

Cholesteryl butyrate solid lipid nanoparticles inhibit adhesion of human neutrophils to endothelial cells

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1 Adhesion of polymorphonuclear cells (PMNs) to vascular endothelial cells (EC) is a critical step in recruitment and infiltration of leukocytes into tissues during inflammation. High doses of butyric acid have been shown to ameliorate inflammation in inflammatory bowel diseases (IBD). Cholesteryl-butyrate solid lipid nanoparticles (chol-but SLN) as prodrug are a possible delivery system for butyric acid.

2 Sodium butyrate or chol-but SLN were coincubated with human PMNs and human umbilical vein EC (HUVEC); adhesion was quantified by computerized microimaging fluorescence analysis. Both chol-but SLN and sodium butyrate displayed antiadhesive effects on FMLP- and IL-1 β -stimulated cells in a concentration–response curve (10^{-8} – 10^{-5} M), but chol-but SLN were in all cases more active. Moreover, chol-but SLN inhibited FMLP-induced adhesion of PMNs to FCS-coated plastic wells, thus showing a direct effect on PMNs, while sodium butyrate had little effect. Confocal microscopy showed that fluorescent SLN entered PMNs and HUVEC after 10 min incubation. Chol-but SLN acted either on activated PMN or HUVEC.

3 Chol-but SLN inhibited O $_2^{\cdot -}$ production and myeloperoxidase release by PMNs evoked by FMLP, in a dose-dependent, but not time-dependent, manner and were more active than sodium butyrate.

4 In conclusion, in all tests chol-but SLN were more active than sodium butyrate. Thus, chol-but SLN might be a valid alternative to sodium butyrate in the anti-inflammatory therapy of ulcerative colitis, avoiding complications related to the administration of sodium butyrate.

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Abbreviations: HUVEC, human umbilical vein endothelial cells; PMNs, polymorphonuclear cells; SLN, solid lipid nanoparticles

Introduction

Migration of leukocytes from the blood stream to sites of infection is a key event in cellular immune and inflammatory responses, mediated by numerous types of molecules including several adhesion receptors (Bendas, 2005). Dysregulation, that is responding to autogenous or nonthreatening agents, can lead to the uncontrolled infiltration of leukocytes into healthy tissue, and such a mechanism contributes, in part, to the pathology of certain inflammatory and autoimmune reactions, such as rheumatoid arthritis, asthma, multiple sclerosis, Crohn's disease, Ulcerative Colitis (UC), psoriasis and many others (Bendas, 2005).

Enteric bacteria do not generally induce a destructive inflammatory response in the gut, and products of bacterial fermentation are known to play an active and beneficial role in epithelial colonic homeostasis. Short-chain fatty acids (SCFAs: acetate, propionate and *n*-butyrate) derived from the enteric bacterial fermentation of nondigested dietary carbohydrates provide an essential energy substrate for the

colonic epithelium: a deficiency of SCFA can trigger the development of diversion colitis (Dyer *et al.*, 2005), resulting from diversion of the fecal stream and inducing crypt abscesses, epithelial cell degeneration, acute and chronic inflammation in the lamina propria, and regenerative changes in the crypts (Glotzer *et al.*, 1981).

Among SCFAs, butyrate is particularly interesting owing to its anti-inflammatory effects, studied in various *in vitro* systems (Segain *et al.*, 2000; Klampfer *et al.*, 2003; Menzel *et al.*, 2004; Scheppach & Weiler, 2004; Zapolska-Downar *et al.*, 2004). Butyrate enemas were found to be cytoprotective in experimental rat colitis (D'Argenio *et al.*, 1996; Venkatraman *et al.*, 2003). Dietary fiber supplementation ameliorated colonic damage in HLA-B7 transgenic rats and this effect was associated with an increased production of SCFAs, which can synergistically inhibit the production of proinflammatory mediators (Rodriguez-Cabezas *et al.*, 2003). A double-blind multicenter clinical trial demonstrated that combined treatment with topical butyrate and 5-ASA is significantly more effective than 5-ASA alone in the management of refractory distal colitis (Vernia *et al.*, 2003). Another pilot study showed that patients with quiescent UC can safely take a diet rich in

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oat bran, specifically to increase the fecal butyrate levels (Hallert *et al.*, 2003).

Unfortunately, the clinical use of sodium butyrate is limited by its short half-life, owing to its rapid metabolism and excretion (Pellizzaro *et al.*, 1999). The requirement for large intravenous doses of the drug means that continuous administration is needed to produce therapeutic blood concentrations. In addition, anemia, headache, nausea, diarrhea, abdominal cramps and a strong odor limit patient compliance (Conley *et al.*, 1998; Patnaik *et al.*, 2002). Prodrugs, such as tributyrin or Pivanex, have been produced to partially overcome some of these problems, and are now in phase I or II clinical trials for their antitumoral effects (Edelman *et al.*, 2003; Reid *et al.*, 2004), but additional studies are needed to further reduce these side effects.

Solid lipid nanoparticles (SLN) are being extensively studied as promising alternative carriers for drugs and diagnostics (Westesen *et al.*, 1997; Gasco, 2001; Manjunath *et al.*, 2005). SLN, prepared from a warm microemulsion and carrying a drug (either hydrophilic or lipophilic) have been tested *in vivo* on laboratory animals *via* duodenal, intravenous and ocular administration. SLN can increase bioavailability and modify pharmacokinetic parameters and tissue distribution of the incorporated drug. Normally, the lipophilic matrix consists of fatty acids and/or triglycerides (Gasco, 2001). Cholesteryl butyrate (chol-but SLN) was employed as SLN lipid matrix (Gasco, 2004) in tests to evaluate its function as a prodrug of butyric acid. In several tumor cell-lines, chol-but SLN displayed a higher antineoplastic effect than butyrate (Pellizzaro *et al.*, 1999; Salomone *et al.*, 2001; Serpe *et al.*, 2004) and fluorescent SLN showed chol-but SLN to be internalized within a few minutes (Pellizzaro *et al.*, 1999; Salomone *et al.*, 2001).

Part of sodium butyrate's activity may be ascribed to a direct effect on leukocytes and microvascular endothelial cells (EC) that are crucial for leukocyte extravasation during the inflammatory response. Indeed, sodium butyrate and other SCFAs exert multiple effects on leukocytes, particularly polymorphonuclear cells (PMNs), altering cytoplasmic pH, calcium concentration, oxygen metabolism, phagocytosis, cell proliferation, cytoskeletal actin distribution and chemotaxis (Eftimiadi *et al.*, 1987; Brunkhorst *et al.*, 1992). These effects may partly be due to sodium butyrate's capacity to interact with the GPR43 receptor (Brown *et al.*, 2003; Le Poul *et al.*, 2003; Nilsson *et al.*, 2003; Xiong *et al.*, 2004), a G-protein-coupled receptor that is highly expressed in PMNs and monocytes (Le Poul *et al.*, 2003; Senga *et al.*, 2003). By contrast, GPR41, another G-protein-coupled receptor that can interact with sodium butyrate, is more widely expressed (Brown *et al.*, 2003), but not present on PMNs or monocytes (Le Poul *et al.*, 2003). Moreover, sodium butyrate may also modulate inflammation by a direct effect on human intestinal microvascular EC, in which it can modulate gene transcription by inhibiting histone deacetylase (Ogawa *et al.*, 2003).

The aim of this study was to compare the effect of sodium butyrate and that of chol-but SLN on PMN function, by assessing their capacity to modulate adhesion to EC, superoxide anion ($O_2^{\cdot-}$) production, and myeloperoxidase release *in vitro*. The results showed chol-but SLN to inhibit all PMN activities and to be much more active than sodium butyrate.

Methods

Cell cultures and reagents

Dextran T500 was from Pharmacia Biotech (Uppsala, Sweden). Bovine calf serum (BCS, endotoxin tested) was from Hyclone Laboratories Inc. (Logan, UT, U.S.A.). Trypsin was from Difco Laboratories Inc., Detroit, MI, U.S.A. Histopaque®1077, fluorescein diacetate, M199 (endotoxin tested), *n*-FMLP, IL-1 β , PMA, cytochrome *c*, superoxide dismutase, cytochalasin B, MTT and HSA were from Sigma-Aldrich (St Louis, MO, U.S.A.).

Epikuron 200 (soya phosphatidylcholine 95%) and sodium taurocholate were kind gifts from Lucas Meyer (Hamburg, Germany) and from PCA (Basaluzzo, Italy), respectively. Cholesteryl butyrate, sodium butyrate and butanol were from Fluka (Buchs, CH). 6-coumarin was from Acros (New Jersey, U.S.A.).

All other reagents and solvents were from Merck (Darmstadt, Germany).

Stock solutions were prepared daily and diluted in M199 to the appropriate concentrations before each experiment.

PMNs were prepared from citrated venous blood obtained from healthy volunteers at a local hospital blood bank, using the standard techniques of dextran sedimentation followed by Histopaque®1077 gradient centrifugation. Residual erythrocytes were removed by hypotonic lysis and PMNs were resuspended in buffered salt solution (BSS) (138 mmol l⁻¹ NaCl, 2.7 mmol l⁻¹ KCl, 8.1 mmol l⁻¹ Na₂HPO₄, 1.5 mmol l⁻¹ KH₂PO₄, 1 mmol l⁻¹ MgCl₂, 1 mmol l⁻¹ CaCl₂, pH 7.4) supplemented with 1 mg ml⁻¹ glucose and 1 mg ml⁻¹ human serum albumin (HSA). Purity of the final cell suspension and cell viability, assessed by the Trypan blue exclusion test, were >95% in all cases. Cell viability was not affected by drug treatment.

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins by collagenase treatment (0.1%) and cultured in M199 with the addition of 20% BCS, 100 U ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin, 5 U.I. ml⁻¹ heparin, 12 μ g ml⁻¹ bovine brain extract and 200 mM glutamine. Purity of the HUVEC preparation, evaluated by morphology and immunostaining for factor VIII, was >95%. Contaminant leukocytes were detected by immunostaining for CD45.

HUVEC were grown to confluence in flasks and used at the III-V passage. Informed consent was obtained from all volunteers.

Preparation of chol-but SLN and sodium butyrate solutions

Chol-but SLN were prepared by the microemulsion method, reported elsewhere (Pellizzaro *et al.*, 1999). Briefly, a warm oil-in-water microemulsion was prepared from cholesterylbutyrate, Epikuron 200, butanol and water. The warm microemulsion was dispersed in cold water and the resulting chol-but SLN aqueous dispersion was washed twice by dialtrafiltration using a TCF2 system Amicon (Danvers, U.S.A.) with a Diaflo YM 100 membrane (cutoff 100,000 Da). The aqueous dispersions of chol-but SLN were sterilized by autoclaving (15 min, 121°C, 2 bar) before use. Fluorescent chol-but SLN

were prepared as above but adding 6-coumarin (0.04% w w⁻¹) to the formulation.

Sodium butyrate solutions were freshly prepared in sterile water or in a mixture of sterile water and ethanol (50:50 v v⁻¹) before each experiment, at a concentration of 5 M.

Characterization and stability of chol-but SLN

The average diameters and polydispersity indices of fluorescent and nonfluorescent chol-but SLN were determined by photon correlation spectroscopy (PCS) using an N4 MD instrument (Coulter) at a fixed angle of 90° and at a temperature of 25°C. Each value reported with its s.e.m. is the average of 10 measurements and is calculated by the Coulter software using cumulant analysis and an exponential sampling method. The polydispersity index is a measure of the size distribution of a nanoparticle population (Koppel, 1972) and is calculated using a software package (Coulter Scientific Instruments, U.S.A.).

The electrophoretic mobility and zeta potential were measured using a 90 PLUS instrument (Brookhaven Instrument Corporation, NY, U.S.A.). To determine the zeta potential, SLN samples were diluted with KCl 0.1 mM and placed in the electrophoretic cell, where an electric field of 15.2 V cm⁻¹ was applied. Each sample was analyzed at least in triplicate. The measured electrophoretic mobility was converted into zeta potential using the Smoluchowsky equation (Müller, 1991). The processing was performed by the software included in the system. The zeta potential is a measure of the surface charge of nanoparticles when they are dispersed in aqueous solutions; the parameter is employed to assess colloidal dispersion stability.

The stability of chol-but SLN was evaluated in physiological phosphate buffer (pH = 7.4) and in BSS. The SLN were dispersed in one or other medium and average diameter and polydispersity index values were determined by PCS after 1, 2, 3 and 4 h.

Quantitative determination of chol-but was by a reverse-phase HPLC method (Duncan, 1979) using a UV detector.

The concentration of chol-but in the SLN was determined on weighed amounts of chol-but SLN dissolved in the mobile phase and then injected.

Visualization of chol-but SLN internalization

Internalization of chol-but-loaded SLN was determined by confocal fluorescence microscopy. 30,000 HUVEC were seeded in 3 ml of culture medium and allowed to attach for 24 h on glass coverslips in a Petri dish. 30,000 PMNs were seeded in 3 ml of culture medium and placed on glass coverslips in a Petri dish. Cells were incubated with 10⁻⁵ M chol-but-loaded SLN for 10 min. HUVEC and PMNs were washed with PBS, then coverslips were inverted and mounted on glass slides. The sections were observed with a fluorescence microscope (Eclipse 800, Nikon, Japan) equipped with a CCD camera (AxioCam HRC; Zeiss) and with a confocal laser scanning microscope (CLSM; Zeiss LSM 510 Meta). The intensity of emission lines (568 nm) was adjusted individually to use the full dynamic range of the photomultiplier. Typically, stacks of six to ten confocal sections (512 × 512 pixels) spaced at 600 nm were acquired at a depth of eight bits, using a × 100

lens (numerical aperture 1.4) and a pinhole set at 1 Airy unit. Cells were analyzed with fluorescence microscopy using the 568 nm laser line as excitation, and fluorescence was collected through a red long-pass filter.

Vitality and proliferation of cells treated with sodium butyrate and chol-but SLN

Sodium butyrate and chol-but SLN (10⁻⁸–10⁻⁵ M) were incubated with PMNs for times between 10 min and 4 h. PMN viability was assessed by Trypan blue exclusion. The effects on cell proliferation were measured by MTT assay, a colorimetric method based on the ability of the HUVEC to convert tetrazolium salt into formazan product (Mosmann, 1983). The cells were treated with chol-but SLN (10⁻⁸–10⁻⁵ M) for 10 min or 4 h. All measurements were performed in triplicate. Cholesterol, equimolarly present in chol-but, does not affect cell growth (Pellizzaro *et al.*, 1999; Serpe *et al.*, 2004).

Adhesion assay

HUVEC were grown to confluence in 24-well plates, washed, and rested for 1 day in M199 plus 10% BCS without bovine brain extract. PMNs (10⁷ cells ml⁻¹) were labeled with fluorescein diacetate (5 µg ml⁻¹) for 30 min at 37°C, washed with BSS, and plated at 10⁶ cells well⁻¹ in a final volume of 0.25 ml BSS on HUVEC. Sodium butyrate or chol-but SLN (both at 10⁻⁸–10⁻⁵ M) and 10⁻⁷ M FMLP were added to HUVEC simultaneously with PMNs, and left in place for 10 min. In the experiments with IL-1β, EC were preincubated for 10 min with sodium butyrate or chol-but SLN (both at 10⁻⁸–10⁻⁵ M) and then stimulated with IL-1β (0.05 ng ml⁻¹) for 4 h, PMNs being added in the last 10 min.

In the time-course test, HUVEC were pretreated with sodium butyrate or chol-but SLN (both at 10⁻⁸–10⁻⁵ M, for 10, 30, 50, 110 or 230 min), and then challenged with 10⁻⁷ M FMLP and PMNs for a further 10 min. After incubation, nonadherent PMNs were removed by washing three times with 1 ml BSS. The center of each well was analyzed by fluorescence image analysis (Dinzani *et al.*, 2003). Adherent cells were counted using Image Pro Plus Software for microimaging (Media Cybernetics, version 5.0). Single experimental points were assayed in quadruplicate, and the standard error of the four replicates was below 10% in all cases. Data are presented as percentage adhesion *versus* the control value, control adhesion being measured on untreated HUVEC. Control adhesion was 51 ± 10 cells microscope fields⁻¹ (*n* = 26).

The direct effect on HUVEC was assessed by pretreating cells with chol-but SLN (10⁻⁵ M) and IL-1β (0.05 ng ml⁻¹) for 4 h, washing three times and challenging with PMNs for a further 10 min.

The direct effect on PMNs was assessed by seeding cells on 24-well ECs-free plates for 10 min at 37°C, in the presence of sodium butyrate or chol-but SLN and 10⁻⁷ M FMLP. The plates had previously been coated with heat-inactivated calf serum for 3 h to reduce spontaneous adhesion to the plastic wells.

Percentage inhibition of adhesion was calculated as follows: (100 – (*a*)/(*b*)) × 100, where *a* is adhesion measured in the presence of butyrate or chol-but SLN plus stimulus minus

basal adhesion, and *b* is adhesion elicited by stimulus minus basal adhesion.

Superoxide anion (O_2^-) production

PMNs (1×10^6 cells ml^{-1}) suspended in a buffered salt solution were pretreated with cytochalasin B ($5 \mu g\ ml^{-1}$) for 5 min at $37^\circ C$, to maximize the measured response, and then challenged with sodium butyrate or chol-but SLN for 5–20 min at $37^\circ C$ before exposure to 10^{-7} M FMLP for a further 5 min, or to 10^{-8} M PMA for 15 min. O_2^- production was determined spectrophotometrically by measuring the superoxide dismutase-inhibitable reduction of cytochrome *c* reduced per 10^6 PMNs min^{-1} . None of the compounds tested interfered with the spectrophotometric assay; they were checked for interference in the assay by measuring their effects on cytochrome *c* reduction with a xanthine oxidase superoxide generating system. Assays were carried out in the same buffer, with $100 \mu M$ cytochrome *c*, $150 \mu M$ hypoxanthine, 0.01 units of xanthine oxidase per milliliter and appropriate concentrations of each drug.

Myeloperoxidase assay

PMNs (2×10^6 cells) were incubated in BSS to a final volume of 1 ml with cytochalasin B and sodium butyrate or chol-but SLN for 5 min, then 10^{-8} M FMLP was added for a further 5 min. Myeloperoxidase release was assayed as described by Henson *et al.* (1978). Release of enzymes was expressed as percentage of total cell enzyme activity.

Statistical analysis

Results are expressed as means \pm s.e.m.; *n* indicates the number of experiments. Data in Figures 3–7 were analyzed by two-way analysis of variance; the Bonferroni multiple comparison post-test was then applied to determine significant differences between specific pairs of means. The molar concentration of a substance that reduces response to the stimulus by 50% (IC_{50}) was calculated with a nonlinear regression model using Origin version 6.0 software (Microcal Software, Northampton, U.S.A.). The IC_{50} data in Figure 6 were analyzed using the Student's *t*-test. Differences were considered to be statistically significant at $P < 0.05$. All statistical analyses were performed using GradPadPrism 3.0 software (GraphPad Software, San Diego, CA, U.S.A.).

Results

Characterization of SLN

The chol-but SLN had a diameter of 160 ± 11 nm and a polydispersity index of 0.2 ± 0.02 with a zeta potential of -28 mV. The zeta potential value is high enough to obtain a stable nanoparticle dispersion; the negative charge on SLN surface favors repulsion among nanoparticles, avoiding their aggregation. Chol-but SLN dispersions had an initial chol-but concentration of 12 mM; their average diameter and polydispersity indices were maintained in BSS for 4 h.

The fluorescent SLN prepared to evaluate internalization of chol-but SLN had a diameter of 130 ± 21 nm, a polydispersity index of 0.28 and a zeta potential of -29 mV (Table 1).

Internalization of fluorescent chol-but SLN was evaluated by confocal microscopy in PMNs; after 10 min incubation, high levels of fluorescence were detectable only in the cytoplasm (Figure 1) and remained there throughout the observation period (10–30 min; data not shown). Similar results were obtained on HUVEC (Figure 2).

Effect of chol-but SLN on cell viability

Chol-but SLN (10^{-8} – 10^{-4} M) incubated with PMNs for times between 10 and 240 min did not have any effect on the viability

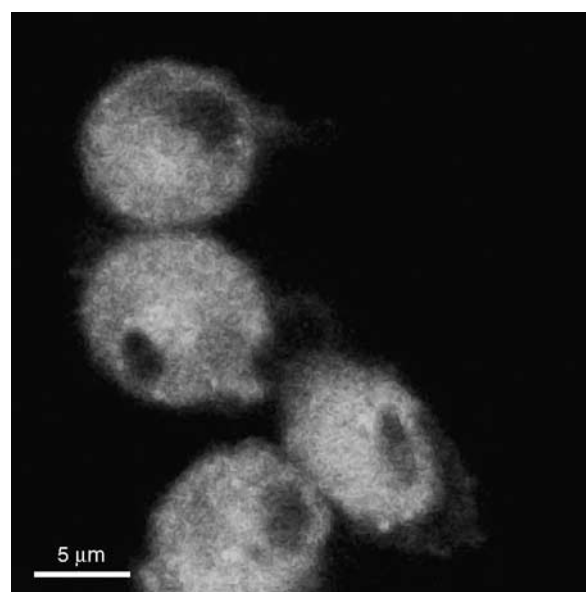


Figure 1 Uptake of 6-coumarin-chol-but SLN on PMNs.

Table 1 Characteristics of chol-but SLN

	Diameter \pm s.e.m. (nm)	Polydispersity index \pm s.e.m.	Zeta potential (mV)	Nanoparticles in 1 ml dispersion (approx. number)*
Chol-but SLN	160 ± 11	0.20 ± 0.02	-28	4.6×10^{14}
Chol-but SLN (fluorescent)	130 ± 21	0.28 ± 0.02	-29	8.7×10^{14}

*Computed from average nanoparticle diameter (spherical shape and density = 1).

SLN aqueous dispersion concentration: 12 mM.

The average diameter and polydispersity index of chol-but SLN dispersions were maintained in BSS solution for 4 h.

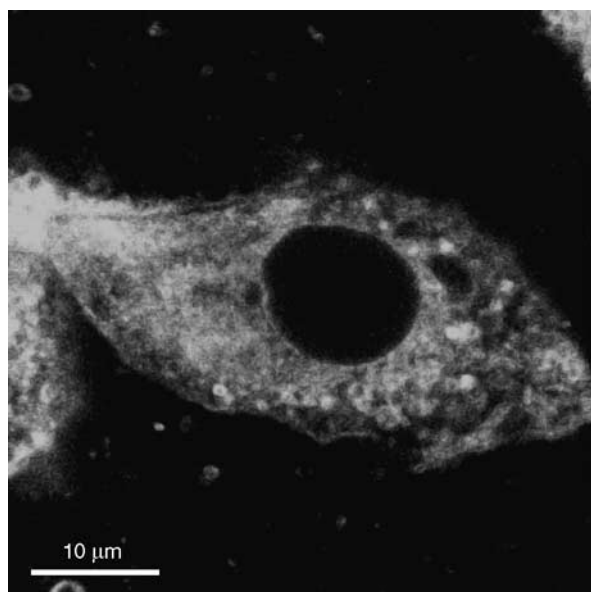


Figure 2 Uptake of 6-coumarin-chol-but SLN on HUVEC.

of PMNs, which was $>98\%$ in all cases. HUVEC treated with chol-but SLN for the same incubation time proliferated with no significant difference *versus* controls.

Effect of sodium butyrate and chol-but SLN on PMN adhesion to HUVEC induced by FMLP

The effects of sodium butyrate and chol-but SLN on PMN adhesion to HUVEC were evaluated by coincubating both cell types for 10 min with each compound (10^{-8} – 10^{-5} M) in the presence of 10^{-7} M FMLP, which mimics bacterial chemotaxin and activates the PMN adhesive machinery. The 10^{-7} M dose of FMLP had previously been found to induce maximal adhesion of PMNs (Avanzi *et al.*, 1998) ($351 \pm 25\%$ of control adhesion). Chol-but SLN strikingly inhibited adhesion in a dose-dependent manner with a maximal inhibition of $71 \pm 7\%$ ($P < 0.001$ *versus* sodium butyrate) at about 10^{-5} M and an IC_{50} at $2.5 \pm 0.5 \times 10^{-7}$ M. The chol-but SLN dose–response curve was not modified by incubation times in the 10–240 min range (data not shown), and thus 10 min incubation was selected for subsequent experiments. By contrast, the inhibitory effect of sodium butyrate was strikingly lower, with a shallow dose–response curve and maximum inhibition of $20 \pm 7\%$ at 10^{-5} M ($n = 5$, Figure 3). Control adhesion (i.e. adhesion in the absence of FMLP) was 51 ± 10 cells/microscope field $^{-1}$ (mean \pm s.e.m., $n = 26$) and was not inhibited by either sodium butyrate or chol-but SLN (data not shown).

Effect of sodium butyrate and chol-but SLN on PMN adhesion to FCS-coated plastic wells

To understand the selective effect of chol-but SLN on the two different cell populations, we evaluated the effect of both formulations on PMN adhesion to FCS-coated plastic wells, using FMLP as stimulus. Comparison of the results obtained in this experimental system with those obtained previously using EC showed that the concentration–response curves were similar, without any significant difference, for both com-

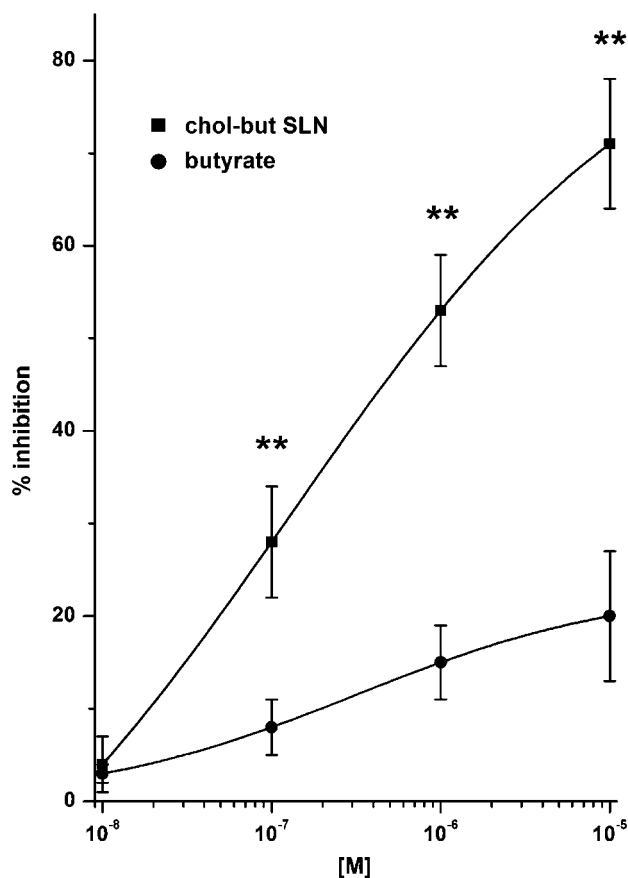


Figure 3 Effects of butyrate and chol-but SLN on PMNs adhesion to HUVEC evoked by FMLP. PMNs and HUVEC were incubated with increasing concentrations (10^{-8} – 10^{-5} M) of the tested compound and with 10^{-7} M FMLP for 10 min at 37°C . Data are expressed as percentage inhibition of control adhesion, as mean \pm s.e.m.; $n = 5$. Asterisks mark statistically significant inhibition of chol-but SLN *versus* butyrate (** $P < 0.001$). Control adhesion (i.e. adhesion in the absence of FMLP) was 51 ± 10 cells/microscope field $^{-1}$ (mean \pm s.e.m., $n = 26$), adhesion induced by 10^{-7} M FMLP was $351 \pm 25\%$ of the control.

pounds (Figures 3 and 4). Indeed, the ability of chol-but SLN to inhibit PMN adhesion can be regarded as a direct effect on PMNs, HUVEC being absent in this experimental model (Figure 4).

Effect of sodium butyrate or chol-but SLN on PMN adhesion to HUVEC induced by IL-1 β

The analysis was then extended to PMN adhesion induced by IL-1 β , which is crucial in the inflammatory response. Experiments were performed using 0.05 ng ml^{-1} IL-1 β , which induced adhesion levels close to those obtained by 10^{-7} M FMLP ($282 \pm 38\%$ of control). Also in this system, chol-but SLN strikingly inhibited adhesion, achieving a plateau at 10^{-6} – 10^{-5} M; maximum inhibition was of $80 \pm 5\%$ and IC_{50} was $1.4 \pm 0.5 \times 10^{-7}$ M (Figure 5). The inhibitory effect of sodium butyrate was significantly weaker, with maximal inhibition at 10^{-5} M of $29 \pm 8\%$ and IC_{50} was $4.4 \pm 0.6 \times 10^{-7}$ M. To assess whether the inhibitory effect could be overcome by increasing the amount of proadhesive stimulus, we used higher doses of IL-1 β (1 ng ml^{-1}) to induce maximal PMN adhesion (500% of

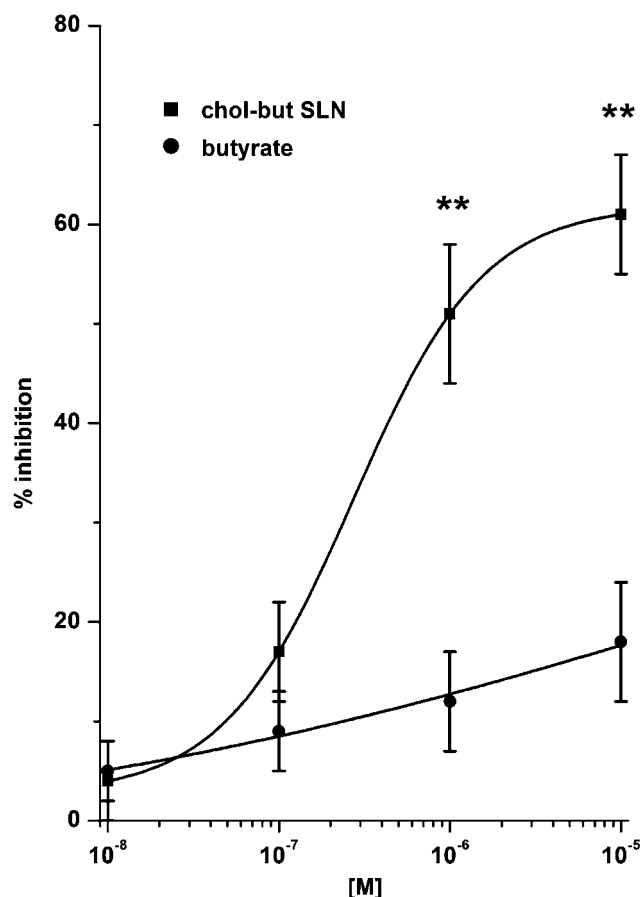


Figure 4 Effects of butyrate and chol-but SLN on PMN adhesion to FCS-coated plastic wells. PMNs were incubated with increasing concentrations (10^{-8} – 10^{-5} M) of the tested compound and with 10^{-7} M FMLP and seeded on 24-well EC-free plates for 10 min at 37°C . Data are expressed as percentage inhibition of control adhesion, as means \pm s.e.m.; $n=5$. Asterisks mark statistically significant inhibition of chol-but SLN versus butyrate ($**P < 0.001$). Control adhesion (i.e. adhesion in the absence of FMLP) was 108 ± 32 cells microscope $^{-1}$ field (mean \pm s.e.m., $n=5$), adhesion induced by 10^{-7} M FMLP was $378 \pm 25\%$ of the control.

control adhesion), but chol-but SLN displayed a similar dose-response inhibitory curve with shape and peak close to those recorded in the presence of 0.05 ng ml^{-1} IL- 1β (data not shown).

Furthermore, we coincubated IL- 1β -stimulated HUVEC with chol-but SLN, washed them three times and added PMNs. In this different situation, chol-but SLN also inhibited PMN adhesion to stimulated HUVEC (% inhibition $76 \pm 4\%$; $n=5$), demonstrating that chol-but SLN can also act on stimulated EC.

Effect of sodium butyrate or chol-but SLN on $\text{O}_2^{\cdot-}$ production and myeloperoxidase secretion induced by FMLP on PMNs

To compare the effect of chol-but SLN on other activities of PMNs, we evaluated their effect on $\text{O}_2^{\cdot-}$ production and myeloperoxidase release evoked by FMLP. Both chol-but SLN and sodium butyrate inhibited $\text{O}_2^{\cdot-}$ production evoked by 10^{-7} M FMLP (2.1 ± 0.15 nmol cytochrome *c* reduced per

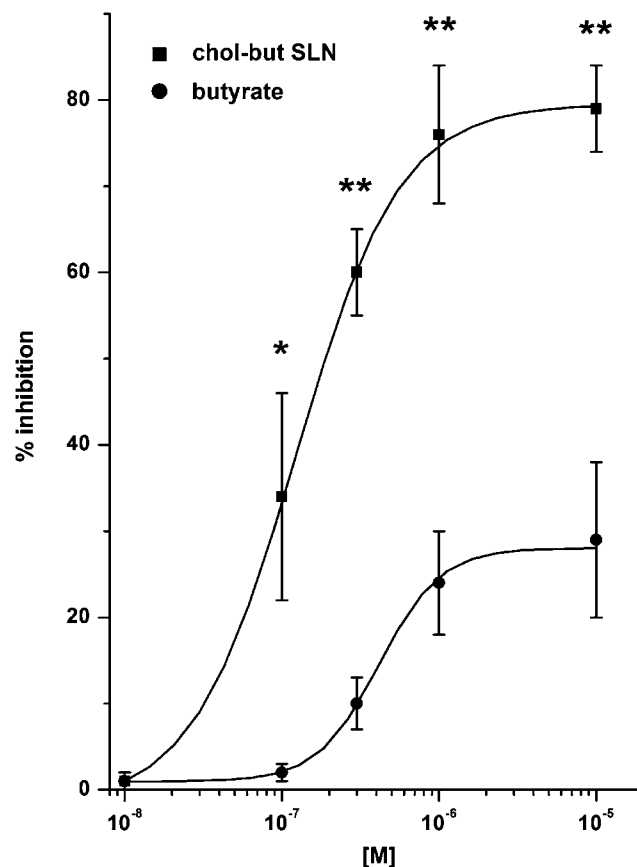


Figure 5 Effects of butyrate and chol-but SLN on PMNs adhesion to HUVEC evoked by IL- 1β . HUVEC were incubated with increasing concentrations (10^{-9} – 10^{-5} M) of the tested compound for 10 min and then stimulated with 0.05 ng ml^{-1} IL- 1β for 4 h at 37°C . Data are expressed as percentage inhibition of control adhesion, as means \pm s.e.m.; $n=5$. Asterisks mark statistically significant inhibition of chol-but SLN versus butyrate ($**P < 0.001$; $*P < 0.01$). Adhesion induced by 0.05 ng ml^{-1} IL- 1β was $282 \pm 38\%$ of the control.

$10^6 \text{ PMNs min}^{-1}$) in a dose-dependent manner, but the former was more active by three orders of magnitude, since IC_{50} values were $2.8 \pm 0.3 \times 10^{-6}$ M for chol-but SLN and $2.8 \pm 0.2 \times 10^{-3}$ M for sodium butyrate (Figure 6). No difference was found when the experiment was prolonged to 25 min (data not shown) or when the amount of FMLP was changed to 10^{-8} or 10^{-6} M (inducing 0.5 and $4.0 \text{ nmol cit}^{-1} \text{ c rid/} 10^6 \text{ cells min}^{-1}$ respectively; data not shown). Neither sodium butyrate (10^{-4} – 10^{-2} M), as also demonstrated by Liu *et al.* (2001), nor chol-but SLN (10^{-7} – 10^{-5} M) inhibited $\text{O}_2^{\cdot-}$ production evoked by 10^{-8} M PMA (3.1 ± 0.25 nmol cytochrome *c* reduced per $10^6 \text{ PMNs min}^{-1}$), showing a stimulus selectivity in their inhibitory activity (data not shown).

Moreover, chol-but SLN strongly inhibited myeloperoxidase release induced by 10^{-8} M FMLP; inhibition was recorded in the concentration range 10^{-6} – 10^{-4} M in a dose-dependent manner and was maximal ($60 \pm 6\%$ inhibition) at 10^{-4} M (Figure 7). By contrast, sodium butyrate slightly inhibited myeloperoxidase secretion ($23 \pm 5\%$ of inhibition) and was active only at concentrations of 10^{-3} – 10^{-2} M. Neither compound induced myeloperoxidase release from nonstimulated PMNs (data not shown).

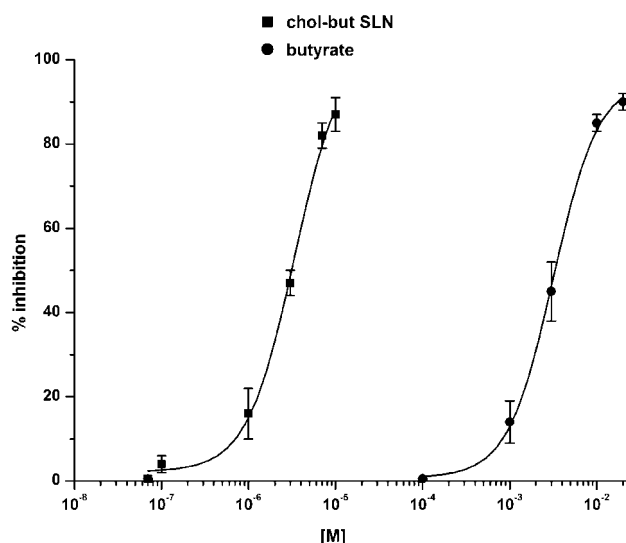


Figure 6 Effects of butyrate or chol-but SLN effects on FMLP-evoked O_2^- production by PMNs. Results are expressed as percentage inhibition of O_2^- production evoked by 10^{-7} M FMLP in the absence of butyrate or chol-but SLN: this production amounted to 2.1 ± 0.15 nmol cytochrome *c* reduced per 10^6 PMNs min^{-1} and was taken as 100%. Data are expressed as means \pm s.e.m.; $n = 5$.

Discussion

The acute phase of UC is characterized histologically by extensive infiltration of PMNs, lymphocytes, and plasma cells into the crypt epithelium. PMNs protect against bacterial and fungal infections by releasing agents that are able to kill the invader microorganisms, but can also be toxic to the host by destroying tissue cells and dissolving the intercellular connective matrix. A key role is played by release of reactive oxygen species, such as O_2^- , and drugs decreasing their production or increasing their clearance can attenuate tissue injury at inflammatory sites (Liu *et al.*, 2001).

Butyrate is known for its anti-inflammatory properties, but its infrequent clinical use is related to the short half-life of butyrate salt derivatives, which impairs long-lasting effects *in vivo* and makes a continuous administration regimen necessary to maintain acceptable plasma levels. To overcome these problems and improve the pharmacological profile, we formulated the drug as SLN, using a butyrate prodrug as lipid matrix. We have shown that incorporation of drugs into SLN changes their pharmacokinetic profile, with increased half-life, area under the concentration *versus* time curve (AUC), and peak plasma concentration, and concomitant decrease of clearance (Cavalli *et al.*, 2002).

Our results show that chol-but SLN exerts a striking inhibitory effect on PMN activation, evaluated in terms of increased adhesiveness, O_2^- production and myeloperoxidase release, and is much more efficient than sodium butyrate. This improvement may be ascribed to the rapid uptake of chol-but SLN, whose cytoplasmic levels are already maximal after 10 min in both PMNs and EC. SLN consist of nontoxic bioacceptable and biodegradable substances, that is solid lipids and phosphatidylcholine, and their rapid internalization is probably due to their size and composition (Pellizzaro *et al.*, 1999; Serpe *et al.*, 2004).

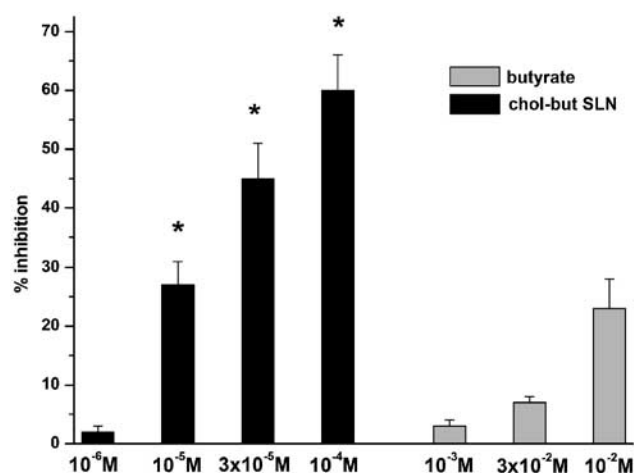


Figure 7 Effects of butyrate or chol-but SLN effects on FMLP-evoked myeloperoxidase release by PMNs. The results are expressed as percentage inhibition of myeloperoxidase release evoked by 10^{-8} M FMLP in the absence of butyrate or chol-but SLN: this release was $31 \pm 5\%$. Data are expressed as means \pm s.e.m.; $n = 5$. Asterisks mark statistically significant inhibition of chol-but SLN *versus* butyrate (* $P < 0.01$).

The higher inhibitory activity of chol-but SLN than sodium butyrate might be ascribed to its accumulation within cells and to the release of higher concentrations of butyric acid from the internalized drug by esterase enzymatic activities, as suggested by Serpe *et al.* (2004) in cancer cells *in vitro*.

The widespread effect of chol-but SLN on different aspects of PMN activation was not due to a general toxic effect on cell function, as it did not affect cell viability nor inhibit PMA-induced superoxide production.

Chol-but SLN appear to act on early events of FMLP-driven activation of PMNs, as their inhibitory activity is already maximal after 10 min incubation. FMLP mimics bacterial chemotaxin and activates PMN by interacting with a surface G-protein receptor, whose triggering initiates a sequence of protein phosphorylation events, including activation of Ras and MAPK p38 and p42/44. Activation of these MAPKs is maximal within 1–2 min, but is maintained for about 10 min and results in a broad range of rapid functional responses, including calcium influx, actin assembly, superoxide production, granule enzyme release, chemokinesis and increased adhesiveness, most of which are mediated by inside-out signals activating $\beta 2$ -integrin function (Nick *et al.*, 1997). Specific inhibition of p38 MAPK results in selective loss of functional responses to FMLP, with inhibition of PMN adhesion, chemotaxis and superoxide release (McLeish *et al.*, 1998). Therefore, together with the recent observation that butyrate inhibits MAPK (Park *et al.*, 2005), these reports strongly suggests that the chol-but SLN inhibitory effect on FMLP-induced activation of PMN is mediated by inhibition of this signaling pathway. This possibility is also supported by our observation that chol-but SLN has no effect on activating PMN induced by PMA, which is a direct activator of protein kinase C (PKC) and activates MAPK only weakly in PMNs (Nick *et al.*, 1997).

Furthermore, chol-but SLN may act not only on PMN, but also on EC. This possibility is suggested by our finding that chol-but SLN is rapidly taken up by HUVEC and inhibits

PMN adhesion to HUVEC more efficiently than their adhesion to FCS-coated plastic wells, moreover chol-but SLN can act even if HUVEC were washed before adding PMNs. EC, indeed, play an active role in PMN recruitment, as they express the ligands for multiple PMN surface molecules involved in adhesion. Moreover, they can actively respond to PMN adhesion due to intracellular signaling events triggered by PMN binding. In cultured EC, ligation of ICAM-1 by β_2 -integrins stimulates activation of several tyrosine kinases and MAPKs, resulting in the recruitment of several actin-binding proteins and cytoskeleton rearrangement (Wang *et al.*, 2005). As a key role has been ascribed to p38 MAPK, it can be suggested that chol-but SLN may also act at the p38 MAPK level in EC.

In summary, our data show that sodium butyrate and chol-but SLN exert rapid anti-inflammatory effects on PMNs, and that the latter is much more potent than the former, as it acts

at three logs lower concentrations and displays a broader inhibitory effect on PMN activation. The stronger effect of chol-but SLN might be ascribed to their rapid internalization in the cytoplasmic compartment, which is maximal within few minutes in both PMN and HUVEC. These results suggest that the use of SLN might be a novel clinical possibility to deliver butyric acid at lower dosage that is still able to produce significant anti-inflammatory effects in patients with UC. Further studies are in progress in our laboratory to elucidate the molecular mechanism of chol-but SLN.

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