules, cholesterol and butyric acid, used as the lipid matrix of SLN could be a suitable pro-drug to deliver butyric acid. *In vitro* chol-but SLN exert an antiproliferative effect on NIH-460, a non-small cell lung carcinoma cell line. In the same research, fluorescence microscopy showed that 6-coumarin-tagged chol-but SLN were almost completely internalized by cells after 5 min of treatment [2] and the effects were achieved at lower concentration than those seen with the use of Na-but [22]. Furthermore, chol-but SLN were able to induce apoptosis on melanoma cell lines, indeed results show a dose/time-dependent effect in melanoma cell apoptosis induction between 3 and 24 h, and a dose-dependent, but not time-dependent, effect after 24 h of treatment [23].

Human leukemic cell lines have widely been used to study the antiproliferative and the differentiative effects of several chemotherapeutic compounds such as of Na-but [13,15], retinoic acid [24,25] and arabino-furanosyl-cytosine [26,27]. Thus, they represent a suitable model to study the effect of SLN on cell growth, viability and cell-cycle distribution.

The aim of the present study was to evaluate the biological activity of SLN, prepared from warm micro-emulsions, and partly composed of chol-but and of phosphatidylcholine, in comparison with Na-but in three human leukemic cell lines derived from acute T cell leukemia (Jurkat), histiocytic lymphoma (U937) and promyelocytic leukemia (HL-60). In particular we investigated cell-cycle distribution and c-*myc* expression, since previous researchers did not evaluate chol-but SLN for these effects in leukemic cells [22,23].

Methods

Cell culture

Jurkat, U937 (ICLC-CBA, Interlab Cell Line Collection-Advanced Biotechnology Center, Genova, Italy) and HL-60 cell lines (DMS, German Collection of Micro-organisms and Cell Cultures, Braunschweig, Germany) were cultured at 37°C in a humidified atmosphere of 5% CO_2/air in RPMI 1640 medium (Biochrom Seromed, Berlin, Germany), supplemented with 2 mM glutamine, antibiotics and 10% fetal calf serum (Biochrom Seromed).

Preparation of chol-but SLN

Chol-but SLN were prepared by Epikuron 200 (containing about 95% of soy phosphatidylcholine; Lucas Meyer, Hamburg, Germany) and chol-but (Fluka, Buchs, Switzerland). Epikuron 200 (15%) and chol-but (12%) were melted at 85°C and a warm water solution (59%) of sodium taurocholate (PCA, Basaluzzo, Italy) (3%) and butanol (Fluka) (11%) were added to obtain a clear system. The microemulsion was immediately dispersed 1:10 in cold water and washed twice with water by the ultrafiltration system (TCF2A; Amicon Grace, Danvers, MA; membrane Diaflo YM 100; Millipore, Bedford, MA). The dispersion was successively sterilized by autoclaving (15 min at 121°C, 1 atm).

Characterization of chol-but SLN

After sterilization, chol-but SLN mean diameter and polydispersity index were measured by photon correlation spectroscopy using a N4MD instrument (Coulter, Hialeah, FL) at a fixed angle of 90°. The wavelength of the laser light (He/Ne) was 632.8 nm and measurements were carried out at 25°C.

Determination of chol-but concentration

Chol-but concentration was determined by high-pressure liquid chromatography (HPLC). A portion of chol-but SLN sterilized dispersion (50–100 µl) was dissolved in the mobile phase and analyzed. Chromatographic conditions were: a reverse-phase column Water Spherisorb 5 µl ODS2 (4.6 × 250 mm), a LC-10AD pump unit control, a SPD-10A UV detector set at 210 nm and a C-R5A Chromatopac integrator (Shimadzu, Kyoto, Japan). The mobile phase consisted of isopropanol:acetonitrile (40:60). The flow rate was 1.2 ml/min.

Cell growth and cell viability

Growth rate and cell viability were monitored daily by the Trypan blue exclusion test. Cell viability data were expressed as percentage of viable cells treated with Na-but and chol-but SLN for 24, 48, 72 and 96 h.

Jurkat, U937 and HL-60 cells were seeded at 2×10^5/ml and cultured for 4 days. At the beginning of each experiment the culture medium was supplemented with increasing concentrations (0.1, 0.25, 0.5 and 1.0 mM) of

sodium salt of butyric acid (Na-but) and chol-but SLN. Since chol-but is equimolarly composed of cholesterol and butyrate, we also evaluated the effect of 0.1 mM free cholesterol on cell growth. We were unable to assay a higher cholesterol concentration because of the cytotoxicity of the corresponding concentration of ethanol (above 0.1%) used to solubilize the organic compound.

Analysis of DNA content with flow cytometry

Both the antiproliferative effect for Na-but and chol-but SLN, and perturbation cell cycle and apoptotic effects were evaluated on the three cell lines at 24 and 48 h.

Single staining with propidium iodide (PI) enables the identification of apoptotic cells and the definition of the distribution of the whole population in the different cell-cycle phases.

The apoptotic nuclei show a diminished PI staining and can be identified as a distinct 'sub-$G_{0/1}$' peak. Cells (10^6) were pelleted, washed twice with Ca^{2+}/Mg^{2+}-free PBS, fixed in 70% ethanol and stored at 4°C. Prior to analysis, they were permeabilized and stained with PI (50 μg/ml) (Sigma, St Louis, MO) in the presence of RNase A (50 μg/ml) (Sigma) for 30 min at 37°C. Finally, 20 000 cells were acquired in linear mode with a FACSCalibur flow cytometer equipped with an argon laser at 488 nm wavelength excitation on CellQuest software (Becton Dickinson, Sunnyvale, CA). Data were gated on pulse-processed PI signals to exclude doublets and larger aggregates as a standard procedure.

Preparation of fluorescent chol-but SLN

Fluorescent chol-but SLN were prepared according to the above described procedure. In this case the warm microemulsion was prepared adding 6-coumarin (Acros, Morris Plain, NJ) (0.04%). The microemulsion was successively dispersed in cold water, washed by ultrafiltration and sterilized.

Fluorescence microscopy

The cellular uptake of 6-coumarin-tagged chol-but SLN by Jurkat, U937 and HL-60 cells was investigated using fluorescence microscopy. Then, 0.5 mM fluorescent chol-but SLN was added to the culture medium. The cells were removed from the flask before the 6-coumarin-tagged chol-but SLN administration, and after 2, 5, 10, 15 and 30 min, washed with phosphate-buffered saline solution (PBS) and centrifuged at 1000 *g* for 7 min. PI solution (5 μg/ml) was added, and the cell suspension was observed and photographed by DIALUX 20 fluorescence microscopy (Leitz, Wetzlar, Germany). Three separate experiments for all the cell lines were performed and at least 100 cells were counted for each condition made in triplicate.

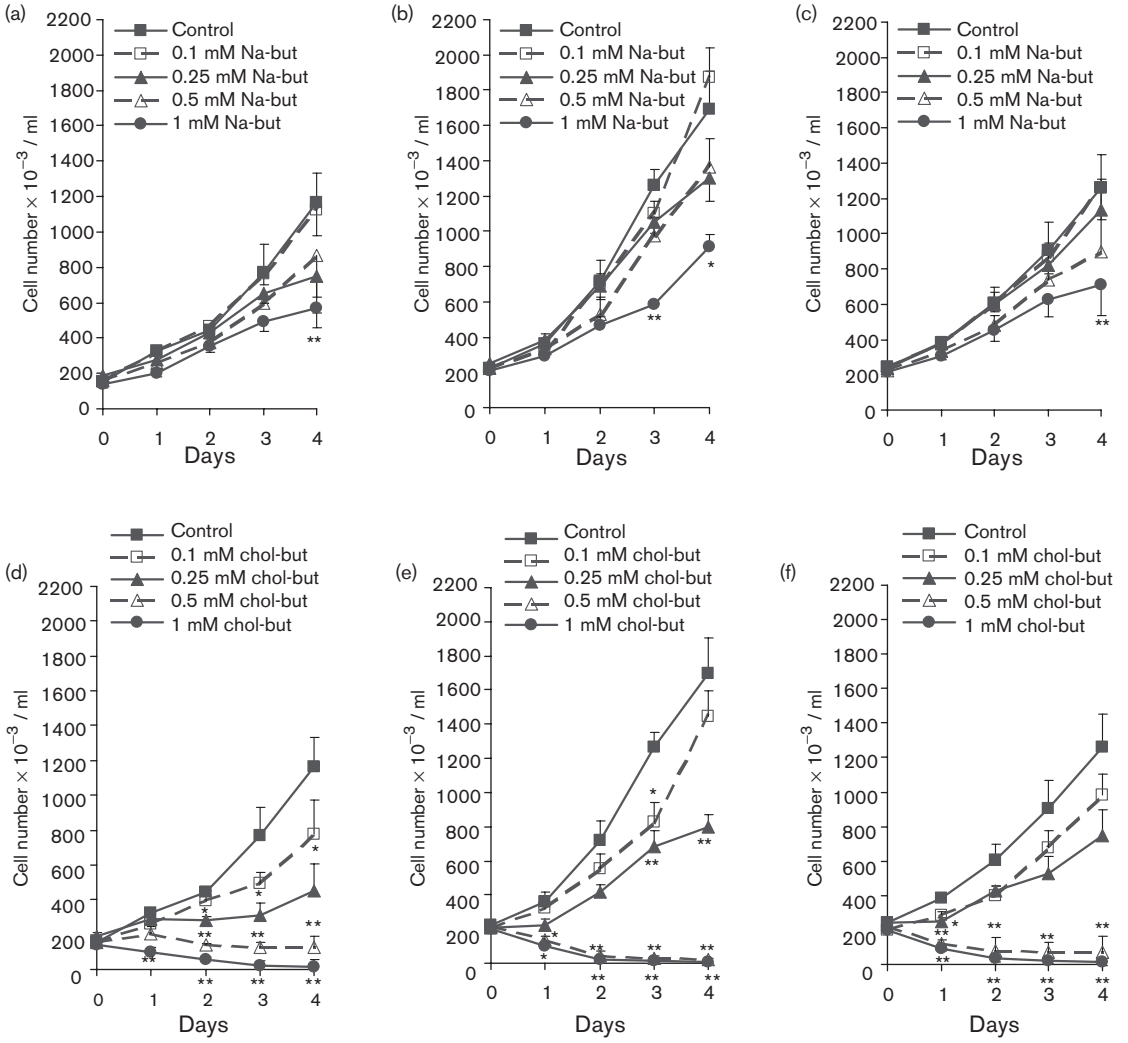
RNA isolation and semi-quantitative RT -PCR analysis

RNA analyses were performed by a semi-quantitative PCR method as previously described [28]. Briefly, the experimental strategy included the following precautions: (i) the number of PCR cycles was kept low in order to obtain an exponential amplification of PCR products; (ii) all results were standardized using the signal obtained with GAPDH; (iii) all experiments were performed with at least three independent cDNA preparations; (iv) to control for DNA contamination, primers were designed to span at least one exon–intron boundary. Total RNA was isolated using the TRIzol Kit (Life Technologies, Milan, Italy). cDNA synthesis was performed with 4 μg of total RNA in a reaction volume of 40 μl containing 4 μl of random primer (50 ng/μl), 0.5 mM of dATP, dGTP, dCTP and dTTP (Invitrogen, San Giuliano Milanese, Italy), 66 U of RNAsin (Promega Italia, Milan, Italy), 8 μl of 5 × first strand buffer, 10 mM DTT, and 200 U of MMLV reverse transcriptase (Invitrogen). Samples were incubated for 1 h at 37°C and the reaction was stopped by heating for 10 min at 95°C. PCR reactions were performed in a GeneAmp PCR System 9600 (Perkin Elmer), with 1 μl of cDNA reaction mixture in a volume of 50 μl containing 200 μM of dATP, dTTP, dGTP and dCTP, 1 μM of 5′ and 3′ primer, and 0.5 U of DNAzyme Taq polymerase (Celbio, Pero, Milan, Italy). Samples were subjected with denaturation at 94°C for 30 s, annealing for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. Negative controls contained water instead of cDNA. The primer pair sequences used for PCR amplification and the numbers of PCR cycles done are indicated as follows. c-*myc*, 28 cycles; (forward primer) 5-GAGACAACGACGGCGGTG-3, (reverse primer) 5-GCTCGTTCCTCCTCTGGC-3, amplifying a 788-bp fragment. GAPDH, 28 cycles; (forward primer) 5-GTCGGAGTCAACGGATTTGG-3, (reverse primer) 5-GGGTGGAATCATATTGGAACATG-3, amplifying a 119-bp fragment. A l0-μl sample of the PCR reaction mixture was separated on a 1% agarose gel and amplification products were stained with GelStar nucleic acid gel staining (FMC BioProducts, Rockland, ME). Densitometric analysis was performed by using the software program Multi-Analyst, version 1.1 (Bio-Rad, Segrate, Italy).

Preparation of total extracts and Western blot analysis

Cells (10×10^6) were washed twice in cold PBS, pH 7.4. Total extracts were prepared by lysis in a buffer containing Tris–HCl buffer, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 0.05% aprotinin. Insoluble proteins were discarded by high-speed centrifugation at 4°C. Protein concentration in the supernatant was measured in triplicate using a commercially available assay (Bio-Rad).

Fig. 1



Cell growth of Jurkat (a and d), U937 (b and e) and HL-60 (c and f) cells. Cells treated with different concentrations from 0.1 to 1.0 mM of Na-but (a–c) and chol-but SLN (d–f). Results are the mean of three separate experiments (±SD). *Significantly different from control values ($p<0.05$). **Significantly different from control values ($p<0.01$).

All proteins were separated by SDS–PAGE and electroblotted on nitrocellulose membrane (Bio-Rad). Membranes were blocked overnight at 4°C in Tris-buffered saline containing 5% milk plus 0.5% Tween 20 and then incubated at room temperature with primary (anti-c-MYC clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA; anti-β-actin, clone AC-15; Sigma, Milan, Italy) and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). Detection was carried out by enhanced chemiluminescence according to the manufacturer's protocol (Amersham-Pharmacia Biotech, Cologno Monzese, Italy). Densitometric analysis was performed by using the software program Multi-Analyst, version 1.1 (Bio-Rad). All results were standardized using the signal obtained with β-actin.

Statistical analysis

Where appropriate, statistical analysis was performed by means of analysis of variance (ANOVA) followed by the Bonferroni post-test.

Results

Chol-but SLN characterization

Sterilized chol-but SLN had an average diameter of 130 nm and a polydispersity index of 0.28. Fluorescent preparation (6-coumarin-tagged chol-but SLN) showed

the same data. Size was also measured before autoclaving, obtaining similar results.

Chol-but concentration determination

The chol-but concentration of both fluorescent and non-fluorescent dispersions of chol-but SLN, determined by HPLC, was 24 mM.

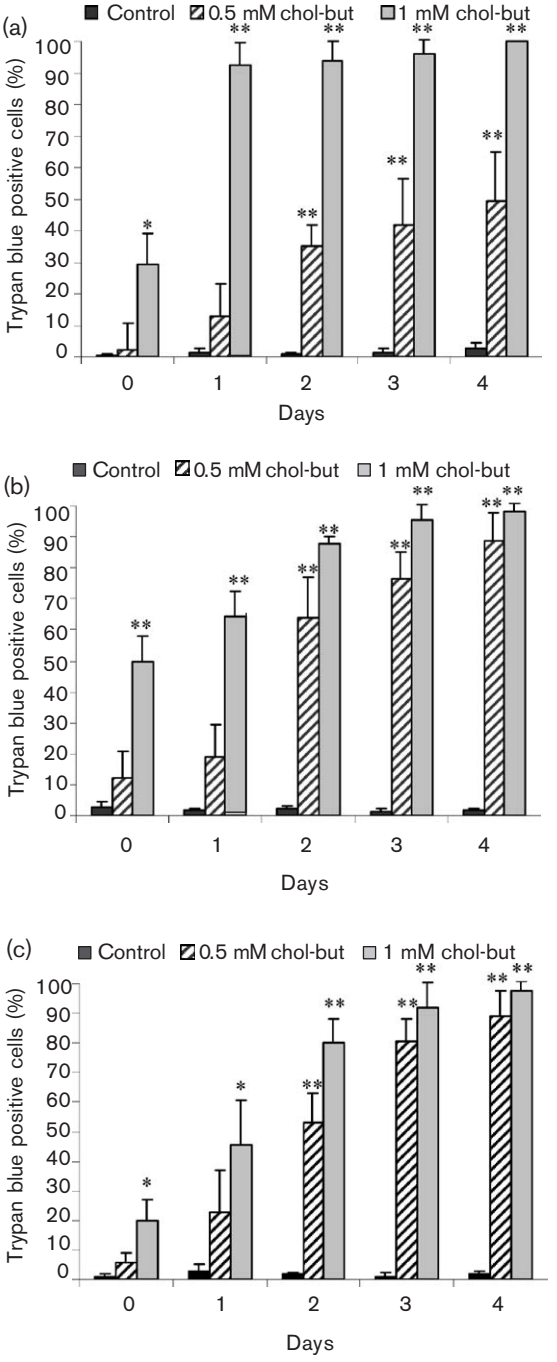
Cell growth and cell viability by Trypan blue uptake

To investigate the antiproliferative effects of Na-but compared with chol-but SLN, we exposed Jurkat, U937 and HL-60 cells to different doses of Na-but and chol-but SLN (0.1, 0.25, 0.5 and 1.0 mM) for 4 days (Fig. 1). A dose of 0.1 mM Na-but was ineffective on cell growth for all the cell lines tested (Fig. 1a–c). A slight, but not significant, reduction of growth rate was observed in Jurkat and U937 cell lines treated with 0.25 and 0.5 mM Na-but (Fig. 1a and b), and in HL-60 cells treated with 0.5 mM Na-but (Fig. 1c). The highest concentration tested of Na-but (1.0 mM), provoked a significant reduction in growth rate in all cell lines (Fig. 1a–c). The Trypan blue dye exclusion test demonstrated that no necrosis occurred at any time, even in cells treated with the highest concentration (1.0 mM) of Na-but (data not shown).

In all the three cell lines treated with chol-but SLN (Fig. 1d–f), a dose-dependent reduction in growth rate was observed after chol-but SLN treatments starting from 0.1 mM concentration. The two highest chol-but SLN concentrations (0.5 and 1.0 mM) provoked a complete block of cell growth in all the cell lines tested (Fig. 1d–f).

The Trypan blue dye exclusion test demonstrated that necrosis occurred after 0.5 and 1.0 mM chol-but SLN treatments (Fig. 2), whereas no necrotic cells were observed after 0.1 and 0.25 mM chol-but SLN treatments (data not shown). In particular, treatment with 0.5 mM chol-but SLN caused a progressive increase of necrotic cells from day 0 to day 4 (from 2.2 to 49.3% in Jurkat cells, Fig. 2a; from 12.4 to 88.5% in U937 cells, Fig. 2b; from 5.8 to 89.3% in HL-60 cells, Fig. 2c). After 1.0 mM chol-but SLN treatment, the percentages of Trypan blue-positive cells at day 1 were 92.3, 64.4 and 45.3% in Jurkat, U937 and HL-60 cells, respectively (Fig. 2a–c). At day 2, with the same chol-but SLN concentration, the mortality was more than 80% in all the lines tested. Cholesterol equimolarly present in chol-but did not affect cell growth at the concentration tested (0.1 mM) in all the cell lines (data not shown).

Fig. 2

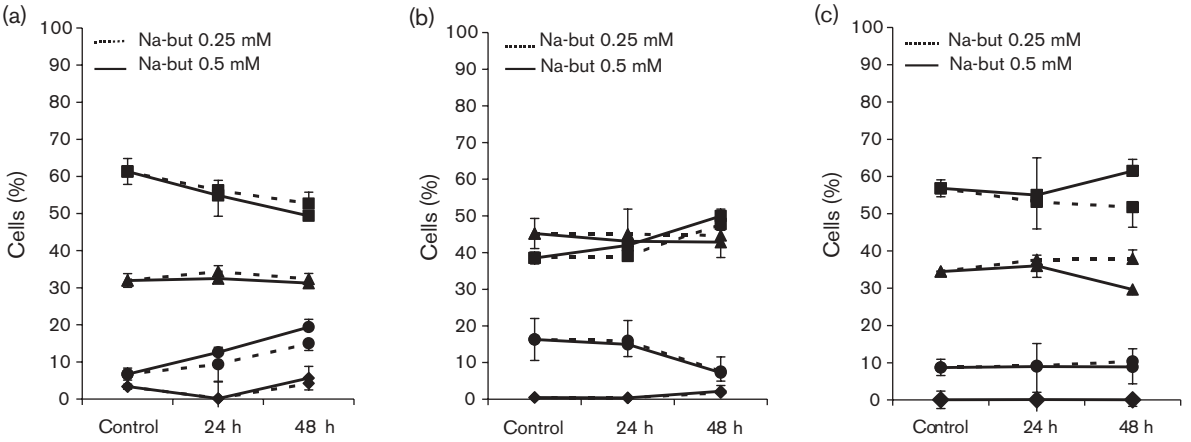


Trypan blue exclusion test of Jurkat (a), U937 (b) and HL-60 (c) cells treated with chol-but SLN at the indicated concentrations. Data are expressed as percentage of positive cells. Results are the mean of three separate experiments (±SD). *Significantly different from control values ($p<0.05$). **Significantly different from control values ($p<0.01$).

Effect of chol-but SLN on apoptosis and cell cycle

The concentrations of chol-but SLN which did not induce a massive necrosis (0.25 and 0.5 mM) were used to assess the apoptosis induction and the cell-cycle distribution. Due to a marked decrease in vitality after 24 h of treatment with 0.5 mM chol-but SLN (Fig. 2), the cell-cycle distribution at 48 h has not been analyzed. In control cells, the proportion of cells in each phase of

Fig. 3



Conditions		Percentage of Jurkat cells				Percentage of U937 cells				Percentage of HL-60 cells			
		G_0/G_1	S	G_2/M	sub-G_0/G_1	G_0/G_1	S	G_2/M	sub-G_0/G_1	G_0/G_1	S	G_2/M	sub-G_0/G_1
Control	24 h	61.3±3.5	31.9±1.8	6.7±1.6	3.3±0.7	38.5±1.6	45.2±4.1	16.3±5.7	0.5±0.1	56.8±2.3	34.5±0.1	8.7±2.2	0.5±0.1
	48 h	59.0±2.7	30.3±1.2	10.7±0.9	1.0±0.3	49.2±2.1	43.8±1.9	7.0±0.8	1.6±1.2	52.4±2.9	37.3±0.8	10.3±1.5	1.6±1.2
Na-but 0.25 mM	24 h	56.3±2.7	34.4±1.6	9.4±1.3	0.2±2.3	39.1±1.2	45.0±6.8	15.9±5.5	0.4±0.0	53.2±7.2	37.7±1.2	9.2±6.0	0.4±0.0
	48 h	52.7±3.1	32.3±1.5	15±2.1	4.2±1.7	47.8±1.7	44.7±3.6	7.5±4.0	1.8±0.2	51.8±5.4	37.9±2.4	10.4±3.4	1.8±0.2
Na-but 0.50 mM	24 h	55.0±5.6	32.5±0.8	12.6±4.8	0.1±4.7	42.0±1.9	43.1±5.2	14.9±3.3	0.4±0.0	55±10.0	36.1±3.1	9.0±7.0	0.4±0.0
	48 h	49.4±0.8	31.2±1.1	19.4±1.9	5.6±3.2	50.0±1.9	42.8±4.2	7.2±2.3	2.2±1.5	61.5±3.1	29.6±1.4	8.9±4.5	2.2±1.5

Effect of Na-but (a–c) on cell-cycle distribution of Jurkat (a), U937 (b) and HL-60 (c) cells. Cells stained with propidium iodide as described in Material and methods and analyzed by flow cytometry. G_0/G_1, squares; S, triangles; G_2/M, circles; sub-G_0/G_1, diamonds. Data expressed as percentage of cells in the different phases of the cell cycle. Results are the mean of three separate experiments (±SD).

the cell cycle did not change appreciably between 24 and 48 h. Little or no effect was observed on cell-cycle distribution after Na-but exposure in the three cell lines. Na-but at 0.25 and 0.5 mM caused a dose-dependent decrease of G_0/G_1 cells in the Jurkat cells and a corresponding increase of G_2/M cells (Fig. 3a). Whereas the same concentrations of Na-but in the U937 cells exerted a slight increase in the percentage of cells in the G_0/G_1 phase and a decrease in the G_2/M phase after 24 h (Fig. 3b). The highest concentration of Na-but in HL-60 cells caused an increase in the G_0/G_1 cells and a decrease in the G_2/M cells at 48 h (Fig. 3c). In the three cell lines treated with 0.25 and 0.5 mM Na-but, flow cytometry did not detect a cell population characterized by a subdiploid DNA fluorescence which correlated with the internucleosomal DNA fragmentation typical of apoptosis.

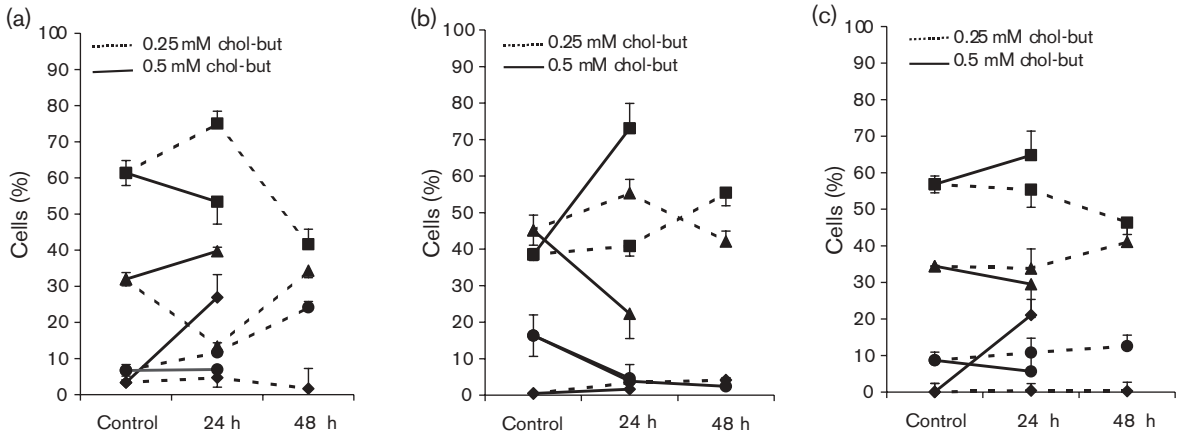
In the Jurkat cells, 0.25 mM chol-but SLN caused an increase in the G_0/G_1 cells at 24 h and a reduction of the $S + G_2/M$ population which correlated with the decrease of cell proliferation (Fig. 4a). The arrest and accumulation of chol-but SLN-treated cells in the G_0/G_1 phase was short-lived; in fact, after 48 h, the proportion of G_0/G_1 cells decreased and a marked accumulation of cells in G_2/M phase occurred, parallel with a decrease of cells in the G_0/G_1 phase. Chol-but SLN at 0.5 mM induced a significant increase of cells having sub-G_0/G_1 DNA content (apoptotic cells) at 24 h (Fig. 4a).

In the U937 cells, the exposure to 0.25 and 0.5 mM chol-but SLN led to a progressive and dose-dependent increase of G_0/G_1 cells, with a decrease of G_2/M-phase cells (Fig. 4b). Chol-but SLN at 0.5 mM led to an increase of cells in the G_0/G_1 phase and at the same time a slight reduction of the percentage of HL-60 cells in the G_2/M phase. Moreover, this concentration provoked a marked increase in the percentage of apoptotic cells (Fig. 4c).

Cellular uptake of fluorescent chol-but SLN

The uptake of 6-coumarin-tagged chol-but SLN by Jurkat, U937 and HL-60 cells was investigated by fluorescence microscopy. With respect to negative control

Fig. 4



Conditions		Percentage of Jurkat cells				Percentage of U937 cells				Percentage of HI-60 cells			
		G_0/G_1	S	G_2/M	sub-G_0/G_1	G_0/G_1	S	G_2/M	sub-G_0/G_1	G_0/G_1	S	G_2/M	sub-G_0/G_1
Control	24 h	61.3±3.5	31.9±1.8	6.7±1.6	3.3±0.7	38.5±1.6	45.2±4.1	16.3±5.7	0.5±0.1	56.8±2.3	34.5±0.1	8.7±2.2	0.0±2.3
	48 h	59.0±2.7	30.3±1.2	10.7±0.9	1.0±0.3	49.2±2.1	43.8±1.9	7.0±0.8	1.6±1.2	52.4±2.9	37.3±0.8	10.3±1.5	0.0±1.1
Chol-but 0.25 mM	24 h	75.0±3.4	13.3±2.6	11.6±2.7	4.6±2.3	40.8±2.7	55.3±3.8	3.8±4.9	3.4±0.6	55.4±4.8	33.8±5.3	10.8±0.5	0.4±1.9
	48 h	41.6±4.3	34.3±2.0	24.1±1.7	1.6±5.6	55.4±3.6	42.1±2.8	2.4±2.4	4.1±0.2	46.3±3.2	41.1±4.5	12.6±3.0	0.3±2.4
Chol-but 0.50 mM	24 h	53.4±6.2	39.7±1.1	6.9±4.9	26.9±6.3	73.1±6.8	22.3±6.8	4.6±0.0	1.6±0.2	64.8±6.6	29.5±9.1	5.6±7.5	21.1±4.2

Effect of chol-but SLN (a–c) on cell-cycle distribution of Jurkat (a), U937 (b) and HL-60 (c) cells. Cells stained with propidium iodide as described in Material and methods and analyzed by flow cytometry. G_0/G_1, squares; S, triangles; G_2/M, circles; sub-G_0/G_1, diamonds. Data expressed as percentage of cells in the different phases of the cell cycle. Results are the mean of three separate experiments (± SD).

(cells with PI only, visualized in red color) 6-coumarin-tagged chol-but SLN (visualized in green color) appeared almost completely internalized by the cells (in more than 80%) after 2 min of incubation (data not shown).

Effect of chol-but SLN on *c-myc* expression

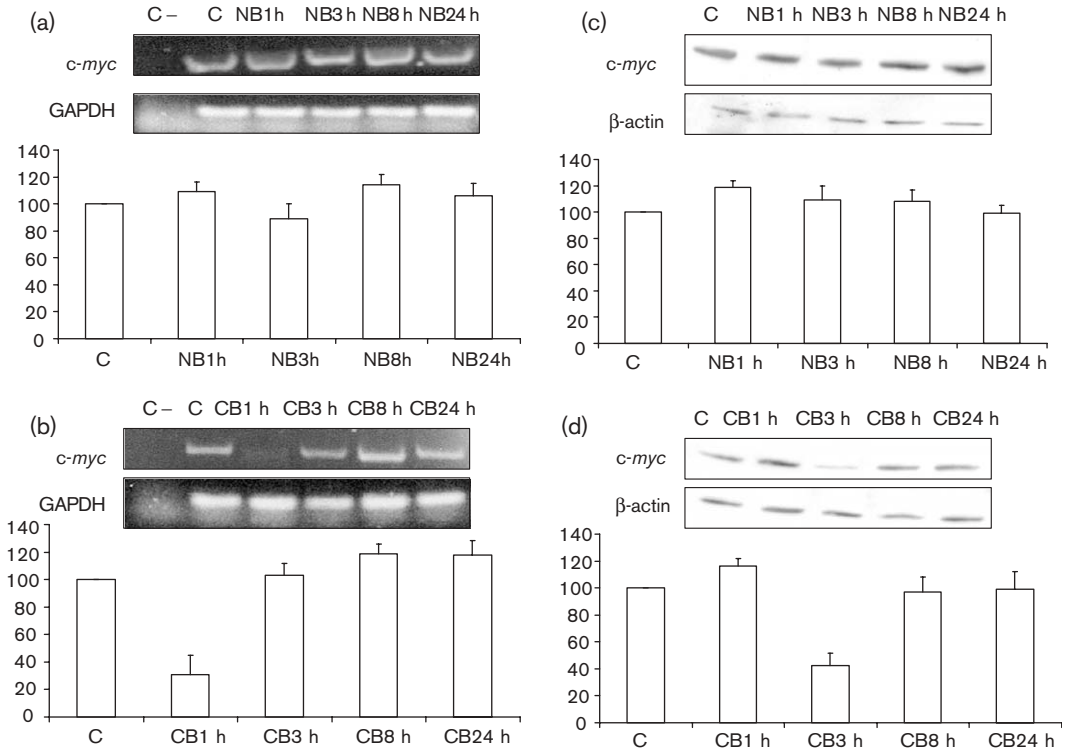
Figures 5–7 show the effect of 1 mM Na-But and 0.25 mM. chol-but SLN on c-*myc* expression in Jurkat, U937 and HL-60 cell lines, respectively. These doses were chosen because they were able to affect cell growth, without apoptotic or necrotic effects. Na-but at 1 mM did not affect mRNA and protein c-*myc* expression in Jurkat (Fig. 5a and c) and HL-60 cell lines (Fig. 7a and c). Only a slight inhibition (less than 50%) of c-*myc* expression was observed in U937 cells after 1 mM Na-but (Fig. 6a and c). The effect of Na-but on c-*myc* expression was transient, mRNA levels were affected after 1 h than returned to the control levels, whereas c-MYC protein decreased after 3 h and then returned to the control levels.

Chol-but SLN at 0.25 mM was able to inhibit (by 40%) c-*myc* mRNA and protein expression in all the three cell lines tested (Figs 5–7b and d). The inhibition of mRNA was observed after 1 h and the inhibition of protein expression at 3 h. The effect was transient and the c-*myc* expression similarly returned to the control values within 8 h.

Discussion

Notwithstanding long-known observations and numerous *in vitro* studies, Na-but or other butyrate derivatives have been rarely employed in hematological malignancies [29]. This infrequent application is not due to side-effects or general toxicity, but to the extremely short half-life of butyrate salt derivatives, which impairs any long-lasting effect *in vivo* and compels a continuous i.v. administration regimen to maintain acceptable plasma levels [13,15,16]. Butyric acid plasma concentrations were increased 6-fold over the endogenous level of 6–8 μM and reached 39–59 μM. In comparison, biologically active concentrations generally reported for *in vitro* studies are at least 20 times higher. Despite these experiences, the interest in butyrate has not declined, and recent phase I trials have explored the possibility of treating high-risk

Fig. 5



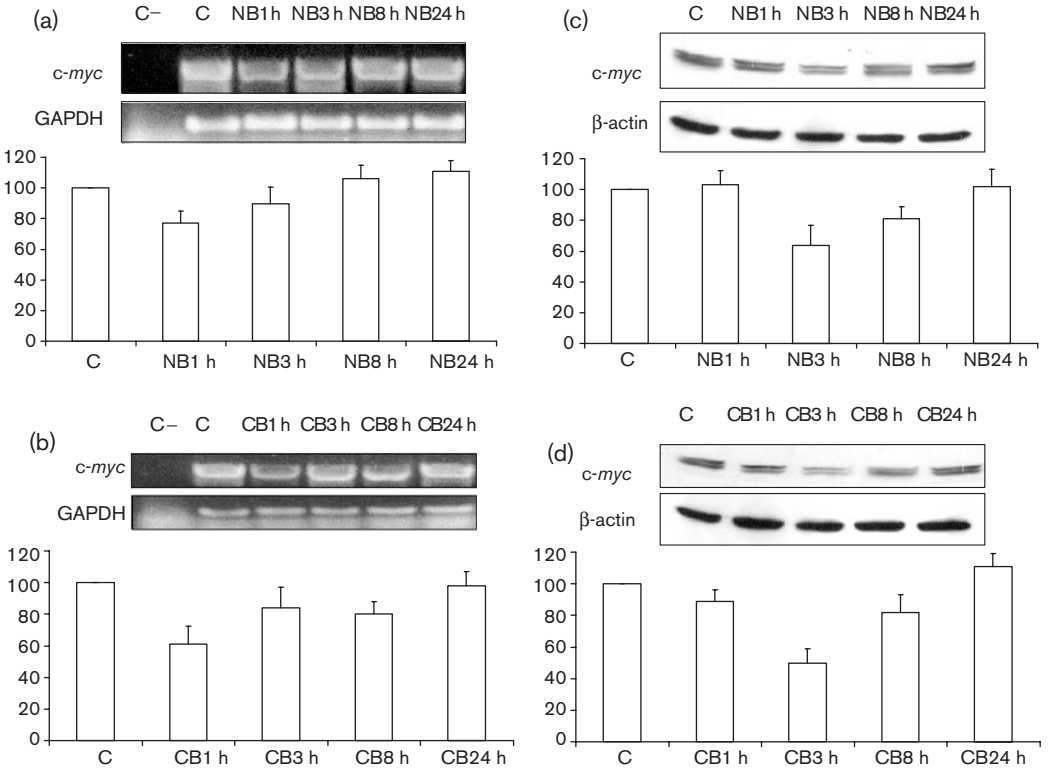
Chol-but SLN down-regulates c-*myc* expression in Jurkat cells. c-*myc* mRNA levels were determined by RT-PCR in Jurkat cells treated with 1 mM Na-but (NB) (a) and 0.25 mM chol-but SLN (CB) (b), collected at the indicated times after the beginning of treatment and the relative densitometric values are shown. Data are normalized using the GAPDH signal and represent the mean of three independent experiments (±SD). Values are expressed as percent of control value (C). To check for DNA contamination, samples without cDNA were also amplified (C–). c-MYC protein levels were determined by Western blot in Jurkat cells treated with 1 mM Na-but (NB) (c) and 0.25 mM chol-but SLN (CB) (d), collected at the indicated times after the beginning of treatment and the relative densitometric values are shown. Equal protein loading was confirmed by exposure of the membranes to the anti-β-actin antibody. Data are normalized using the β-actin signal and are indicated as mean of three independent experiments (±SD) and are expressed as percent of control value (C).

myelodysplastic syndromes and AML with sodium phenylbutyrate. In almost all the subjects treated an improvement in some hematological parameters has been observed, showing a significant clinical activity of sodium phenylbutyrate, with no toxicity [13]. Over the past decade, several approaches have been addressed to achieve effective concentrations of butyrate *in vivo*, as well as reduce metabolic side-effects of associated cations. Sodium salts of butyric acid, arginine, butyril-cAMP, phenylbutyrates, acetates of butyric esters and butyric amides have been used with different frequency *in vitro* and *in vivo* [18].

Efforts have been made to devise a pro-drug of butyric acid that can be hydrolyzed to achieve effective whole concentrations *in vitro* and *in vivo* over a sustained period of time. Tributyrin, a triacylglycerol form of butyric acid, is found to be about 4-fold more potent than butyrate sodium salt in the induction of HL-60 cell differentiation [30]. Pivalyloxymethyl butyrate (AN-9) affects cells at about a 10-fold lower concentration and at a faster rate than does butyric acid. Propionate, isobutyrate and valerate esters, as well as isobutyryloxymethyl butyrate, have been prepared, but they do not elicit effects similar to those of AN-9 [31]. The amphiphile monoacetonide structure of monobutyric esters derived from glucose (MAG-3But) and mannose (MAM-1But) has good affinity for the tumor cell membrane, allowing a beneficial antineoplastic effect *in vitro* and *in vivo* [32]. The numerous studies conducted with butyric acid delivery systems, such as through the use of a pro-drug, suggest the feasibility of infusion-free administration, consistent with long-term chronic therapy [18].

SLN present several advantages as a drug-delivery system such as the use of chol-but as a butyric acid pro-drug, the small and colloidal size of the SLN, and the possibility of being a suitable tool for several *in vivo* administration routes, such as the ocular, i.v. or duodenal route [33]. Furthermore, in previous animal studies we have shown that the incorporation of anticancer drugs in SLN can change their pharmacokinetic profile with an increase in

Fig. 6



Chol-but SLN down-regulates c-*myc* expression in U937 cells. c-*myc* mRNA levels were determined by RT-PCR in U937 cells treated with 1 mM Na-but (NB) (a) and 0.25 mM chol-but SLN (CB) (b), collected at the indicated times after the beginning of treatment and the relative densitometric values are shown. Data are normalized using the GAPDH signal and represent the mean of three independent experiments (± SD). Values are expressed as percent of control value (C). To check for DNA contamination, samples without cDNA were also amplified (C–). c-MYC protein levels were determined by Western blot in U937 cells treated with 1 mM Na-but (NB) (c) and 0.25 mM chol-but SLN (CB) (d), collected at the indicated times after the beginning of treatment and the relative densitometric values are shown. Equal protein loading was confirmed by exposure of the membranes to the anti-β-actin antibody. Data are normalized using the β-actin signal and are indicated as mean of three independent experiments (± SD) and are expressed as percent of control value (C).

half-life, area under the concentration versus time curve (AUC), peak plasma concentration and a concomitant decrease of the clearance [34]. We also observed an improvement in oral bioavailability of idarubicin and doxorubicin-loaded SLN administered duodenally and a significant change in the biodistribution of the drug with the passage through the blood–brain barrier [35,36].

Based on this and other previous *in vitro* [2,23] and *in vivo* studies, we were interested in evaluating the anti-leukemic activity *in vitro* of chol-but SLN in order to investigate whether chol-but SLN was endowed with a major effect on leukemic cell growth compared with Na-but. The experiments were designed in accordance with the reports of *in vitro* effects (differentiation and cell death) of butyrate in myeloid and lymphoid model systems that depend on prolonged exposure to a minimum concentration of the agent (0.5–5.0 mM) [27].

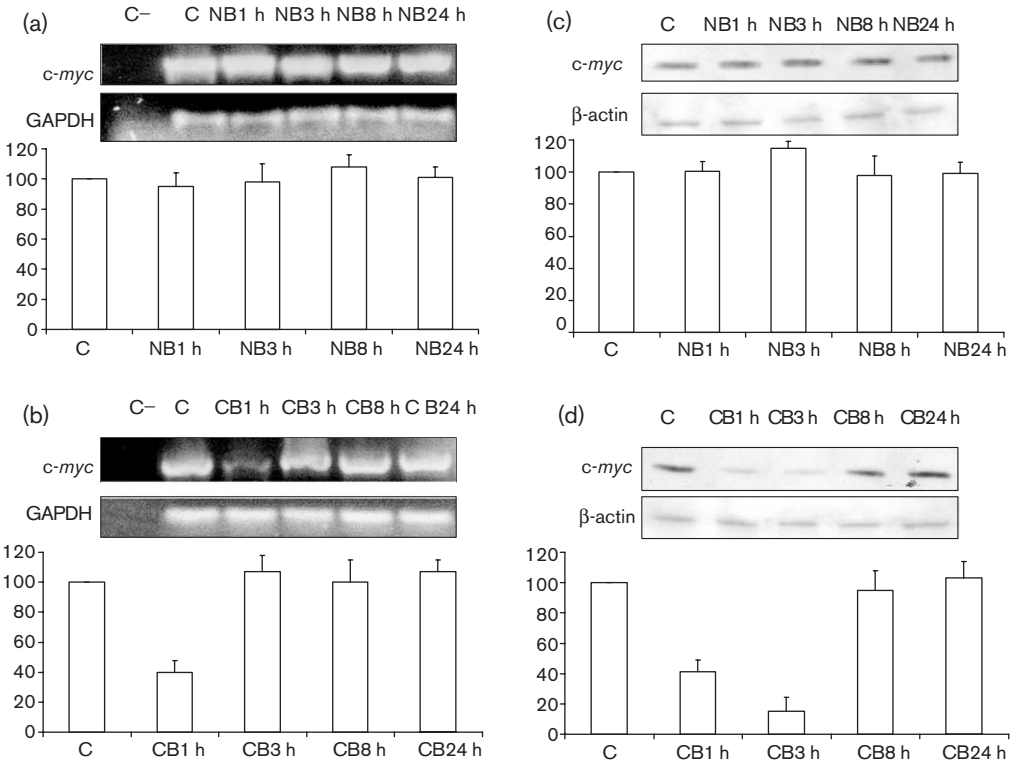
The results presented herein show that chol-but SLN inhibits Jurkat, U937 and HL-60 cell growth to a greater extent than Na-but.

The arrest in the G_1 phase of the cell cycle was observed in the myeloid model (HL-60 and U937) and in the G_2/M phase in the lymphoid model (Jurkat) treated with chol-but SLN. Moreover, we observed a dose-dependent apoptosis in the HL-60 and Jurkat cells at 24 h, and programmed cell death was paralleled by a significant inhibition of cell proliferation.

The cell-cycle arrest in G_2/M was unexpected, because in most cells investigated butyrate caused a block in the G_0/G_1 phase, as reported from several authors. In fact, butyrate-induced G_0/G_1 arrest has been ascribed to c-*myc* repression and/or $p21^{WAF1}$ up-regulation [31].

However, other authors found a different G_2/M block, suggesting that the induction of $p21^{WAF1}$ by Na-but leads

Fig. 7



Chol-but SLN down-regulates c-*myc* expression in HL-60 cells. c-*myc* mRNA levels were determined by RT-PCR in HL-60 cells treated with 1 mM Na-but (NB) (a) and 0.25 mM chol-but SLN (CB) (b), collected at the indicated times after the beginning of treatment and the relative densitometric values are shown. Data are normalized using the GAPDH signal and represent the mean of three independent experiments (±SD). Values are expressed as percent of control value (C). To check for DNA contamination, samples without cDNA were also amplified (C–). c-MYC protein levels were determined by Western blot in HL-60 cells treated with 1 mM Na-but (NB) (c) and 0.25 mM chol-but SLN (CB) (d), collected at the indicated times after the beginning of treatment and the relative densitometric values are shown. Equal protein loading was confirmed by exposure of the membranes to the anti-β-actin antibody. Data are normalized using the β-actin signal and are indicated as mean of three independent experiments (±SD) and are expressed as percent of control value (C).

to the inhibition of the sequential activation of cyclin A- and B_1-dependent kinases, resulting in the inhibition of G_2 progression [23].

In the human CCRF-CEM cell line, acute T lymphoblastic leukemia cells, butyrate inhibited cell proliferation, accumulated cells in the G_2/M phase of the cell cycle and induced typical apoptotic cell death. This effect has been ascribed to a deletion of the cell-cycle inhibitor $p16^{INK4A}$ in these cells [37]. However, other mechanisms may be involved.

In our experimental system Na-but (0.25 and 0.5 mM) did not show any apoptotic effect in the three cell lines tested, since we used concentrations lower than 2–5 mM, which are the levels able to induce apoptosis in several cell lines, whereas 0.5 mM chol-but SLN induced apoptosis in HL-60 and Jurkat, but not in U937 cells. In agreement with our results, other authors observed that apoptosis may be involved or not by using the same compound (butyrate or its derivatives) in different cell lines [6,11,38]. An explanation to understanding the difference in leukemic cell susceptibility to undergo apoptosis may be their proliferative characteristics. The decision to undergo apoptosis is a cell-cycle-dependent event. Induction of leukemic cell differentiation towards neutrophils or monocytes may also trigger the apoptotic process as a consequence of the differentiation process [38].

As we have shown, c-*myc* expression was rapidly down-regulated both in terms of mRNA and protein levels with 0.25 mM chol-but SLN in all three leukemic cell lines. Moreover, there was an increase in the level of c-*myc* mRNA and protein after 8 h of incubation that may have been due to the growth of cells that were resistant to the effect of this concentration of butyric acid incorporated in chol-but SLN. Na-but at 1 mM was unable to influence c-*myc* expression, although a slight, but not significant, down-regulation was observed in U937 cell lines.

In this study we noted that c-*myc* expression was rapidly down-regulated in all the three cell lines by chol-but SLN irrespective of whether or not apoptosis was induced and the chol-but SLN cell-cycle effects were achieved after 24 h. In the HL-60 cell line, Barrera *et al.* observed after treatment with HNE (4-hydroxynonenal), a product of lipid peroxidation, a rapid and transient c-*myc* down-regulation prior to the cell growth arrest [39]. Moreover recent studies confirm that Na-but down-regulation of c-*myc* oncogene is reversible in 180 min [40]. Therefore we speculate in agreement with the experimental data of other authors that c-*myc* is an early gene modulator certainly related to a cascade of phenomena that lead to growth inhibition [38,41]. Our results show how the c-*myc* effect of chol-but SLN was evident at lower concentrations than those reported in the literature with the use of Na-but [18,31].

Our data showed that chol-but SLN inhibit growth and induce apoptosis in human leukemic cell lines to an extent greater than that of concentrations of natural butyrate. Their biological effects are achieved at lower concentrations and after a lower incubation time than those seen with the use of Na-but. This higher biological activity may depend on the very fast internalization of chol-but SLN into the cells as shown with the 6-coumarin-tagged chol-but SLN, likely due to their small size and composition.

In conclusion, we think that the higher sensitivity of the cells to chol-but SLN compared to the free butyric acid may be related to the marked uptake and accumulation of chol-but SLN in the cells; the SLN releases the drug, so enhancing its action. Further studies are in progress in our laboratory to develop a method to measure butyrate concentration inside the cells and in the blood to verify the ability of chol-but SLN to release butyric acid.

Acknowledgments

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