

Tributylin, a Stable and Rapidly Absorbed Prodrug of Butyric Acid, Enhances Antiproliferative Effects of Dihydroxycholecalciferol in Human Colon Cancer Cells¹

Tanja Gaschott, Dieter Steinhilber,* Vladan Milovic and Jürgen Stein²

2nd Department of Medicine and *Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University, Frankfurt/Main, Germany

ABSTRACT Tributylin, a prodrug of natural butyrate, has been evaluated with an aim to overcome pharmacokinetic drawbacks of natural butyrate as a drug, i.e., its rapid metabolization and inability to achieve pharmacologic concentrations in neoplastic cells. We studied the effects of tributylin on growth, differentiation and vitamin D receptor expression in Caco-2 cells, a human colon cancer cell line. Tributylin was more potent in inhibiting growth and inducing cell differentiation than natural butyrate. The effect was further enhanced after addition of physiologic concentrations of dihydroxycholecalciferol [(OH)₂D₃]. The synergistic effect of tributylin and (OH)₂D₃ in Caco-2 cells was due to tributylin-induced overexpression of the vitamin D receptor, as measured by reverse transcriptase-polymerase chain reaction. Treatment with tributylin increased binding of (OH)₂D₃ to its receptor 1.5-fold, without any change in receptor affinity. We conclude that tributylin may, at least in part, exert its growth-reducing and differentiation-inducing effect in Caco-2 cells by an upregulation of the vitamin D receptor; this may provide a useful therapeutic approach in chemoprevention and treatment of colorectal cancer by the two nutrients occurring naturally in human diet. *J. Nutr.* 131: 1839–1843, 2001.

KEY WORDS: • colon cancer • dihydroxycholecalciferol • tributylin • vitamin D receptor

Butyrate, a normal constituent of the colonic luminal contents, is formed by bacterial fermentation of unabsorbed complex carbohydrates in the mammalian digestive tract. In normal colonic mucosa, butyrate serves as a primary energy source, promotes growth of normal colonic epithelial cells in vivo and in vitro and plays a role in preventing certain types of colitis (1). In contrast, in a wide variety of neoplastic cells, butyrate acts as a potent antineoplastic agent, i.e., it inhibits growth and induces differentiation, restoring normal phenotype and function (2).

The studies done during the last decade provide multiple lines of evidence that butyrate indeed interferes with the pathogenesis of colorectal cancer. Butyrate inhibits DNA synthesis and arrests growth of neoplastic colonocytes in G₁ (3), modifies expression of genes involved in chemotherapy resistance (4) and in cell proliferation/differentiation (5,6), and induces apoptosis by a p53-independent pathway (7). At least some of butyrate's antineoplastic effects in colon cancer cells may be due to its synergistic action with another antiproliferative agent, 1,25-dihydroxyvitamin D₃ [dihydroxycholecalciferol; (OH)₂D₃]. In various cancer cell lines it has been shown that butyrate and (OH)₂D₃ act synergistically in reducing proliferation and enhancing differentiation of neoplastic cells (8–10).

In spite of its early promise, butyrate is not among the drugs used for cancer treatment. The major problem has been to achieve and maintain its millimolar concentrations in blood. Butyrate is metabolized rapidly as soon as it enters the colonocyte via its active transport system (11–13), and its plasma concentrations are far below those required to exert its antiproliferative/differentiating actions. A prodrug of natural butyrate, tributylin, is a neutral short-chain fatty acid triglyceride that is likely to overcome the pharmacokinetic drawbacks of natural butyrate as a drug (14). Because it is rapidly absorbed and chemically stable in plasma, tributylin diffuses through biological membranes and is metabolized by intracellular lipases, releasing therapeutically effective butyrate over time directly into the cell. Compared with butyrate, tributylin has more favorable pharmacokinetics (14–16) and is well tolerated (17). Liquid tributylin filled into gelatin capsules and administered orally resulted in millimolar concentrations of butyrate both in plasma and inside the cell (17). In vitro, tributylin has potent antiproliferative, proapoptotic and differentiation-inducing effects in neoplastic cells (18–20). In this study, human colon cancer cells (Caco-2) were used to investigate the effects of tributylin on growth and differentiation.

MATERIALS AND METHODS

Chemicals and supplies. Disposable cell culture ware was purchased from Nalge Nunc International (Wiesbaden, Germany). Dul-

¹ This study was supported by Else Kröner-Fresenius Foundation.

² To whom correspondence should be addressed.

E-mail: J.Stein@em.uni-frankfurt.de.

becco's modified Eagle's medium (DMEM),³ fetal calf serum (FCS), sodium pyruvate, nonessential amino acids and PBS were obtained from GIBCO BRL (Eggenstein, Germany). Penicillin/streptomycin was from Biochrom (Berlin, Germany). Tributyrin was purchased from Sigma (Deisenhofen, Germany) and butyric acid (sodium salt) from Merck-Schuchardt (Munich, Germany). [³H]-(OH)₂D₃ (6.6 · 10¹² Bq/mmol) and (OH)₂D₃ were obtained from Immundiagnostik (Bensheim, Germany), and Lipofundin 10% N from B. Braun (Melsungen, Germany).

Cell culture. Caco-2 cells were obtained from the German Cancer Research Center (Heidelberg, Germany). The stock was maintained in 175 cm² flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO₂. The medium consisted of DMEM, supplemented with 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids. The cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed three times per week. Passages 44–53 were used in all experiments. The cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. Before treatment, the cells were synchronized in medium containing 1% FCS.

Tributyrin was incorporated into Lipofundin to yield a 20% (v/v) stock emulsion. The emulsion was mixed vigorously for ~5 min. The stock emulsion of tributyrin was freshly prepared each day. (OH)₂D₃ was dissolved in ethanol (final maximal concentration of ethanol in medium was 0.1%) to yield a 1 mmol/L stock solution, which was stored at -20°C. Butyrate was dissolved in PBS. To assess whether the solvents might influence the experimental conditions, control cells were treated with either 0.1% ethanol or 10.0% PBS Lipofundin in concentrations used in stock solution. No differences were observed.

Cell proliferation. Cell proliferation was assessed by cell counting after staining with crystal violet (21). To investigate changes in DNA synthesis, incorporation of 5-bromo-2'-deoxyuridine (BrdU) was done additionally within the initial 48 h in culture, using the Cell Proliferation ELISA Kit (Boehringer Mannheim, Germany).

Cell differentiation. Alkaline phosphatase activity was used to assess differentiation of Caco-2 cells. For the assay, the cells were washed with cold PBS, scraped, sonicated (2 × 5 s) and centrifuged at 1500 × g for 10 min. Alkaline phosphatase activity in the supernatant was measured by hydrolysis of *p*-nitrophenyl phosphate at pH 9.8 and 25°C (Ecoline Alkaline Phosphatase Assay, Merck, Darmstadt, Germany). Cellular protein was determined by Coomassie blue assay using a commercial kit (Bio-Rad Laboratories GmbH, Munich, Germany). Enzyme activity was expressed as milliunits per milligram of protein, one unit representing the enzyme activity hydrolyzing 1 μmol of substrate/min.

Vitamin D receptor assay. Synchronized subconfluent cells were treated with tributyrin at a concentration of 0.5 mmol/L for 48 h (1% FCS in the medium). Then they were washed twice with 0.5 mL culture medium and incubated at 16°C for 8 h with increasing concentrations (0.2–2 nmol/L) of [³H]-labeled (OH)₂D₃ in the absence or presence of unlabeled (OH)₂D₃ (1 μmol/L). Incubation was terminated by placing the dishes on ice and by aspiration of the medium followed by washing with cold Hanks' buffered saline. The cells were solubilized by a 30-min incubation at room temperature with 0.5 mL of 1 mol/L NaOH, and radioactivity was measured by liquid scintillation counting. Saturation kinetics were analyzed by Scatchard plots.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Caco-2 cells were grown either serum free or with 1% FCS and then cultured in the presence of 5–500 μmol/L tributyrin for 12 h. Total RNA was isolated by RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions and quantitated by measuring the absorbance at 260 nm. RT-PCR was performed as described (22). RT and PCR primers were deduced from the vitamin D receptor

sequence (23). After DNase digestion, 2 μg of total RNA were reverse transcribed into cDNA using oligo d(T)-primer. After cDNA synthesis, PCR amplification was performed using the cDNA template with the specific primer pair (F1: 5'-GCCCCACCACAAGACTAT-3' and R2: 5'-CCTTTTGGATGCTGTAAGT-3'). The amplification profile consisted of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s after a 5-min denaturation step at 94°C in a Perkin Elmer Gene Amp PCR System 9600 (Wellesley, MA). The amplified products (50 cycles, 297 bp) were detected by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and ultraviolet transillumination. As control, β-actin mRNA was used, resulting in a PCR product of 405 bp. The optical densities of the PCR products were analyzed by a commercially available computer program (Bio-1 D V.96., Vilber Lourmat, France), normalized for the density of β-actin, and verified by sequencing.

Statistics. All data presented in this paper are means ± SD, *n* = 3–8 different experiments. Unpaired one-way ANOVA was used to compare means; *P*-values were corrected by the Bonferroni method for multiple comparisons, and *P* < 0.05 was the level of significance required to reject the null hypothesis.

RESULTS

Tributyrin was more potent than butyrate in inhibiting Caco-2 cell growth when cell counting was used to evaluate proliferation. After 96 h of treatment and within the concentration range of up to 5 mmol/L butyrate or tributyrin, the 50% inhibitory concentration values were 0.85 ± 0.9 mmol/L for tributyrin, and 2.2 ± 0.5 mmol/L for butyrate (Fig. 1). Tributyrin was also a more potent growth inhibitor than either (OH)₂D₃ alone or butyrate over time, i.e., after 48–120 h of treatment, tributyrin (1 mmol/L) reduced Caco-2 cell growth by 22–53% compared with 8–15% for (OH)₂D₃ alone (1 μmol/L) and 11–23% for butyrate (2 mmol/L). The combination of tributyrin (1 mmol/L) and 1 μmol/L (OH)₂D₃ further reduced Caco-2 cell growth, i.e., after 120 h of treatment, this combination reduced growth to 55 ± 4% (*P* < 0.01 vs. butyrate and *P* < 0.05 vs. tributyrin alone, mean ± SD, *n* = 4).

DNA synthesis rate (as measured by BrdU incorporation) was influenced less markedly than cell counts (indicating changes when the cells were driven through the cycle completely). After 48 h of incubation, DNA synthesis was reduced to 84.3 ± 3.1% by 1 μmol/L (OH)₂D₃ alone, to 80.9 ± 2.1% by 2 mmol/L butyrate and 76.7 ± 7.0% by 1 mmol/L tributyrin alone. The combination of tributyrin (1 mmol/L) and (OH)₂D₃ (1 μmol/L) reduced the BrdU incorporation rate to 64.8 ± 4.9%, significantly lower than untreated cells (mean ± SD, *n* = 8; *P* < 0.001).

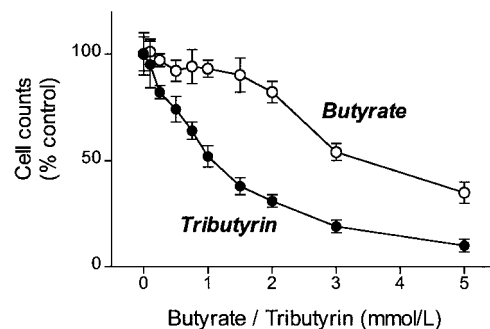


FIGURE 1 Effect of butyrate and tributyrin on Caco-2 cell growth. Cells were seeded at a density of 28,500 cells/cm² and treated daily with medium supplemented with 0–5 mmol/L butyrate or tributyrin. Cell counts were calculated by staining with crystal violet after 96 h of treatment. Values are means ± SD, *n* = 8.

³ Abbreviations used: BrdU, 5-bromo-2'-deoxyuridine; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; (OH)₂D₃, dihydroxycholecalciferol; RT-PCR, reverse transcriptase-polymerase chain reaction.

TABLE 1

Effect of butyrate, tributyrin, dihydroxycholecalciferol and the combination on Caco-2 cell differentiation, as assessed by alkaline phosphatase (AP) activity^{1,2}

Treatment, d	0	3	6	9
	<i>AP, mU/mg cell protein</i>			
None	9 ± 0.8	14 ± 0.7	49 ± 10	82 ± 4
Dihydroxycholecalciferol	9 ± 0.7	22 ± 0.2	69 ± 6.7	125 ± 3
Butyrate	9 ± 0.3	113 ± 16	294 ± 11	301 ± 15
Tributyryn	9 ± 0.8	143 ± 5 ^a	368 ± 21 ^a	393 ± 28 ^a
Tributyryn + Dihydroxycholecalciferol	9 ± 1.0	188 ± 6 ^a	523 ± 19 ^b	535 ± 31 ^b

¹ Caco-2 cells were treated daily with medium supplemented with 1 μmol/L dihydroxycholecalciferol, or 2 mmol/L butyrate, or 1 mmol/L tributyrin, or with medium supplemented with the combination of 1 mmol/L tributyrin and 1 μmol/L dihydroxycholecalciferol.

² Values are means ± SD, n = 3. ^aP < 0.05 vs. butyrate; ^bP < 0.01 vs. butyrate.

(OH)₂D₃, butyrate, tributyrin and the combination [tributyryn + (OH)₂D₃] all stimulated differentiation in Caco-2 cells 2- to 10-fold compared with spontaneous differentiation occurring in untreated cells. The order of potency was (OH)₂D₃ < butyrate < tributyrin < [tributyryn + (OH)₂D₃] (Table 1). Compared with butyrate, both tributyrin and [tributyryn + (OH)₂D₃] were significantly more potent inducers of Caco-2 cell differentiation.

Tributyryn markedly increased binding of (OH)₂D₃ in Caco-2 cells. Maximum specific binding of [³H]-(OH)₂D₃ increased > 100% upon preincubation with tributyrin (0.5 mmol/L) for 48 h (control, 14,321 bound molecules/cell; tributyrin, 31,657 bound molecules/cell), whereas receptor affinity was unaffected (control cells, K_D = 3.1 × 10⁻¹⁰ mol/L; cells treated with tributyrin, K_D = 4.8 × 10⁻¹⁰ mol/L) (Fig. 2). As assessed by RT-PCR, tributyrin dose-dependently enhanced vitamin D receptor expression in Caco-2 cells (Fig. 3). Incubation of Caco-2 cells with tributyrin at two different concentrations, within the range shown to have an effect on Caco-2 cell growth (5 × 10⁻⁶ and 5 × 10⁻⁴ mol/L), increased vitamin D receptor mRNA by 70% and 150%, respectively.

DISCUSSION

Nutrition and nutrients have long been postulated to play a role in colon carcinogenesis. Broad experimental evidence

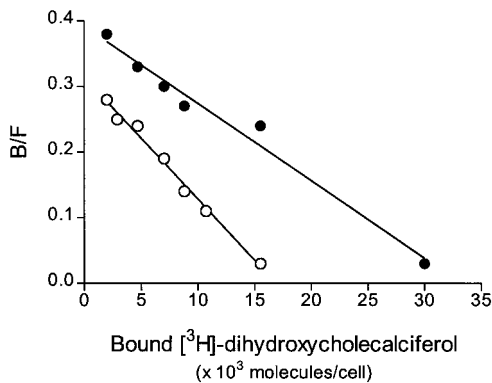


FIGURE 2 Enhanced [³H]-dihydroxycholecalciferol [(OH)₂D₃] binding by tributyrin in Caco-2 cells. Subconfluent Caco-2 cells, synchronized in 0.1% fetal calf serum (FCS) for 24 h, were then cultured in Dulbecco's modified Eagle's medium with 1% FCS. Tributyrin (0.5 mmol/L) was added for a 48-h culture period. [³H]-(OH)₂D₃ was incubated for 8 h at 16°C. Specific binding of [³H]-(OH)₂D₃ was determined as described in the Methods section. Open circles, untreated cells; full circles, tributyrin. B/F = bound/free.

exists that various nutrients, within a certain concentration range, may either act as cocarcinogens or prevent cancer (24–27); however, an adequate dose level for drug use is not known. Most nutrients are metabolized rapidly, eliminated from the lumen or chemically altered before reaching the systemic circulation in concentrations sufficient to inhibit cancer growth. Tributyrin, a prodrug of natural butyrate, may overcome the poor bioavailability of natural butyrate, a compound with well-established usefulness in cancer prevention (17).

Our data provide several lines of evidence that tributyrin exerts antiproliferative actions on colon cancer cells more potently than butyrate itself. Furthermore, our study shows

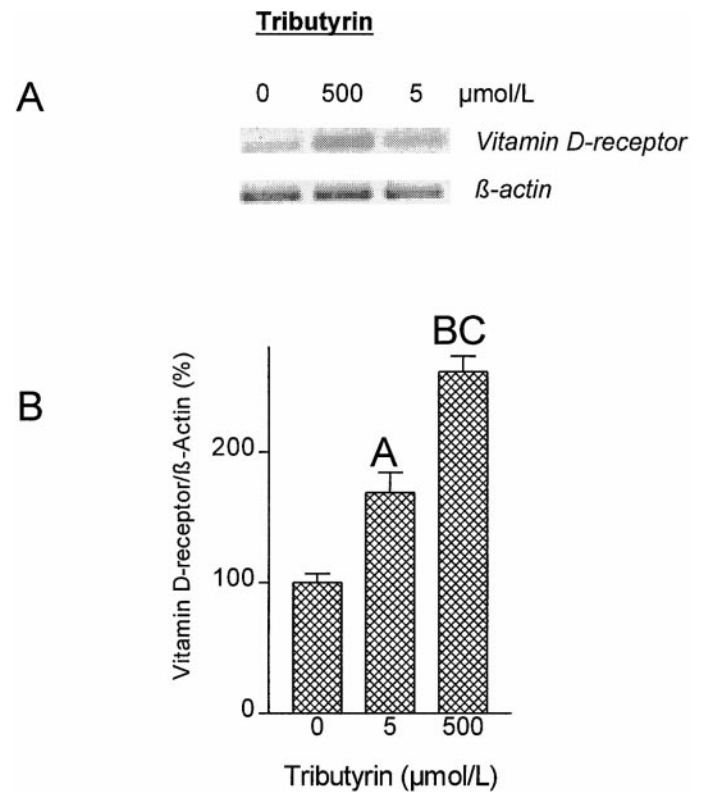


FIGURE 3 (A) Effect of tributyrin on vitamin D receptor expression in Caco-2 cells. Cells were grown for 12 h in serum-free medium in the absence or presence of tributyrin. Total RNA from each cell culture was analyzed by reverse transcriptase-polymerase chain reaction for vitamin D receptor-mRNA content. (B) *Ratio of Vitamin D-receptor-mRNA/β-actin-mRNA. Values are means ± SD, n = 3. ^AP < 0.01 vs. control; ^BP < 0.001 vs. control; ^CP < 0.01 vs. tributyrin (5 μmol/L).

that tributyrin applied with (OH)₂D₃ inhibits growth more potently than any of the substances given alone. The antiproliferative and prodifferentiating action of tributyrin, further enhanced by the addition of (OH)₂D₃, likely occurs due to upregulation of vitamin D receptor expression by tributyrin and the subsequent enhanced binding of (OH)₂D₃ to Caco-2 cells. These data may be of a considerable clinical relevance because they suggest a possible therapeutic approach in patients with colorectal cancer.

Tributyrin was more potent than butyrate in reducing the number of Caco-2 cells in culture. Impairment of DNA synthesis during the initial phases of proliferation was apparently not the reason for the potent growth-inhibitory effect of tributyrin, i.e., BrdU incorporation was highly variable; after 48 h, the effect of tributyrin was slightly ($P = 0.0687$) greater than that of butyrate. However, tributyrin was a more potent inducer of Caco-2 cell differentiation than natural butyrate, and this effect was enhanced a further 40% by the addition of (OH)₂D₃. These data suggest that it is not only the amount of available butyrate in the cell that is responsible for enhanced differentiation after treatment with tributyrin, but that the explanation for the effect may lie in a fine regulatory mechanism involving the action of butyrate (as a potent differentiating agent) on the upregulation of the vitamin D receptor in colon cancer cells.

The antineoplastic properties of (OH)₂D₃ have been well established. (OH)₂D₃ inhibits growth and induces differentiation in a number of neoplastic cell lines such as the HL-60 human promyelocytic cell line (28), in breast cancer cells (29), colon cancer cells (30–32), malignant melanoma (33) and histiocytic lymphoma (34). Enhanced differentiation after administration of (OH)₂D₃ was also observed in nontransformed cells, such as those of normal human bone (35) and monocytes (36). Suppression of neoplastic growth was not observed in tumors lacking the vitamin D receptor, nor did it occur after administration of a structurally related metabolite, 1,24,25-trihydroxycholecalciferol, suggesting that the antiproliferative effect of (OH)₂D₃ is specific and exclusively receptor mediated (37).

The vitamin D receptor, present in "classical" vitamin D-responsive organs such as bone, kidney and intestine has also been localized in a variety of other normal tissues and several cancer cell lines, including the Caco-2 cell line (30–32). The actions of (OH)₂D₃ in regulating transcription of the genes involved in calcium and phosphate regulation, metabolism of vitamin D, DNA replication and differentiation, have all been attributed to the high affinity binding of (OH)₂D₃ to its receptor (38). An alternative mode of action may be that (OH)₂D₃ inactivates estrogen, which has been implicated in the development of colorectal cancer (39). Therefore, it is logical to propose that the substance causing overexpression of the vitamin D receptor in neoplastic cells may provide a valuable tool to enhance its antiproliferative effect in cancer. Our results, which show that butyrate (in the form of its prodrug, tributyrin) upregulates the expression of the vitamin D receptor and enhances the antiproliferative action of (OH)₂D₃ in human colon cancer cells, may therefore point out a novel method to impair colon cancer cell growth. The improved bioavailability of butyrate (in the form of tributyrin) may create an opportunity for its possible therapeutic and chemopreventive applications, especially if synergy *in vivo*, confirming *in vitro* studies, can be demonstrated with (OH)₂D₃ and its analogs. Combinations of tributyrin and (OH)₂D₃ (or its fluorinated analogs) may allow such a drug to be used in cancer patients. A promising application ensuring enhanced delivery may include encapsulation of both tribu-

tyrin and (OH)₂D₃ into liposomes or their conjugation to an appropriate antibody delivery system. Studies designed to investigate this approach are in progress.

LITERATURE CITED

- Scheppach, W. (1994) Effects of short chain fatty acids on gut morphology and function. *Gut* 35 (suppl. 1): S35–S38.
- Gillet, R., Jeannesson, P., Sefraoui, H., Arnould-Guerin, M. L., Kirkiacharian, S., Jardillier, J. C. & Pieri, F. (1998) Piperazine derivatives of butyric acid as differentiating agents in human leukemic cells. *Cancer Chemother. Pharmacol.* 41: 252–255.
- Toscani, A., Soprano, D. R. & Soprano, K. J. (1988) Molecular analysis of sodium butyrate-induced growth arrest. *Oncogene Res.* 3: 223–238.
- Mickle, L. A., Bates, S. E., Richert, N. D., Currier, S., Tanaka, S., Foss, F., Rosen, N. & Fojo, A. T. (1989) Modulation of the expression of a multidrug resistance gene (mdr-1/P-glycoprotein) by differentiating agents. *J. Biol. Chem.* 264: 18031–18040.
- Taylor, C. W., Kim, Y. S., Childress-Fields, K. E. & Yeoman, L. C. (1992) Sensitivity of nuclear c-myc levels and induction to differentiation-inducing agents in human colon tumor cell lines. *Cancer Lett.* 62: 95–105.
- Wächtershäuser, A., Loitsch, S. M. & Stein, J. (2000) PPAR- γ is selectively upregulated in Caco-2 cells by butyrate. *Biochem. Biophys. Res. Commun.* 272: 380–385.
- Hague, A., Diaz, G. D., Hicks, D. J., Krajewski, S., Reed, J. C. & Paraskeva, C. (1997) Bcl-2 and bak may play a pivotal role in sodium butyrate-induced apoptosis in colonic epithelial cells; however overexpression of bcl-2 does not protect against bak-mediated apoptosis. *Int. J. Cancer* 72: 898–905.
- Tanaka, Y., Bush, K. K., Eguchi, T., Ikekawa, N., Taguchi, T., Kobayashi, Y. & Higgins, P. J. (1990) Effects of 1,25-dihydroxyvitamin D₃ and its analogs on butyrate-induced differentiation of HT-29 human colonic carcinoma cells and on the reversal of the differentiated phenotype. *Arch. Biochem. Biophys.* 276: 415–423.
- Yoneda, T., Aya, S. & Sakuda, M. (1984) Sodium butyrate (SB) augments the effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) on neoplastic and osteoblastic phenotype in clonal rat osteosarcoma cells. *Biochem. Biophys. Res. Commun.* 121: 796–801.
- Yoshida, M., Tanaka, Y., Eguchi, T., Ikekawa, N. & Saijo, N. (1992) Effect of hexafluoro-1,25-dihydroxyvitamin D₃ and sodium butyrate combination on differentiation and proliferation of HL-60 leukemia cells. *Anticancer Res.* 12: 1947–1952.
- Stein, J., Schröder, O., Milovic, V. & Caspary, W. F. (1995) Mercaptopropionin inhibits butyrate uptake in isolated apical membrane vesicles of the rat distal colon. *Gastroenterology* 108: 673–679.
- Schröder, O., Opritz, J. & Stein, J. (2000) Substrate and inhibitor specificity of butyrate uptake in apical membrane vesicles of the rat distal colon. *Digestion* 62: 152–158.
- Stein, J., Zores, M. & Schröder, O. (2000) Short-chain fatty acid (SCFA) uptake into Caco-2 cells by a pH-dependent and carrier mediated transport mechanism. *Eur. J. Nutr.* 39: 121–125.
- Schröder, C., Eckert, K. & Maurer, H. R. (1998) Tributyrin induces growth inhibitory and differentiating effects on HT-29 colon cancer cells *in vitro*. *Int. J. Oncol.* 13: 1335–1340.
- Egorin, M. J., Yuan, Z. M., Sentz, D. L., Plaisance, K. & Eiseman, J. L. (1999) Plasma pharmacokinetics of butyrate after intravenous administration of sodium butyrate or oral administration of tributyrin or sodium butyrate to mice and rats. *Cancer Chemother. Pharmacol.* 43: 445–453.
- Heerd, B. G., Houston, M. A., Anthony, G. M. & Augenlicht, L. H. (1999) Initiation of growth arrest and apoptosis of MCF-7 mammary carcinoma cells by tributyrin, a triglyceride analogue of the short-chain fatty acid butyrate, is associated with mitochondrial activity. *Cancer Res.* 59: 1584–1591.
- Conley, B. A., Egorin, M. J., Tait, N., Rosen, D. M., Sausville, E. A., Dover, G., Fram, R. J. & Van Echo, D. A. (1998) Phase I study of the orally administered butyrate prodrug, tributyrin, in patients with solid tumors. *Clin. Cancer Res.* 4: 629–634.
- Chen, Z. X. & Breitman, T. R. (1994) Tributyrin: a prodrug of butyric acid for potential clinical application in differentiation therapy. *Cancer Res.* 54: 3494–3499.
- Rocchi, P., Ferreri, A. M., Magrini, E. & Perocco, P. (1998) Effect of butyrate analogues on proliferation and differentiation in human neuroblastoma cell lines. *Anticancer Res.* 18: 1099–1103.
- Watkins, S. M., Carter, L. C., Mak, J., Tsau, J., Yamamoto, S. & German, J. B. (1999) Butyric acid and tributyrin induce apoptosis in human hepatic tumour cells. *J. Dairy Res.* 66: 559–567.
- Westergren-Thorsson, G., Onnervik, P. O., Fransson, L. A. & Malmstrom, A. (1991) Proliferation of cultured fibroblasts is inhibited by L-iduronate-containing glycosaminoglycans. *J. Cell Physiol.* 147: 523–530.
- Klaus, G., May, T., Hügel, U., Von Eichel, B., Rodriguez, J., Fernandez, P., Reichrath, J., Ritz, E. & Mehls, O. (1997) Parathyroid hormone prevents 1,25(OH)₂D₃ induced down-regulation of the vitamin D receptor in growth plate chondrocytes *in vitro*. *Kidney Int.* 52: 45–51.
- Burmester, J. K., Maeda, N. & Deluca, H. F. (1988) Isolation and expression of rat 1,25-dihydroxyvitamin D₃ receptor cDNA. *Proc. Natl. Acad. Sci. U.S.A.* 85: 1005–1009.
- Delzenne, N. M., Kok, N., Deloyer, P. & Dandriofosse, G. (2000) Dietary

fructans modulate polyamine concentration in the cecum of rats. *J. Nutr.* 130: 2456–2460.

25. Durantón, B., Nsi-Emvo, E., Schleiffer, R., Gosse, F., Galluser, M. & Raul, F. (1997) Suppression of preneoplastic changes in the intestine of rats fed low levels of polyamines. *Cancer Res.* 57: 573–575.

26. Ellmerich, S., Scholler, M., Durantón, B., Gosse, F., Galluser, M., Klein, J. P. & Raul, F. (2000) Promotion of intestinal carcinogenesis by *Streptococcus bovis*. *Carcinogenesis* 21: 753–756.

27. Murphy, G. M. (1996) Human bile acid kinetics: if a straight line justifies the means is the 'intestinal factor' confined to the small intestine? *Eur. J. Gastroenterol. Hepatol.* 8: 7–9.

28. Miyaoura, C., Abe, E., Kuribayashi, T., Tanaka, H., Konno, K., Nishii, Y. & Suda, T. (1981) 1,25-Dihydroxyvitamin D₃ induces differentiation of human myeloid leukemia cells. *Biochem. Biophys. Res. Commun.* 102: 937–943.

29. Colston, K. W., Berger, U. & Coombes, R. C. (1989) Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet* 1: 188–191.

30. Giuliano, A. R., Franceschi, R. T. & Wood, R. J. (1991) Characterization of the vitamin D receptor from the Caco-2 human colon carcinoma cell line: effect of cellular differentiation. *Arch. Biochem. Biophys.* 285: 261–269.

31. Hulla, W., Kallay, E., Krugluger, W., Peterlik, M. & Cross, H. S. (1995) Growth control of human colon-adenocarcinoma-derived Caco-2 cells by vitamin-D compounds and extracellular calcium in vitro: relation to c-myc-oncogene and vitamin-D-receptor expression. *Int. J. Cancer* 62: 711–716.

32. Cross, H. S., Hulla, W., Tong, W. M. & Peterlik, M. (1995) Growth

regulation of human colon adenocarcinoma-derived cells by calcium, vitamin D and epidermal growth factor. *J. Nutr.* 125 (suppl. 7): 2004S–2008S.

33. Colston, K. W., Colston, M. J. & Feldman, D. (1981) 1,25-Dihydroxyvitamin D₃ and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology* 108: 1083–1086.

34. Olsson, I., Gullberg, U., Ivhed, I. & Nilsson, K. (1983) Induction of differentiation of the human histiocytic lymphoma cell line U-937 by 1,25-dihydroxycholecalciferol. *Cancer Res.* 43: 5862–5867.

35. Beresford, J. N., Gallagher, J. A. & Russell, R.G.G. (1986) 1,25-Dihydroxyvitamin D₃ and human bone-derived cells in vitro: effects on alkaline phosphatase, type I collagen and proliferation. *Endocrinology* 119: 1776–1785.

36. Brungs, M., Radmark, O., Samuelsson, B. & Steinhilber, D. (1995) Sequential induction of 5-lipoxygenase gene expression and activity in Mono Mac 6 cells by transforming growth factor β and 1,25-dihydroxyvitamin D₃. *Proc. Natl. Acad. Sci. U.S.A.* 92: 107–111.

37. Halline, A. G., Davidson, N. O., Skarosi, S. F., Sitrin, M. D., Tietze, C., Alpers, D. H. & Brasitus, T. A. (1994) Effects of 1,25-dihydroxyvitamin D₃ on proliferation and differentiation of Caco-2 cells. *Endocrinology* 134: 1710–1717.

38. Segaert, S. & Bouillon, R. (1998) Vitamin D and regulation of gene expression. *Curr. Opin. Clin. Nutr. Metab. Care* 1: 347–354.

39. English, M. A., Hughes, S. V., Kane, K. F., Langman, M. J., Stewart, P. M. & Hewison, M. (2000) Oestrogen inactivation in the colon: analysis of the expression and regulation of 17 β -hydroxysteroid dehydrogenase isozymes in normal colon and colonic cancer. *Br. J. Cancer* 83: 550–558.