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Apoptotic Death in Adenocarcinoma Cell Lines Induced by Butyrate and Other Histone Deacetylase Inhibitors

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ABSTRACT. *n*-Butyrate inhibits the growth of colon cancer cell lines. In the HCT 116 cell line, butyrate-induced growth inhibition is almost fully reversible, whereas in the VACO 5 cell line, a subpopulation undergoes apoptosis within 30 hr of treatment with butyrate. Concurrent treatment of VACO 5 cells with butyrate and the phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) accelerates and increases the incidence of cell death to nearly 100% of the population, whereas HCT 116 cells largely remain alive during treatment with this combination. The action of butyrate as an inhibitor of histone deacetylase was assessed in these cell lines by examining extracted core histones for their electrophoretic mobility in Triton/acid/urea gels. The concentrations of butyrate that were effective for inducing apoptosis were similar to the concentrations that caused hyperacetylation of core histones in the VACO 5 cell line. Furthermore, an examination of other carboxylic acids for induction of apoptosis revealed a rank order that corresponded to the order of potency in causing hyperacetylation of core histones. Specifically, the active acids were 3–5 carbons in length and lacked substitution at the 2-position. Isovaleric and propionic acids, in particular, proved to be effective inducers of both hyperacetylation and apoptosis at 5 mM concentrations, a finding of potential relevance to the unusual pancytopenia occurring after acidotic episodes in isovaleric and propionic acidemias. The duration of butyrate treatment required for chromatin fragmentation (10–20 hr) corresponded to the time required for histone H4 to become predominantly tetraacetylated. Furthermore, trichostatin A, a structurally dissimilar inhibitor of histone deacetylase, mimicked butyrate-induced apoptosis of VACO 5 cells and growth inhibition of HCT 116 cells. The dramatic enhancement of VACO 5 cell death by TPA, and the high level resistance of HCT 116 cells to butyrate were not evident from histone acetylation determinations. Thus, applications of butyrate for cytoreduction therapy will benefit from pharmacodynamic assessment of histone acetylation, but will require additional work to predict susceptibility to butyrate-induced death. BIOCHEM PHARMACOL 53;9:1357–1368, 1997.

KEY WORDS. apoptosis; butyric acid; phorbol ester; colon cancer; isovaleric acid; histone acetylation; trichostatin

Butyrate and other SCFA** induce phenotypic maturation in many eukaryotic cell types, and have been demonstrated repeatedly to cause growth arrest and reversal of neoplastic characteristics in cultured cells [1]. Butyrate has considerable appeal for therapy, given the lack of acute cytotoxicity in normal tissues even at high concentrations. In fact, butyrate promotes the survival of normal colonic epithelia, in culture and *in vivo* [2–6]. Many cell lines derived from neoplastic tissues have also been found to survive butyrate treatment despite a reversible growth arrest [7–10]. However, some cell lines, especially several derived from lymphoma, ovarian cancer, and adenocarcinoma of prostate, colon, and breast, have been found to undergo terminal differentiation and/or apoptosis when treated with millimolar concentrations of sodium *n*-butyrate [9–18].

Butyrate (at 1–10 mM) and the other SCFA (acetate, propionate, and valerate, at nearly 100 mM in aggregate) are present within the colonic lumen as a result of bacterial fermentation of fiber and non-digested starch [19, 20]. These endogenous SCFA are active on colonic mucosal cells, as evident from analysis of core histone acetylation

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** *Abbreviations:* NIB, nucleochromatin isolation buffer; PKC, protein kinase C; SCFA, short-chain fatty acid(s); TAU, Triton–acetic acid–urea polyacrylamide gel; TPA, 12-O-tetradecanoylphorbol 13-acetate; and TsA, trichostatin A.

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[20] (see below). Animals maintained germ-free or on total parenteral nutrition suffer from atrophy of the intestinal mucosa, a situation that can be ameliorated by instillation of butyrate and other SCFA into the colorectum [4]. In addition, bovine milk fat is approximately 10% butyrate triglyceride by weight. In humans, sodium butyrate is an effective therapy for certain types of ulcerative colitis, allowing the healing of mucosal ulcers and promoting the survival of colonic epithelial cells [2, 3]. In consideration of the death-inducing actions of SCFA on neoplastic cells, fermentation-derived SCFA have been suggested to be responsible for some of the preventive actions of dietary fiber against colorectal cancer [13, 20]. The mechanisms by which SCFA might determine this differential maintenance of survival by normal and neoplastic cells are experimentally accessible in cultured cells.

The many actions of fatty acids on cells can be distinguished on the basis of structure–activity relationships, indicating independent subcellular targets. Such actions include alteration of cell volume and intracellular pH [21, 22], modulation of cytoskeletal structures [23], inhibition of histone deacetylase [24–26], stimulation of phospholipid synthesis [27], activation of mitochondrial gene expression [28], and metabolism as acyl-CoA species via numerous pathways (e.g. beta-oxidation or alkyl chain elongation). While propionate, *n*-butyrate, and isobutyrate are roughly equivalent in several of these respects, *n*-butyrate is the most active SCFA in inhibiting the histone deacetylase [24].

Nucleosomal histone acetylation is determined by the opposing actions of acetyltransferase and deacetylase enzymes [24–26]. The charge neutralization of core histones (H2A, H2B, H3, and H4) and certain high mobility group proteins, which is effected by lysine-acetylation, is thought to relax chromatin supercoils and allow access to DNA by proteins such as transcription factors and DNA repair/replication enzymes. Physiologically relevant histone acetylation may be limited to short-lived, region-specific hyperacetylation, superimposed upon low level acetylation of bulk histones. By inhibiting the deacetylase with butyrate (or with the antibiotic TsA [29]), the acetyltransferase is allowed to proceed unopposed, leading to progressive hyperacetylation of histones in bulk chromatin. Despite the profound, global changes in chromatin conformation accompanying this non-specific hyperacetylation, most cells exhibit only a reversible growth inhibition and modest alteration of gene expression. However, some cell types survive with a differentiated phenotype upon butyrate removal, while others rapidly undergo apoptotic cell death.

As an initial investigation into the mechanism by which butyrate causes death in certain colon cancer cell lines, we examined acetylation of core histones after treating cells with a series of SCFA species. These results, and others characterizing histone acetylation as an indicator of butyrate action, point to this chromatin modification as a central element of the mechanism of apoptosis-inducing activity of SCFA.

MATERIALS AND METHODS

Materials

Organic acids were purchased from the Aldrich Chemical Co., Milwaukee, WI. Sodium salts of each were prepared by neutralizing aqueous solutions with NaOH. TPA was purchased from LC Services, Inc., Woburn, MA, dissolved in 95% ethanol, and stored at −20° for up to 3 years without loss of biological activity. TsA was obtained from Dr. Minoru Yoshida, Faculty of Agriculture, University of Tokyo, and was dissolved in 95% ethanol and stored at −20°. Proteinase K and Triton X-100 were purchased from Boehringer Mannheim, Indianapolis, IN. Triton X-114 and soybean trypsin inhibitor were purchased from the Fluka Chemical Corp., Ronkonkoma, NY. Protamine sulfate, histone III-S, and cytochrome *c* were purchased from the Sigma Chemical Co., St. Louis, MO.

Cell Culture

The VACO 5 colon cancer cell line was established from the primary tumor of a patient with Duke's stage D colon adenocarcinoma [30]. The HCT 116 cell line was obtained from the American Type Culture Collection, Rockville, MD. Both cell lines were grown in DMEM with 2% fetal bovine serum (GIBCO, Inc., Grand Island, NY). Cells were disaggregated for passage by incubating in calcium-free medium (S-MEM) for 1–6 hr, then shaking the cells loose, and scraping adherent cells from the flask. For the seeding of multiwell dishes, cell suspensions were additionally sieved through 42 μm Nitex (Tetko, Elmsford, NY). Cultures for assay were typically set up at 2–5 × 10^5 cells/1.5 mL in 10 cm^2 wells and grown for 2–4 days before use. Incubations were usually initiated by adding the indicated agents as a 4-fold concentrated solution.

Cell Number Determination

Both floating and adherent cells were rinsed and incubated for 1 hr in S-MEM to loosen cell aggregates, and then treated with trypsin [final concentrations-0.1% trypsin (1:200 strength, GIBCO), 0.01% ethylene glycol *O,O′* bis (2-aminoethyl) *N,N,N′,N′* tetraacetic acid, and 0.01% dextran sulfate 8000] for 30 min to dissociate residual cell clusters, and to discriminate viable cells from debris and damaged cells. Trypsin action was terminated by adding soybean trypsin inhibitor (final concentration 100 μg/mL plus calf serum at 5% and trypan blue at 200 μg/mL). The cell suspensions were kept on ice for 1–5 hr until counting by a hemocytometer. Cells were distinguished from apoptotic fragments by being larger than ~10 μm in diameter, and distinguished from debris and necrotic cells by phase brightness, resistance to trypsin [31], and trypan blue exclusion. In experiments to assess the growth abilities of cells that had been treated for defined periods of time, the non-adherent cells were collected by centrifugation (500 *g*, 10

min, 4°), rinsed with complete culture medium to remove the indicated agents, and added back to dishes containing adherent cells in fresh culture medium.

Statistical analyses for treatment differences were done with Instat statistical software (version 2.0.3 for Macintosh, GraphPad Software, San Diego, CA). All P values were two-tailed and calculated using Student's t-test.

Histone Acetylation

The extraction and electrophoretic separation of chromatin proteins were performed by modified versions of the methods of Loidl and Gröbner [32] and Zweidler [33], respectively. Cells were collected by scraping and centrifugation (500 g, 10 min, 4°), lysed with 0.5% Triton X-100 in NIB (60 mM KCl, 15 mM NaCl, 3 mM $MgCl_2$, 15 mM MOPS, 0.1 mM phenylmethylsulfonyl fluoride, 0.25 M sucrose, 5 mM n-butyrate), and centrifuged (15,000 g, 5 min, 4°) to obtain a crude chromatin and cytoskeletal fraction. These pellets were washed in NIB and stored in 25% glycerol:75% NIB at −80°. Histones were displaced from DNA using protamine; pelleted nuclear residues were treated with approximately 0.2 mg of protamine sulfate per million cells (estimated by measuring detergent-soluble protein on each sample and on a known number of cells) after chelating Mg^{2+} with 10 mM EDTA, and displaced proteins were collected by adding 8 M urea in 5% acetic acid. An aliquot of the extract corresponding to approximately 200,000 cells was electrophoresed immediately on continuous 12% polyacrylamide gel (0.32% bis-acrylamide) in Triton/acid/urea (6 mM Triton X-114, 5% acetic acid, and 8 M urea) using 5% acetic acid as tank buffer. Gels were preelectrophoresed twice, including a mercaptoethylamine sweep [33]. Pyronin Y was employed as a marker dye, and cytochrome c (20 μg) and histone III-S (2 μg) were used as migration markers; when the cytochrome c (naturally brown in color) had electrophoresed 80% of the way to the bottom, the run was terminated and proteins were fixed with 25% trichloroacetic acid, and stained with 0.1% Coomassie blue R-250. After destaining in 20% ethanol/5% acetic acid, the gel was impregnated with 5% glycerol in 5% acetic acid and dried between cellophane sheets.

DNA fragmentation

Apoptotic cleavage of DNA was assessed by agarose gel electrophoresis as previously described [34]. Briefly, adherent cells were scraped from the dish and together with previously detached cells were collected from the culture medium by centrifugation (500 g, 10 min, 4°) and then counted. One million cells each were placed in sample slots of a 2% agarose gel, where they were lysed and digested with SDS, proteinase K, and ribonuclease A. After electrophoresis in 40 mM Tris-acetate with 2 mM EDTA, at 2 V/cm for 18 hr, DNA was stained with ethidium bromide and photographed under UV light.

RESULTS

Inhibition of Growth and Induction of Apoptosis by SCFA

The VACO 5 cell line grows as poorly differentiated round cells with a doubling time of approximately 35 hr. This cell line was assessed for growth inhibition and death during continuous exposure to butyrate (Fig. 1). n-Butyrate caused a combination of growth arrest and cell loss over the first 48 hr, but continuing butyrate treatment beyond 48 hr led to little additional reduction in cell survival. Cultures treated with butyrate for the first 24 hr and then refed with butyrate-free medium also exhibited high rates of cell death during the subsequent 24 hr, indicating that events relevant to cell death were instated in a large proportion of cells during the initial 24-hr period. Cells that survived butyrate exposure resumed growth after butyrate removal, as indicated by the increase in cell number after 48 hr.

n-Butyrate and a series of other 2, 4, or 6 carbon fatty acids (SCFA) were tested for effects upon growth and survival. Figure 2 demonstrates that n-butyrate was the only acid in this series that completely blocked cell replication; furthermore, the final cell yield after 4 days of growth in 5 mM butyrate was 65% of the starting cell number, indicating the death of ≥ 35% of the cells. Among the other SCFA, only caproate treatment decreased the cell yield, but such cells nonetheless exhibited substantial growth despite the continued presence of this 6-carbon fatty acid.

The combination of n-butyrate and TPA can lead to the death of nearly 100% of cells in certain colon cancer lines, accelerating and increasing the incidence of butyrate-

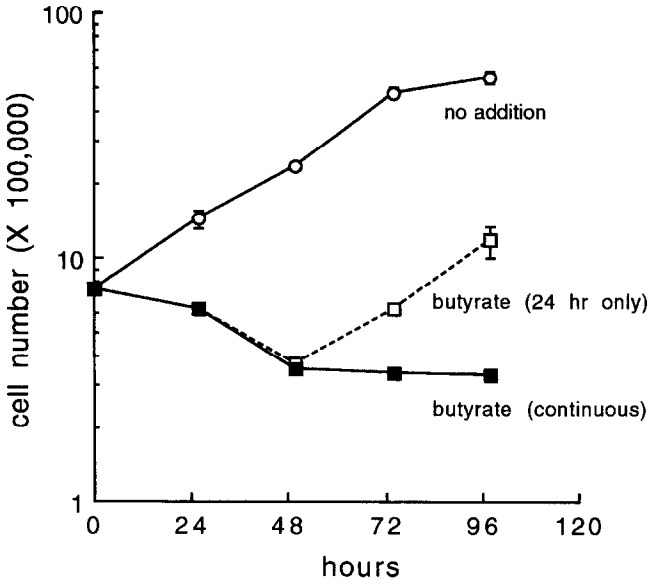

FIG. 1. Effect of butyrate on growth and survival of VACO 5 cells. Sodium n-butyrate was added to logarithmically growing VACO 5 cells for a final concentration of 5 mM. After 24 hr, some dishes were washed and refed with butyrate-free culture medium and reincubated. Cells were counted at the indicated time. The average cell number and range (bars) for two dishes each are presented.

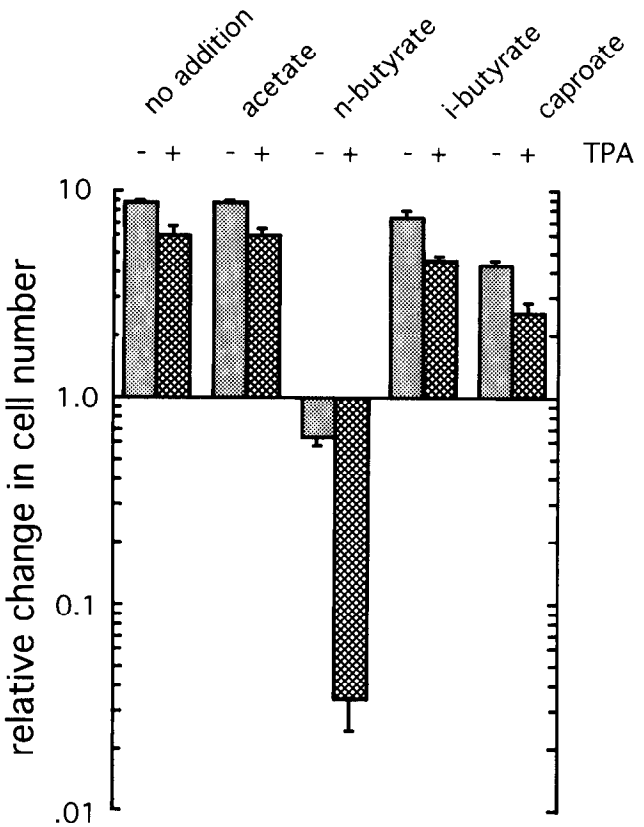

FIG. 2. Structure–activity relationship of SCFA for growth and death of VACO 5 cells. Cultures were treated with sodium salts of each fatty acid (5 mM each) in the absence (stippled bars) or in the presence (cross-hatched bars) of 100 nM TPA. Cells were counted after 4 days of continuous treatment. i-Butyrate-iso-butyrate. Data are the average and standard deviation of the ratio of the final cell number relative to the starting cell number (200,000/per 10 cm^2 dish) for three dishes. Significant differences were found by Students *t*-test (two-tailed) between final control cell number and final cell number for cells treated with *n*-butyrate ($P < 0.0001$), caproate ($P < 0.0001$), and iso-butyrate ($0.025 < P < 0.05$). *n*-Butyrate also effected a significant loss of cells relative to the starting number ($P < 0.02$), especially in the presence of TPA ($P < 0.0001$).

induced apoptosis [10]. To determine whether any of the other SCFA which had proven inactive in growth suppresion could nonetheless induce cell death in the presence of TPA, cultures were treated concurrently with 5 mM of each neutralized acid and 100 nM TPA (Fig. 2). Although TPA by itself had little effect on cell yield, the combined presence of *n*-butyrate and TPA decreased the cell number by 97% within 4 days. In the presence of the other SCFA, TPA decreased cell yields by substantially less: cell yields were reduced by 30–40% relative to cultures treated with each SCFA alone, including caproate. Thus, within this limited series, *n*-butyrate was again the only SCFA that caused appreciable levels of cell death, despite additional treatment with TPA.

DNA fragmentation analysis can be used as a sensitive qualitative screening method for determining the relative activity among agents for induction of apoptotic cell death [34]. Expanding on the findings in Fig. 2, a larger series of short, medium, and branched chain-carboxylic acids was assessed to establish a rank-ordered structure–activity relationship for VACO 5 cell death. For each of the SCFA, a 5 mM concentration was applied for 18 or 30 hr, alone or in the additional presence of 20 nM TPA. Cells were then harvested, lysed, and analyzed electrophoretically for DNA integrity. The results shown in Fig. 3 demonstrate that *n*-butyrate was the only acid that as a single agent yielded prominent internucleosomal fragmentation. In the additional presence of TPA, apoptotic DNA cleavage and loss of high molecular weight genomic DNA were pronounced, even at concentrations of 1.6 mM *n*-butyrate. In the presence of TPA, several other short- and branched-chain acids (valeric, isovaleric, and propionic acids) also induced DNA fragmentation. Caproic acid and those SCFA with methyl substitution at the 2-position (isobutyric, pivalic, and 2-methylbutyric acids) yielded little increase in DNA fragmentation at 5 mM.

Histone Acetylation after Treatment with SCFA

The aforegoing studies indicated that butyrate was nearly unique among carboxylic acids in its ability to induce apoptosis. Even then, treatment for extended periods of time and/or concurrent treatment with TPA was required to obtain high levels of apoptosis. One target of SCFA action which has been reported to have a corresponding specificity for *n*-butyrate is inhibition of histone deacetylase. Accordingly, we compared the relative ability of each of the SCFA to cause histone hyperacetylation in VACO 5 cells. Histones were extracted from cells and separated on TAU gels to resolve core histone acetylforms (Fig. 4). Histone H4 and its acetylforms have been used as indicators of histone acetylation in general, as these proteins manifest acetylation-dependent charge variation characteristic of the entire family of nucleosomal core histones, and can be separated readily as a family from other chromatin proteins [33]. In the absence of butyrate, the majority of H4 from VACO 5 cells was present as nonacetylated and the mono-acetylated species. After treatment with butyrate at concentrations of 5 mM for 24 hr, tetraacetylated H4 was the major isoform among the H4 species (Fig. 4a); concentrations as low as 0.5 mM *n*-butyrate also caused increased H4 tetraacetylation within 24 hr. The time–course for hyperacetylation was also examined during treatment with 5 mM butyrate (Fig. 4b). Tetraacetylated H4 species became faintly visible after 30 min, prominent after 90 min, and predominant after 24 hr of treatment with 5 mM *n*-butyrate. Upon removal of butyrate, the rate of deacetylation was extremely rapid, leading to predominantly singly-acetylated H4 within 30 min and non-acetylated H4 within 90 min. Similar concentration–response and time–course characteristics were evident for H3, H2B, and H2A.

This sensitive measure of *n*-butyrate response was used to compare the other SCFA for their relative abilities to induce histone hyperacetylation. VACO 5 cells were treated

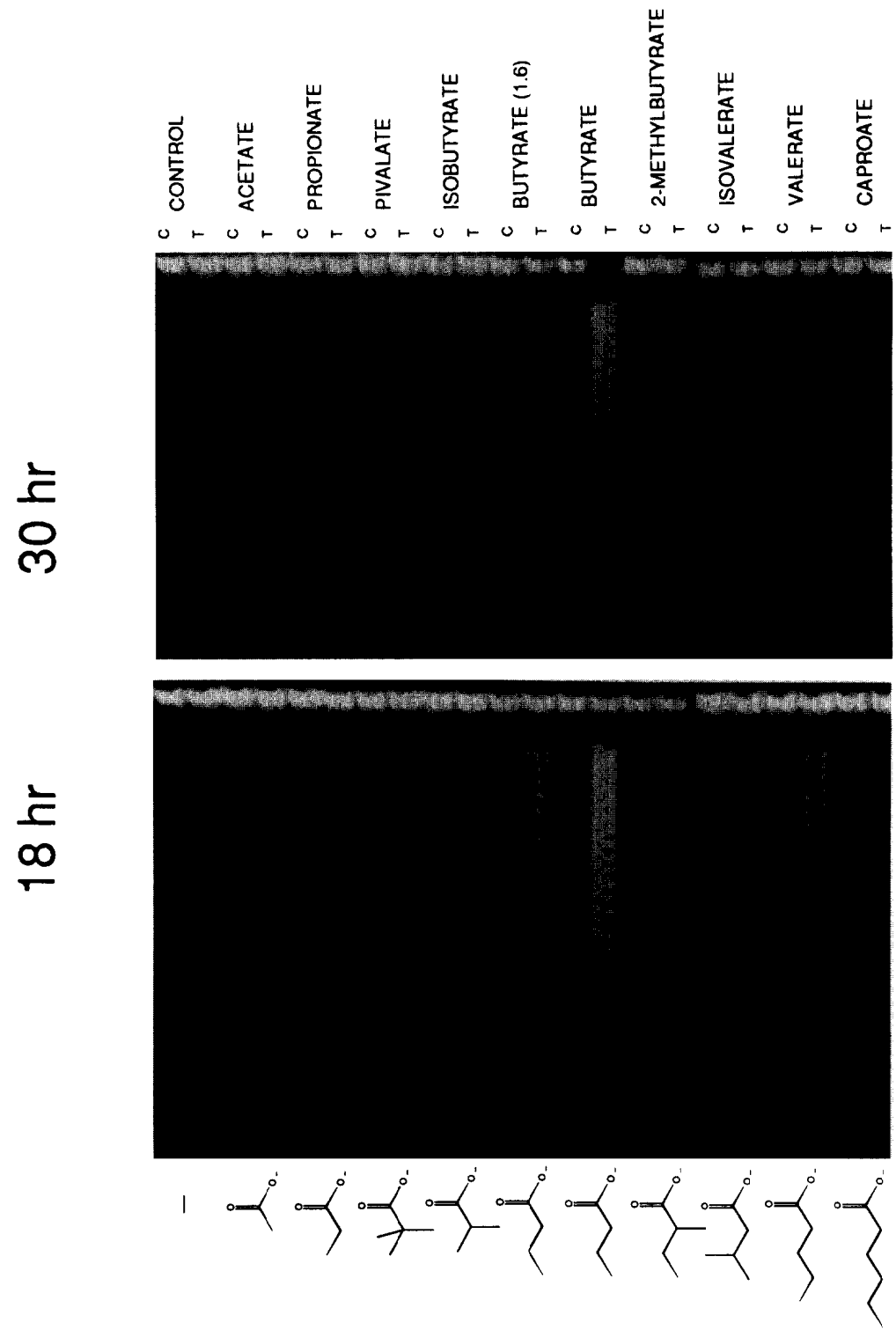

FIG. 3. Structure–activity relationship of short- and branched-chain carboxylic acids on DNA cleavage in VACO 5 cells. Cultures were treated with 5 mM concentrations of sodium salts for each of the SCFA shown; 1.6 mM *n*-butyrate was also included, as indicated. For each treatment, duplicate dishes also received either 20 nM TPA or a vehicle control addition at time 0 hr. At 18 or 30 hr, cells were harvested and analyzed for DNA fragmentation. Results are typical of 2–4 experiments for each fatty acid.

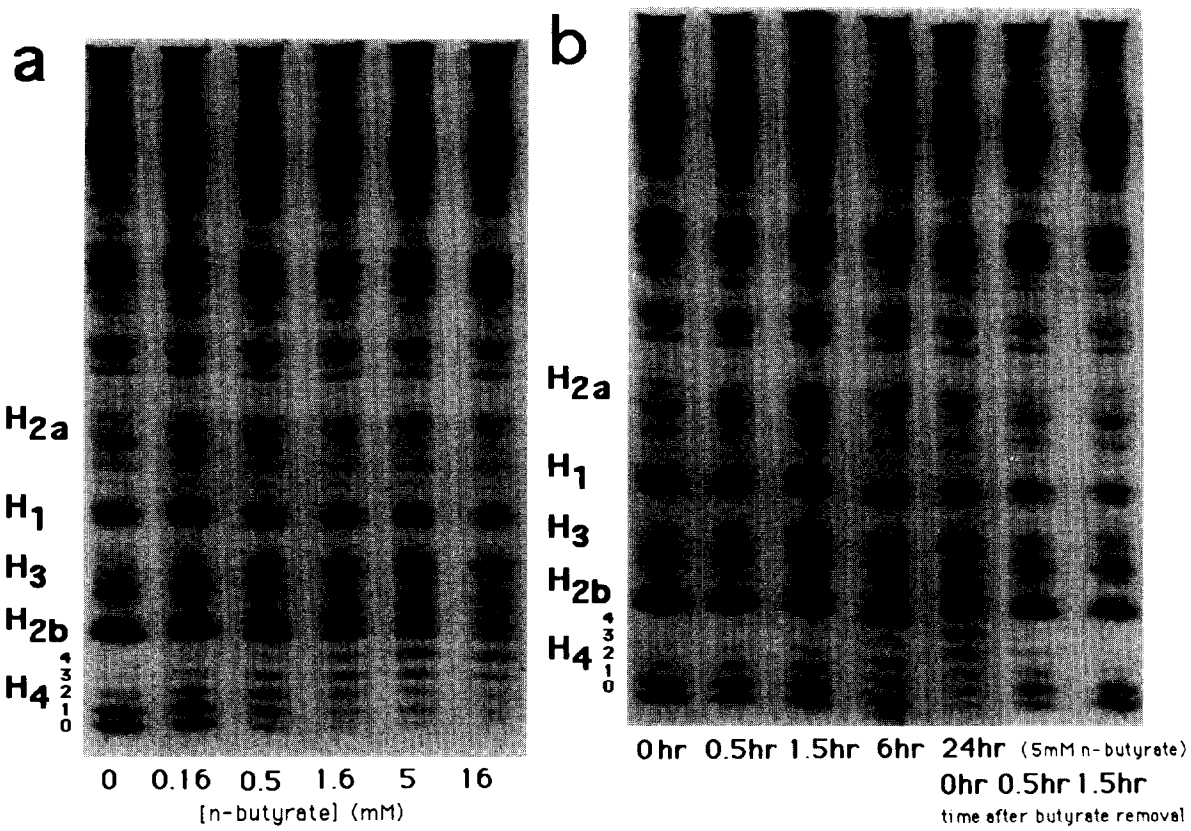

FIG. 4. Concentration and time relationship of butyrate treatment on histone acetylation and deacetylation in VACO 5 cells. (a) Cultures were treated with 0–16 mM *n*-butyrate for 24 hr. Cells were harvested for histone extraction, and analyzed for histone by Triton-acetic acid-urea gel electrophoresis as detailed in Materials and Methods. Superimposable concentration–response curves were obtained in three other experiments. Migration positions of the major histone classes are indicated. (b) Cultures were treated with 5 mM butyrate for the indicated periods of time. Some cultures treated for 24 hr were washed and reincubated in the absence of butyrate for 0.5 or 1.5 hr before harvest. Time–course results for acetylation onset and decay are typical of two and three experiments, respectively.

for 24 hr with 0.5 and 5 mM concentrations of each of the acids examined previously for apoptosis-inducing ability. Cells were extracted and the histone protein fractions electrophoresed on TAU gels; regions corresponding to H4 are indicated (Fig. 5). As was shown for chromatin degradation, *n*-butyrate was the most active of these acids at inducing histone hyperacetylation, with substantial accumulation of hyperacetylated species evident at both 5 and 0.5 mM. Isovalerate, valerate, and propionate were also active at 5 mM. SCFA that were substituted at the 2-position (2-methylbutyrate, isobutyrate, or pivalate) caused little or no increase in histone hyperacetylation. Thus, those SCFA that induced apoptosis were capable of causing hyperacetylation of core histones; furthermore, the extent of hyperacetylation following treatment with 5 mM SCFA was proportional to the extent of DNA cleavage shown in Fig. 3.

The possibility that TPA caused increases in cell death by increasing the extent or rate of hyperacetylation was tested. As shown in Fig. 6, the pattern of core histones resolved by TAU–PAGE indicated that TPA had little effect on histone acetylation, either in the presence or absence of butyrate. These results were obtained at 11 hr of treatment, prior to the period when extensive apoptotic chromatin fragmentation could potentially skew the results. TPA also had no notable effect on the level of butyrate-induced hyperacetylation at shorter (2 or 6 hr) or longer (24 hr) treatment periods, and failed to alter histone acetylation patterns of cells treated with lower concentrations of butyrate (data not shown). Thus, TPA treatment increased the extent of apoptosis without increasing the extent of hyperacetylation, reducing the concentration of butyrate at which H4 tetraacetylation became prominent, or shortening the duration of butyrate treatment required for cells to display maximally acetylated core histones.

Inhibition of Growth and Induction of Apoptosis after Treatment with TsA

Since the action of butyrate as an inhibitor of histone deacetylase appeared to be necessary, if not sufficient for butyrate-induced apoptosis, TsA, a structurally distinct inhibitor of the deacetylase, was compared with butyrate for its growth-modulating activities on VACO 5 cells. As shown in Fig. 7 (top panel), cells treated with 1 μM TsA were growth-inhibited to an extent similar to that of buty-

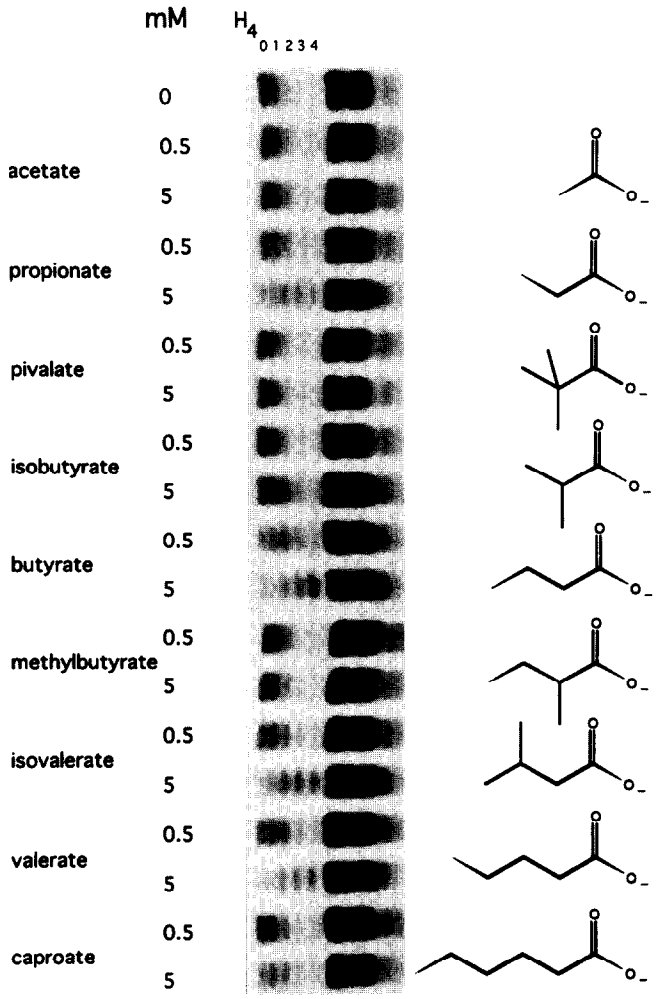

FIG. 5. Structure–activity relationship for SCFA on histone acetylation in VACO 5 cells. Cultures were treated for 24 hr and then harvested for histone extraction. Gel electrophoresis of histones was performed as in Fig. 4, except that only the H4 region of the Triton-acetic acid-urea gel are indicated. Results are typical of at least two experiments for each acid.

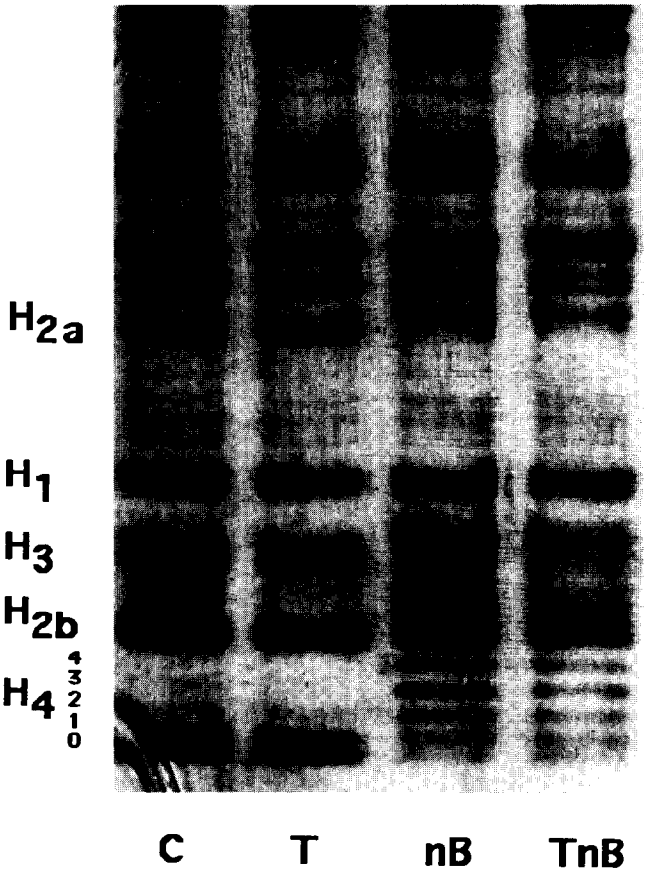

FIG. 6. Effect of TPA on butyrate-induced and basal histone acetylation in VACO 5 cells. Cultures were treated for 11 hr with 0 or 5 mM *n*-butyrate and 0 or 20 nM TPA. Extracted chromatin proteins were analyzed by TAU gel electrophoresis. Key: C, no treatment; T, TPA alone; nB, *n*-butyrate alone; and TnB, TPA/*n*-butyrate combination. Results are typical of three experiments spanning the time from 2 to 24 hr.

rate-treated cells. In this experiment, TsA caused a net 35% reduction in cell number. Upon removal of either agent, surviving cells resumed replication. If instead, cells were treated with either butyrate or TsA in the presence of TPA, then, respectively, a 76 or 91% reduction in cell number was obtained after the 42-hr treatment period. Furthermore, cell death continued after removing these agents from the culture medium: after 3 additional days of culture in the absence of TsA or TPA, cell yields were 0.3% of the starting cell numbers. VACO 5 cultures treated for 18 hr with TsA in the absence or presence of TPA exhibited signs of apoptosis: cell detachment, shrinkage, and internucleosomal DNA cleavage with prominent loss of high M_r DNA (Fig. 8).

Butyrate and TsA Responsiveness of HCT 116 Cells

We next considered the possibility that resistance of some cell lines to butyrate-induced death may be reflected in a lower degree of core histone acetylation during butyrate treatment. In contrast to VACO 5 cells, HCT 116 undergoes reversible growth arrest but little cell death in 5 mM butyrate even after TPA addition (Fig. 7, bottom panel, and [10]). Treatment of HCT 116 cultures with TsA gave results comparable to those seen following butyrate treatment, i.e. growth was inhibited in the presence of the deacetylase inhibitor, but resumed upon removal of the agent. TPA caused insignificant changes in cell number. TPA in conjunction with either butyrate or TsA treatment led to decreased cell yields after subsequent culture when compared with cultures treated with the deacetylase inhibitors alone (Fig. 7). However, DNA fragmentation at 24 hr of treatment was minimal compared with that of VACO 5 cells (Fig. 8), and such cultures instead exhibited morphological indications of intercellular contact-inhibited growth* [10]. As shown in Fig. 9, core histone acetylation in HCT 116 cells was, in fact, strongly accentuated by

* McBain JA, unpublished results.

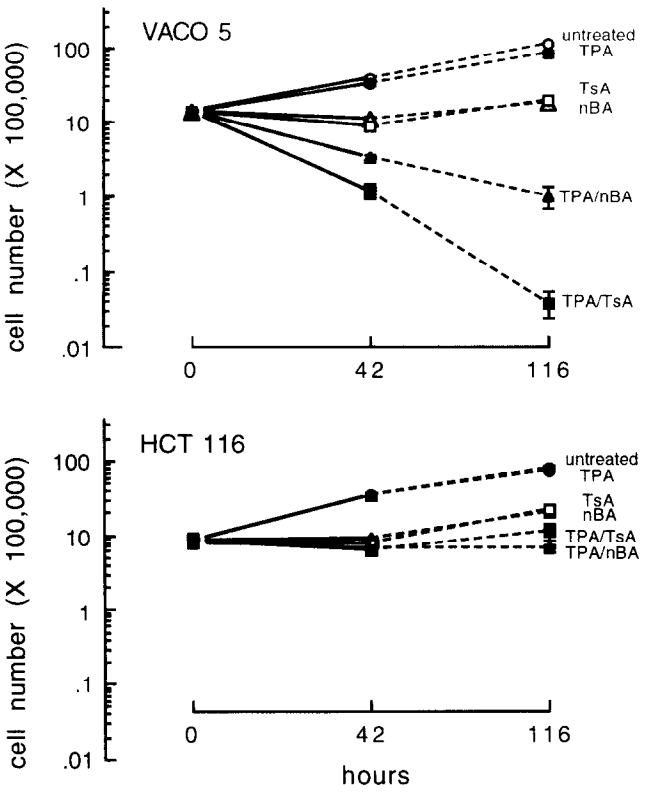

FIG. 7. Reversible growth arrest or cell death in VACO 5 and HCT 116 cultures induced by *n*-butyrate (nBA) or trichostatin A (TsA). Cell cultures were treated with 0 or 5 mM *n*-butyrate, or 1 μM TsA, for 42 hr in the continuous presence of 20 nM TPA and/or 0.025% ethanol (vehicle). Cells were then washed, refed with drug-free culture medium, and either counted or recultured for an additional 74 hr, after which they were again counted. Data are the average number of cells ± SD. The solid line represents incubation in the continuous presence of the agent(s), during which time nBA or TsA effected a highly significant loss of VACO 5 cells ($0.001 > P > 0.0001$), but a non-significant loss of HCT 116 cells ($P > 0.4$). The combination of either nBA or TsA with TPA effected an extremely significant loss of VACO 5 cells ($P < 0.0001$), but a non-significant loss of HCT 116 cells ($P > 0.1$). The difference between cell numbers in cultures treated with vehicle alone and with TPA was slightly significant for VACO 5 cells ($P = 0.024$), but not for HCT 116 ($P > 0.4$) in which the growth curves are essentially superimposed. The dashed line represents the subsequent growth of cells in culture medium alone. Closed symbols, 20 nM TPA; open symbols, absence of TPA. Circles and triangles, 0 and 5 mM nBA, respectively; squares, 1 μM TsA.

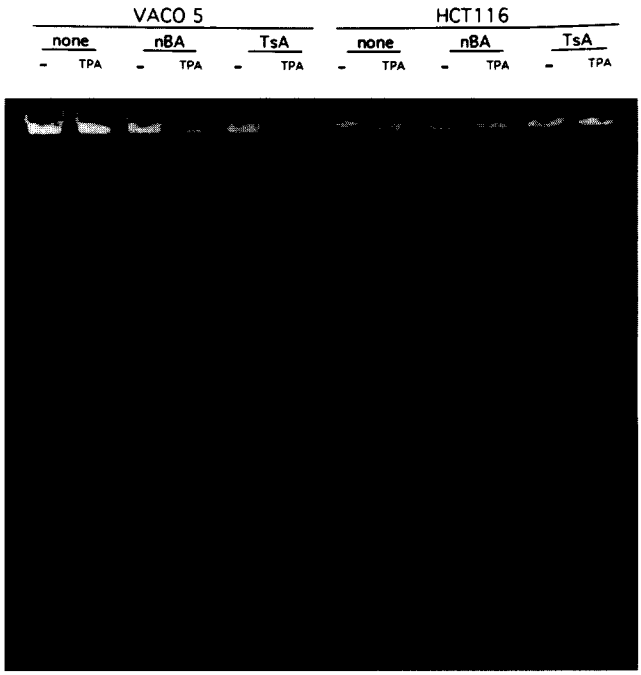

FIG. 8. Comparison of *n*-butyrate (nBA) and trichostatin A (TsA) for induction of DNA cleavage in VACO 5 and HCT 116 cell cultures. Cultures were treated with 5 mM nBA or 1 μM TsA, either in the absence or presence of 20 nM TPA. At 24 hr, cells were harvested and analyzed for DNA fragmentation. Results are typical of two experiments.

butyrate. In HCT 116 cells treated with 5 mM butyrate, tetraacetylated H4 accumulated to a proportionately greater extent in comparison with that seen in VACO 5 cells. Hence, histone hyperacetylation alone is insufficient to precipitate apoptosis despite auxiliary treatment with TPA.

DISCUSSION

The data presented in this paper indicate that butyrate-induced apoptosis in the VACO 5 colon cancer cell line requires nearly maximal levels of acetylation of core histones. In addition to dependence on millimolar *n*-butyrate concentrations, a lag period is evident during treatment in which little cell death occurs and during which histone acetylation progressively increases. In contrast to this correlation of histone hyperacetylation and apoptosis, the accompanying inhibition of growth is often tolerated by the cells. In VACO 5 and several other adenocarcinoma cell lines, the incidence of death during an initial exposure to butyrate typically remains below 50%, but can be increased to nearly 100% by concurrent treatment with a PKC agonist, TPA [10]. In spite of this, several poorly differentiated adenocarcinoma cell lines (e.g. HCT 116) are nearly fully resistant to butyrate-induced death even if TPA is included during treatment. In such cell lines, core histones become hyperacetylated and growth ceases during butyrate treatment, indicating butyrate responsiveness. These findings indicate that sensitivity or resistance to butyrate-induced death in adenocarcinoma cells is determined by factors beyond the overall balance of histone acetylating and deacetylating enzymes. Nonetheless, structure/activity relationships among SCFA, the coordinate timing of hyperacetylation and death, and the ability to mimic butyrate effects with TsA suggest that histone hyperacetylation (to a level equivalent to tetraacetylation of histone H4) is a critical factor in the induction of apoptosis by butyrate.

n-Butyrate appears to be universally active as a growth inhibitor for naturally occurring vertebrate cell types. Primary cultures, cultivated cell strains, and non-selected cell

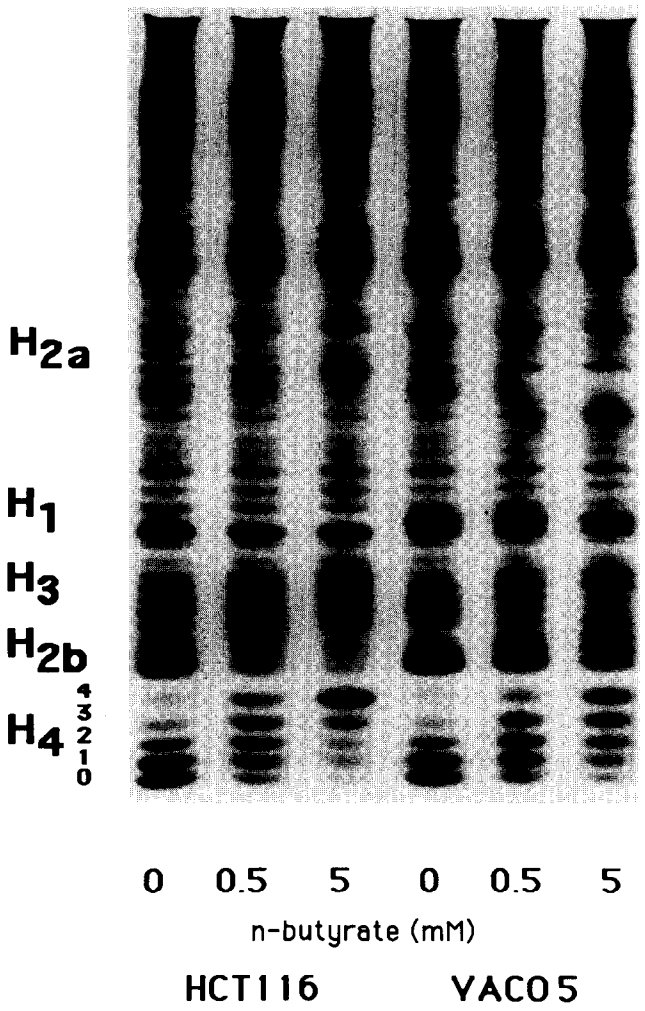

FIG. 9. Comparison of acetylation pattern and histone H1 species in VACO 5 and HCT 116 cells after incubation with butyrate. HCT 116 and VACO 5 were treated for 24 hr with 0, 0.5, or 5 mM *n*-butyrate and then analyzed for histone acetylation. Results are typical of two comparison gels.

lines typically hyperacetylate core histones and undergo growth arrest in the presence of millimolar concentrations of butyrate. Resistance to both the growth inhibition and histone hyperacetylation, however, is seen in certain embryonic cell types [35]. Cell lines that fail to hyperacetylate histones have been obtained by repetitively selecting for growth in high concentrations of butyrate [36, 37]. A similar phenotype has been obtained through selection for resistance to TsA [29]. SCFA other than butyrate have been less well studied as growth inhibitors, but our limited assessment suggests that relative deacetylase inhibition with these agents correlates with either growth inhibition, apoptosis, or differentiation of cells, depending upon the cell type.

Although our data support the hypothesis that histone deactylase is the target of *n*-butyrate with respect to depressed growth and survival, butyrate and other SCFA act at extranuclear sites such as cytoskeletal structures [23], the plasma membrane [21–23], and mitochondria [28]. These alternative targets are of interest with respect to apoptosis because of findings that cells undergoing apoptosis exhibit early changes attributable to altered cytoskeletal organization [38], intracellular pH regulation [39], and mitochondrial transmembrane potential [40]. However, the rank order of efficacy of SCFA for action upon each of these potential targets is less consistent with the growth effects than is histone hyperacetylation. For example, treatment of cells with SCFA increases the expression of cytochrome oxidase components by mitochondria, with a rank order: caproate > valerate > butyrate >> isobutyrate [28]. Conversely, SCFA treatment stabilizes the neutrophil actin cytoskeleton, in rank order: propionate > acetate > butyrate > valerate > isobutyrate > caproate [23]. In the later paper, neutrophils exhibited a cytoplasmic acidification of up to 0.5 pH unit, in a rank order: valerate > caproate > butyrate > acetate > propionate, demonstrating distinct mechanisms for the two actions. Cytoplasmic acidification of over 0.5 pH unit was also noted after treatment of colonocytes with 65 mM acetate, *n*-butyrate, or isobutyrate [22]. The death-inducing effects of 40 mM acetate found by Hague *et al.* [14] may result, in part, from this extranuclear action of high concentration SCFA. Similarly, the moderate growth inhibitory effects of caproate on VACO 5 (Fig. 2) may be due to membrane actions attributable to its amphipathic nature. Our finding that growth arrest, TPA-augmented induction of apoptosis, and histone hyperacetylation were each induced by *n*-butyrate > propionate = isovalerate = valerate > caproate >> isobutyrate > methylbutyrate argues for actions involving chromatin. The nearly indistinguishable action of 1 μM TsA toward these growth/survival phenomena, presumably without direct actions on the other targets of SCFA, is further evidence for the histone deacetylase (or a highly related enzyme) as the target of relevance to both growth arrest and apoptosis.

It appears that apoptosis induction requires not inhibition of the deacetylase *per se*, nor hyperacetylation of the rapidly acetylated fraction of nucleosomes, but rather the hyperacetylation of bulk chromatin. Thus, deacetylase inhibition for ≥ 12 hr was required to obtain both a maximally acetylated chromatin and a substantial frequency of cell death [10]. The subset of histones which are evident on Coomassie blue-stained TAU gels are largely the highly abundant, slowly-acetylated fraction of histones [26]. The action of the histone acetyltransferase on such bulk chromatin is thought to be limited by infrequent exposure of specific histone domains or some other kinetic barrier involving the acetyltransferase/chromatin complex. We thus considered the possibility that TPA might be enhancing apoptosis induction by facilitating acetylation of this fraction of histones. Using bryostatin 1 as an antagonist of TPA actions, we have shown that such actions are required as late as 1–3 hr before the times at which apoptosis is detected, i.e. between 12 and 30 hr of TPA/*n*-butyrate treatment [10]. Contrary to expectation, histones displayed by TAU–PAGE analyses of such cells were indistinguishable

from histones of cells treated with butyrate alone. Thus, the chromatin of butyrate-treated cells became enriched in hyperacetylated species at a similar rate in the presence or absence of TPA. It remains possible that hyperacetylation of a minor fraction of chromatin, critical for cell survival, was accelerated by TPA actions. Evidence of this possibility would require a far more incisive analysis for minor populations of nucleosomes. In addition, the histone analysis used herein is limited to proteins that can be recovered from sedimentable chromatin assemblies, structures that are rendered non-sedimentable during apoptotic DNA cleavage. Therefore, it remains formally possible that cells bearing fully acetylated nucleosomes appeared more frequently in the presence of TPA, but were subject to immediate chromatin cleavage, obviating analysis of their histones.

Several specific roles for histone hyperacetylation in death induction would be consistent with our results. Much of the existing literature on histone acetylation assumes this system to have a primary role in facilitating (or silencing) gene transcription. The phenotypic differentiation that accompanies butyrate treatment of many cell types has been attributed to such altered gene expression [1]. The nature of genes that could precipitate apoptosis is, however, a matter of conjecture at this point. Moreover, several of our findings are inconsistent with a transcriptional model. Higher concentrations of butyrate are needed to induce apoptosis than are sufficient to effect dramatic stimulation of gene transcription in many cell types. In murine erythroleukemia cells, 0.1 to 1 mM *n*-butyrate stimulated transcription of globin genes and induced differentiation, while concentrations over 3 mM were required for substantial hyperacetylation of bulk chromatin [1, 16, 41]. Changes in gene expression are thought to result from modest increases in acetylation of chromatin, possibly localized to promoter/enhancer regions [25, 26]. Hyperacetylation of bulk chromatin, in contrast, may have consequences beyond transcriptional facilitation. It has been suggested that several other apoptosis inducers directly alter chromatin conformation as part of their action [42]. Butyrate-induced hyperacetylation has long been known to sensitize chromatin to direct DNase action *in vitro* [1, 24–26, 43–45], and such DNase hypersensitivity is seen in silent as well as transcriptionally competent genes [43]. Although it is unclear whether butyrate could confer similar lability to endogenous nucleases, butyrate-treated cells do exhibit increased sensitivity to the action of introduced, exogenous nucleases [44]. Finally, apoptosis may follow as a cell type-specific epiphenomenon to changes such as growth arrest, which appears to occur as a general, if unexplained response to chromatin hyperacetylation. However, VACO 5 cells survived for several days while growth-arrested after withdrawing serum, or during treatment with aphidicolin or high concentrations of thymidine, even when cultured in the additional presence of TPA.*

Most cell lines tested for growth effects of *n*-butyrate have proven tolerant of the resulting growth inhibition despite histone hyperacetylation. Indeed, some butyrate-tolerant cell lines exhibited an even more extensive acetylation than did cell lines with susceptibility to butyrate-induced death (e.g. HCT 116 in comparison with VACO 5, Fig. 9). Schröter *et al.* [46] termed the proportional distribution of acetylforms of H4 resulting from 20 hr of culture in 5 mM butyrate, as the "limit acetylation pattern." The pattern shown by the L132 subline of HeLa, which was similar to the limit acetylation pattern we found for HCT 116, was suggested to be characteristic of oncogenically "transformed" cell lines. The limit acetylation pattern of H4 in our butyrate-treated cultures of VACO 5 was said to be characteristic of normal cells, such as diploid human fibroblasts or lymphocytes [46]. We have, however, seen this latter acetylation pattern in many cancer-derived cell lines, irrespective of sensitivity to butyrate-induced death.* Clearly, the conformation of hyperacetylated chromatin can be additionally determined by other chromatin structural proteins, e.g. H1 isotypes [45] which also distinguish HCT 116 (and several other butyrate-tolerant colon and breast cell lines) from VACO 5 (Fig. 9) and COLO 201.* Apart from these chromatin distinctions, and as evident in the dramatic enhancement of butyrate sensitivity by TPA, we have postulated [10] that elements of the PKC system play a determining role in sensitivity to butyrate-induced death. Other proteins with roles in cell growth and survival (oncogene products, etc.) are also obvious candidates for such response determinants. However, several cell lines that are mutant in the p53 gene have been shown to be among the most sensitive to butyrate-induced death [9–11, 13, 17, 19]. Thus, cell-specific determinants of butyrate-responsiveness appear to be multi-fold, and continuing investigations may point out a number of clinically useful response predictors.

In several metabolic disorders, specific SCFA accumulate to millimolar concentrations in plasma. Isovaleric acidemia is an autosomal recessive disorder caused by a deficiency of isovaleryl-CoA dehydrogenase, a flavoenzyme of leucine catabolism. Individuals with this disorder, after ingestion of high protein meals, suffer from episodes of acute metabolic acidosis manifested as vomiting and loss of consciousness. Less explicably, and relatively unique to acidemias of isovaleric and propionic acids, are hair loss, pancytopenia, and thymic involution [47, 48]. Plasma isovaleric acid concentrations associated with these severe occurrences remain as high as 10 mM for many hours, suggesting that the organic acid itself may be responsible for the cell losses. In similarity to this disorder, ingestion of hypoglycin, an unusual amino acid contained in unripe breadfruit (*Bligha sapida*), can result in impaired beta-oxidation of SCFA [49]. The active hypoglycin metabolite, methylenecyclopropylacetyl CoA, is a suicide substrate of short chain- and isovaleryl-CoA dehydrogenases [50]. As a consequence, *n*-butyric and isovaleric acids accumulate to millimolar concentrations in

* McBain JA, unpublished results.

plasma. As with isovaleric acidemia, hypoglycin ingestion can lead to thymic and splenic involution, apoptosis of liver ("piecemeal necrosis"), and hematological disturbances [49]. Thus, certain SCFA may cause cell type specific apoptosis *in vivo*, as sequelae of genetic or toxicological deficiencies of fatty acid catabolism. Each of these cell loss phenomena proceed in the apparent absence of an exogenous PKC modulator.

Our studies have addressed several issues related to the requirements for use of butyrate and other histone deacetylase inhibitors in cancer therapy. The results presented in the current report indicate that assessment of core histone acetylation levels in the tumor, or even in peripheral blood mononuclear cells, may be a reasonable pharmacodynamic means for monitoring these therapies. Clinical trials of butyrate salts and esters have shown these agents to be effective for instituting butyric acidemia [51–53], and reactivating fetal hemoglobin production in persons with thallasemia [52]. However, plasma butyrate in these trials may not have reached the sustained, high concentrations required for cytoreduction therapies, primarily because of metabolic consumption (plasma half-life of 6 min with butyrate salts [51]). Our studies on cell lines from colorectal cancer (herein and Ref. 10) and breast cancer* have indicated that millimolar concentrations of butyrate must be maintained continuously for 24–48 hr. Even then, the histone deacetylase inhibitor may need to be combined with a PKC modulator to effect a greater than 50% reduction in cell survival. Considering the potential sequelae of these therapies, akin to organic acidemias or hypoglycin poisoning (above), such treatments may best be reserved for tumors with inherent sensitivity to butyrate-induced apoptosis. Molecular definition of the factors contributing to the susceptibility of a particular tumor to butyrate-induced death, currently under investigation in our laboratories, may thus facilitate the design of clinical cancer trials of histone deacetylase inhibitors.

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References

1. Kruh J, Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem* **42:** 65–82, 1982.
2. Harig JM, Soergel KH, Komorowski RA and Wood CM, Treatment of diversion colitis with short-chain fatty acid irrigation. *N Engl J Med* **20:** 23–28, 1989.
3. Scheppach W, Sommer H, Kirchner T, Paganelli G-M, Bartram P, Christl S, Richter S, Dusel F and Kasper H, Effect of butyrate enemas on the colonic mucosa in distal ulcerative colitis. *Gastroenterology* **103:** 51–56, 1992.
4. Koruda MJ, Rolandelli RH, Bliss DZ, Hastings J, Rombeau JL and Settle RG, Parenteral nutrition supplemented with short-chain fatty acids: Effect on the small bowel mucosa in normal rats. *Am J Clin Nutr* **51:** 685–689, 1990.
5. Scheppach W, Bartram P, Richter A, Liepold H, Dusel G, Hofstetter G, Ruthlein J and Kasper H, Effect of short-chain fatty acids on the human colonic mucosa *in vitro*. *J Parenter Enteral Nutr* **16:** 43–48, 1992.
6. Roediger WEW, Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology* **83:** 424–429, 1982.
7. Barnard JA and Warwick G, Butyrate rapidly induces growth inhibition and differentiation in HT29 cells. *Cell Growth Differ* **4:** 495–501, 1993.
8. Foss FM, Veillette A, Sartor O, Rosen N and Bolen JB, Alterations in the expression of $pp60^{c\text{-}src}$ and $pp56^{c\text{-}lck}$ associated with butyrate-induced differentiation of human colon carcinoma cells. *Oncogene Res* **5:** 13–23, 1989.
9. Chung YS, Song IS, Erickson RH, Sleisenger MH and Kim YS, Effect of growth and sodium butyrate on brush border membrane-associated hydrolases in human colorectal cancer cell lines. *Cancer Res* **45:** 2976–2982, 1985.
10. McBain JA, Eastman A, Simmons DL, Pettit GR and Mueller GC, Phorbol ester augments butyrate-induced apoptosis of colon cancer cells. *Int J Cancer* **67:** 715–723, 1996.
11. Bell PA and Jones CN, Cytotoxic effects of butyrate and other 'differentiation inducers' on immature lymphoid cells. *Biochem Biophys Res Commun* **104:** 1202–1208, 1982.
12. Ho SB, Yan P-S, Dahiya R, Neuschwander-Teri BA, Basbaum C and Kim YS, Stable differentiation of a human colon adenocarcinoma cell line by sodium butyrate is associated with multidrug resistance. *J Cell Physiol* **160:** 213–226, 1994.
13. Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D and Paraskeva C, Sodium butyrate induces apoptosis in human colonic tumour cell lines in a p53-independent pathway: Implications for the possible role of dietary fibre in the prevention of large-bowel cancer. *Int J Cancer* **55:** 498–505, 1993.
14. Hague A, Elder DJ, Hicks DJ and Paraskeva C, Apoptosis in colorectal tumour cells: Induction by the short chain fatty acids butyrate, propionate, and acetate, and by the bile salt deoxycholate. *Int J Cancer* **60:** 400–406, 1995.
15. Langdon SP, Hawkes MM, Hay FG, Lawrie SS, Schol DJ, Hilgers J, Leonard RCF and Smyth JF, Effect of sodium butyrate and other differentiation inducers on poorly differentiated human ovarian adenocarcinoma cell lines. *Cancer Res* **48:** 6161–6165, 1988.
16. Friend C, Zajak-Kaye M, Holland JG and Pogo BG-T, Depletion of sodium butyrate from the culture medium of Friend erythroleukemia cells undergoing differentiation. *Cancer Res* **47:** 378–382, 1987.
17. Graham KA and Buick RN, Sodium butyrate induces differentiation in breast cancer cell lines expressing the estrogen receptor. *J Cell Physiol* **136:** 63–71, 1988.
18. Halgunset J, Lamvik T and Espevik T, Butyrate effects on growth, morphology, and fibronectin production in PC-3 prostatic carcinoma cells. *Prostate* **12:** 65–77, 1988.
19. Cummings JH, Pomare EW, Branch WJ, Naylor CPE and MacFarlane GT, Short chain fatty acids in human large intestine, portal, hepatic, and venous blood. *Gut* **28:** 1221–1227, 1987.
20. Boffa LC, Lupto JR, Mariani MR, Ceppi M, Newmark HL, Scamati A and Lipkin M, Modulation of colonic epithelial cell proliferation, histone acetylation, and luminal short chain fatty acids by variation of dietary fiber (wheat bran) in rats. *Cancer Res* **52:** 5906–5912, 1992.
21. Diener M, Peter A and Scharrer E, The role of volume-sensitive Cl^- channels in the stimulation of chloride absorption by short-chain farry acids in the rat colon. *Acta Physiol Scand* **151:** 385–394, 1994.
22. Chu S and Montrose MH, An Na^+-independent short-chain

* McBain JA, unpublished results.

fatty acid transporter contributes to intracellular pH regulation in murine colonocytes. *J Gen Physiol* **105:** 589–615, 1995.
23. Brunkhorst BA, Kraus E, Coppi M, Budnick M and Niederman R, Propionate induces polymorphonuclear leukocyte activation and inhibits formylmethionyl-leucyl-phenylalanine-stimulated activation. *Infect Immun* **60:** 2957–2968, 1992.
24. Cousens LS, Gallwitz D and Alberts BM, Different accessibilities in chromatin to histone acetylase. *J Biol Chem* **254:** 1716–1723, 1979.
25. Csordas A, On the biological role of histone acetylation. *Biochem J* **265:** 23–38, 1990.
26. Davie JR and Hendzel MJ, Multiple functions of dynamic histone acetylation. *J Cell Biochem* **55:** 98–105, 1994.
27. Anderson KE, Whitlon DS and Mueller GC, Role of fatty acid structure in the reversible activation of phosphatidylcholine synthesis in lymphocytes. *Biochim Biophys Acta* **835:** 360–368, 1985.
28. Heerdt BG and Augenlicht LH, Effects of fatty acids on expression of genes encoding subunits of cytochrome *c* oxidase and cytochrome c oxidase activity in HT29 human colonic adenocarcinoma cells. *J Biol Chem* **266:** 19120–19126, 1991.
29. Yoshida M, Kijima M, Akita M and Beppu T, Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J Biol Chem* **265:** 17174–17179, 1990.
30. McBain JA, Weese JL, Meisner LF, Wolfberg WH and Willson JKV, Establishment and characterization of human colorectal cancer cell lines. *Cancer Res* **44:** 5813–5821, 1984.
31. De Luca C, The use of trypsin for the determination of cellular viability. *Exp Cell Res* **40:** 186–188, 1965.
32. Loidl P and Gröbner P, Biosynthesis and posttranslational acetylation of histones during spherulation of *Physarum polycephalum*. *Nucleic Acids Res* **14:** 3745–3762, 1986.
33. Zweidler A, Resolution of histones by polyacrylamide gel electrophoresis in the presence of nonionic detergents. *Methods Cell Biol* **17:** 223–234, 1978.
34. Barry MA, Behnke CA and Eastman A, Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins, and hyperthermia. *Biochem Pharmacol* **40:** 2353–2362, 1990.
35. Almouzni G, Khochbin S, Dimitrov S and Wolffe AP, Histone acetylation influences both gene expression and development of *Xenopus laevis*. *Devl Biol* **165:** 654–669, 1994.
36. Chalkley R and Shires A, The isolation of HTC variant cells which can replicate in butyrate. Change in histone acetylation and tyrosine aminotransferase induction. *J Biol Chem* **260:** 7698–7704, 1985.
37. Ohlsson-Wilhelm BM, Farley BA, Kosciolek B, La Bella S and Rowley PT, K562 human erythroleukemia cell variants resistant to growth inhibition by butyrate have deficient histone acetylation. *Am J Hum Genet* **36:** 1225–1238, 1984.
38. Wyllie AH, Kerr JFR and Currie AR, Cell death: The significance of apoptosis. *Int Rev Cytol* **68:** 251–306, 1980.
39. Li J and Eastman A, Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line associated with intracellular acidification. Role of the Na^+/H^+ antiport. *J Biol Chem* **270:** 3203–3211, 1995.
40. Zamzami N, Marchetti P, Castedo M, Decaudin D, Macho A, Hirsch T, Susin SA, Petit PX, Mignotte B and Kroemer G, Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *J Exp Med* **182:** 367–377, 1995.
41. Glauber JG, Wandersee NJ, Little JA and Ginder GD, 5′-Flanking sequences mediate butyrate stimulation of embryonic globin gene expression in adult erythroid cells. *Mol Cell Biol* **11:** 4690–4697, 1991.
42. Alnemri ES and Litwack G, Activation of internucleosomal DNA cleavage in human CEM lymphocytes by glucocorticoid and novobiocin. Evidence for a non-Ca^{2+} requiring mechansim. *J Biol Chem* **265:** 17323–17333, 1990.
43. Birren BW, Taplitz SJ and Herschman HR, Butyrate-induced changes in nuclease sensitivity of chromatin cannot be correlated with transcriptional activation *Mol Cell Biol* **7:** 3863–3870, 1987.
44. Hojo ETS, Balajee AS and Natarajan AT, Modulatory effect of sodium butyrate on Alu I-induced chromosomal aberrations in CHO cells. *Mutat Res* **309:** 165–173, 1994.
45. Perry CA and Annunziato AT, Histone acetylation reduces H1-mediated nucleosome interactions during chromatin assembly. *Exp Cell Res* **196:** 337–345, 1991.
46. Schröter H, Gómez-Lira MM, Plank K-H and Bode J, The extent of histone acetylation induced by butyrate and the turnover of acetyl groups depend on the nature of the cell line. *Eur J Biochem* **120:** 21–28, 1981.
47. Sweetman L, Branched chain organic acidurias. In: *Metabolic Basis of Inherited Disease* (Ed. Stanbury JB), pp. 791–819. McGraw Hill, New York, 1989.
48. Kelleher JF, Yudkoff M, Hutchinson R, August CS and Cohn RM, The pancytopenia of isovaleric acidemia. *Pediatrics* **65:** 1023–1027, 1980.
49. Tanaka K, Jamaican vomiting sickness. In: *Handbook of Clinical Neurology*, (Eds. Vinken PJ and Bruyn GW), Vol. 37, pp. 791–819. Elsevier, Amsterdam, 1979.
50. Ikeda Y and Tanaka K, Selective inactivation of various acyl-CoA dehydrogenases by (methylenecyclopropyl) acetyl-CoA. *Biochim Biophys Acta* **1038:** 216–221, 1990.
51. Miller AA, Kurschel E, Osieka R and Schmidt CG, Clinical pharmacology of sodium butyrate in patients with acute leukemia. *Eur J Cancer Oncol* **23:** 1283–1287, 1987.
52. Sher GD, Ginder GD, Little J, Yang S, Dover GJ and Olivieri NF, Extended therapy with intravenous arginine butyrate in patients with β-hemoglobinopathies. *N Engl J Med* **332:** 1606–1610, 1995.
53. Yuan Z, Eiseman J, Plaisance K, Sentz D, Bigora S, Fosler M, Young D and Egorin M, Plasma pharmacokinetics of butyrate after the administration of tributyrin and sodium butyrate to mice and rats. *Proc Am Assoc Cancer Res* **35:** 429, 1994.