Exposure To A Deuterated Analogue Of Phenylbutyrate Retards S-phase Progression In HT-29 Colon Cancer Cells

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ABSTRACT: Differentiation agents that induce neoplastic cells to regain a normal phenotype and/or cause growth arrest without significantly affecting normal cells represent an attractive option for cancer treatment. Analogues of short chain fatty acids, such as phenylbutyrate (PB), have been studied as clinically relevant agents. In an attempt to improve its pharmacokinetic profile, structural modifications of PB and other fatty acids have been studied. We hypothesize that strategic isotopic modification of PB would result in a longer half-life and thus translate into a more potent differentiation agent for clinical use. Using a colon cancer model, we demonstrated that 2,2,3,3-tetradeuterated PB (D4PB) significantly increased induction of apoptosis and inhibition of cell proliferation as compared with PB and butyrate. Difference in potency could not be explained by the effect of D4PB on the expression of specific regulatory proteins of the apoptotic cascade or from the inhibitory effect of D4PB on histone deacetylase activity. Interestingly, exposure of HT-29 colon cancer cells to D4PB resulted in a slowing of S transit, in contrast to butyrate and PB, which induced a G2/M cell cycle block. This difference in cell cycle effect may explain the differences seen in the potency of the phenotypic changes seen with treatment with D4PB. Further studies are needed to elucidate the mechanisms underlying effects of D4PB on the cell cycle. J Pharm Sci 91:1054–1064, 2002
Keywords: fatty acid; phenylbutyrate; cell cycle; colon cancer

INTRODUCTION

The short chain fatty acid (SCFA) butyrate has been reported to inhibit proliferation and stimulate differentiation in multiple cancer cell lines.[1–3] Although it has been known for years that butyrate is an effective differentiation agent *in vitro*, clinical trials evaluating butyrate as a therapeutic agent for malignancy have been disappointing. The lack of a clinical response is believed to be related to the rapid metabolism and very short plasma half-life (6 min) of butyrate, leading to the inability to achieve adequate serum concentrations (1–5 mM).[4] Based on encouraging preclinical data, there has been an interest in developing derivatives of butyrate as clinically applicable differentiating agents.[5,6]

In the search for SCFA derivatives that may provide the differentiation effects of butyrate but with improved pharmacokinetics, phenylbutyrate (PB) has emerged in phase I trials as a promising

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anticancer agent. PB is an oral, nontoxic agent that can achieve millimolar concentrations in humans and is presently approved for treatment of hyperammonemia in individuals with in-born errors of metabolism.[7] Although the literature suggests that PB acts as a prodrug for phenylacetate (PA), others suggest that PB may exert its effect through a PA-independent manner.[8] Although clinically appealing, PB and PA still require maintenance of millimolar concentrations to exert differentiating effects, and these doses may lead to certain side effects, including neurotoxicity.[9] Attempts have been made to design analogues of PB that would increase its plasma half-life as well as improve its anticancer effects. One approach is through strategic isotopic modifications of PB, which would theoretically hinder the conversion of PB to PA via the β-oxidation pathway.

Therefore, the purpose of this study was to investigate the antiproliferative and pro-apoptotic effects of the PB analog, 2,2,3,3-tetradeuterated PB (D4PB). We demonstrated that D4PB is more potent than PB and butyrate with respect to inducing apoptosis and inhibiting cell proliferation. Interestingly, exposure of HT-29 colon cancer cells to D4PB induced an S-phase slowing, in contrast to butyrate and PB, which induce a G2/M cell cycle block.

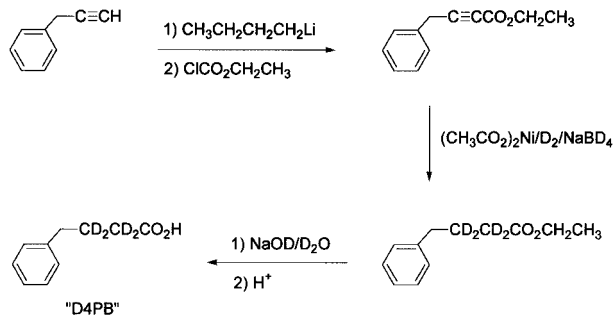
MATERIALS AND METHODS

Materials and Cell Culture

Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich Chemical Company (Milwaukee, WI and St. Louis, MO) and all antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PB was a kind gift from Dr. Hokan Cederberg (Triple Crown America, Philadelphia, PA). HT-29 Cells were maintained in McCoy's Media supplemented with 10% heat-inactivated bovine calf serum. Butyrate and phenylbutyrate were dissolved in phosphate buffered saline and D4PB was dissolved in dimethyl sulfoxide (DMSO). DMSO did not exceed a final concentration of 0.1% and by itself, had no effect on any endpoint measured (data not shown).

D4PB Synthesis

The synthesis of D4PB was accomplished through a series of three steps (Scheme 1).



Scheme 1. Synthesis of D4-phenylbutytrate.

4-Phenylbut-2-ynoic Acid Ethyl Ester

The following is based on a literature procedure for the synthesis of a related compound.[10] Under an atmosphere of N_2, *n*-butyl lithium (50 mmol, 20 mL of a 2.5 M solution in hexanes) was added in a dropwise manner to a cooled (−79°C, acetone/dry ice bath) solution of 3-phenyl-1-propyne (48 mmol, 5.5 g) in dry ether (42 mL). The reaction solution was stirred for 1 h at −79°C and then ethylchloroformate (52 mmol, 5.0 mL, Aldrich Chemical Company) was added. The mixture was stirred for 5 min at −79°C and then for 1 h at 5°C (ice bath). The reaction was quenched by the addition of saturated, aqueous NH_4Cl solution (50 mL) at ice bath temperature. After dilution with water (20 mL), the organic layer was separated and the aqueous layer was extracted with ether (3×50 mL). The combined organic layers were washed with saturated NaCl solution (1×30 mL), dried ($MgSO_4$), filtered, and concentrated on a rotary evaporator. The residual oil was flash chromatographed on silica gel (230–400 mesh, 400 mL, 7-in. column height) using ether–hexanes (3:97 followed by 5:95) as eluent. The product ($C_6H_5CH_2C{\equiv}CCO_2CH_2CH_3$) was obtained as a yellow oil in 68% yield (6.0 g, R_f 0.40 in ether-hexanes, 1:9): ^{1}H NMR ($CDCl_3$) δ 7.25–7.34 (m, 5H, aromatic), 4.23 (q, $J = 7$ Hz, 2H, OCH_2), 3.74 (s, 2H, $C_6H_5C\underline{\mathbf{H}}_2$), and 1.31 (t, 3H, $J = 7$ Hz, CH3); ^{13}C NMR ($CDCl_3$): δ 153.5 (C=O), 133.9, 128.6, 127.9, and 127.1 (aromatic), 86.19 and 74.81 (alkyne), 61.90 (OCH_2), 25.06 ($C_6H_5\underline{\mathbf{C}}H_2$), and 14.11 ($CH_3$).

2,2,3,3-Tetradeutero-4-phenylbutanoic Acid Ethyl Ester

Based on a published procedure for the selective reduction of olefins with a nickel catalyst,[11] nickel(II) acetate tetrahydrate [$(CH_3CO_2)_2Ni{\cdot}4H_2O$, 4.0 m mol, 0.99 g] was dissolved in 96% CH_3CH_2OD

(24 mL of ethanol-*d* and 1 mL of D_2O) and then placed under an atmosphere of D_2 (using a balloon filled with D_2 gas). A freshly prepared solution of 1 M $NaBD_4$ in CH_3CH_2OD (4 mL) was added quickly via syringe to the nickel acetate solution resulting in the formation of the catalyst (nickel boride) as a black suspension. To this mixture was added 4-phenylbut-2-ynoic acid ethyl ester (32 mmol, 6.0 g). The flask and syringe used in the transfer of the alkyne were rinsed with CH_3CH_2OD (2×2 mL) and these washings were added to the reaction mixture. The reaction flask was evacuated (~150 mmHg) and refilled ($3\times$) with D_2 gas using a balloon. Finally, the reaction mixture was stirred vigorously under D_2 (balloon pressure) for 16 h. The mixture was then vacuum filtered through Celite, and the filtrate was concentrated on a rotary evaporator. The residual material was flash chromatographed on silica gel (230–400 mesh, 50 mL, 6-in. column height) using ether–hexanes (3:7) as eluent. The crude product was again flash chromatographed on silica gel (230–400 mesh, 100 mL, 6-in. column height) using ether–hexanes (1:9) as eluent. The product ($C_6H_5CH_2CD_2CD_2CO_2CH_2CH_3$) was obtained as a yellow oil in 90% yield (5.6 g, $R_f = 0.4$ in ether–hexanes, 1:9): ^1H NMR ($CDCl_3$) δ 7.14–7.25 (m, 5H, aromatic), 4.10 (q, $J = 7.1$ Hz, 2H, OCH_2), 2.61 (br s, 2H, $C_6H_5C\underline{\mathbf{H}}_2$), and 1.22 (t, $J = 7.1$ Hz, 3H, CH_3); ^{13}C NMR ($CDCl_3$) δ 173.1 (C=O), 141.1, 128.2, 128.1, and 125.7 (aromatic), 60.01 (OCH_2), 34.81 ($C_6H_5\underline{\mathbf{C}}H_2$), and 14.18 ($CH_3$). (Note: The ^{13}C signals for the CD_2 moieties were only visible with long accumulation times).

2,2,3,3-Tetradeutero-4-phenylbutanoic acid ("D4-PB")

A turbid mixture of 2,2,3,3-tetradeutero-4-phenylbutanoic acid ethyl ester (29 mmol, 5.6 g) and 2 M $NaOD/D_2O$ (30 mL, 60 mmol) was refluxed (2.5 h), and the resultant clear solution was washed with ether (3×30 mL) to remove unreacted starting material. The pH of the reaction mixture was adjusted to 7 using conc. HCl, and the aqueous layer was washed again with ether to remove impurities (1×30 mL). The pH of the aqueous layer was then adjusted to 2 (conc. HCl) and the product was extracted with ether (2×30 mL). The final ether extracts were combined, dried ($MgSO_4$), filtered, and concentrated on a rotary evaporator to give the product ($C_6H_5CH_2CD_2CD_2CO_2H$), which solidified on standing (26 mmol, 4.3 g, 90% yield, mp 48–50°C): ^1H NMR ($CDCl_3$) δ 11.78 (br s, 1H, CO_2H), 7.17–7.30 (m, 5H, aromatic), and 2.65 (s, 2H, CH_2); ^{13}C NMR ($CDCl_3$) δ 180.1 (C=O), 141.2, 128.4, 128.4, and 126.0 (aromatic), 34.72 (CH_2).

Anal. calcd. for $C_{10}H(D)_{12}O_2$ (deuterium analyzed as hydrogen): C, 71.42; H(D), 7.19. Found: C, 71.18; H(D), 7.17.

[^{3}H]Thymidine

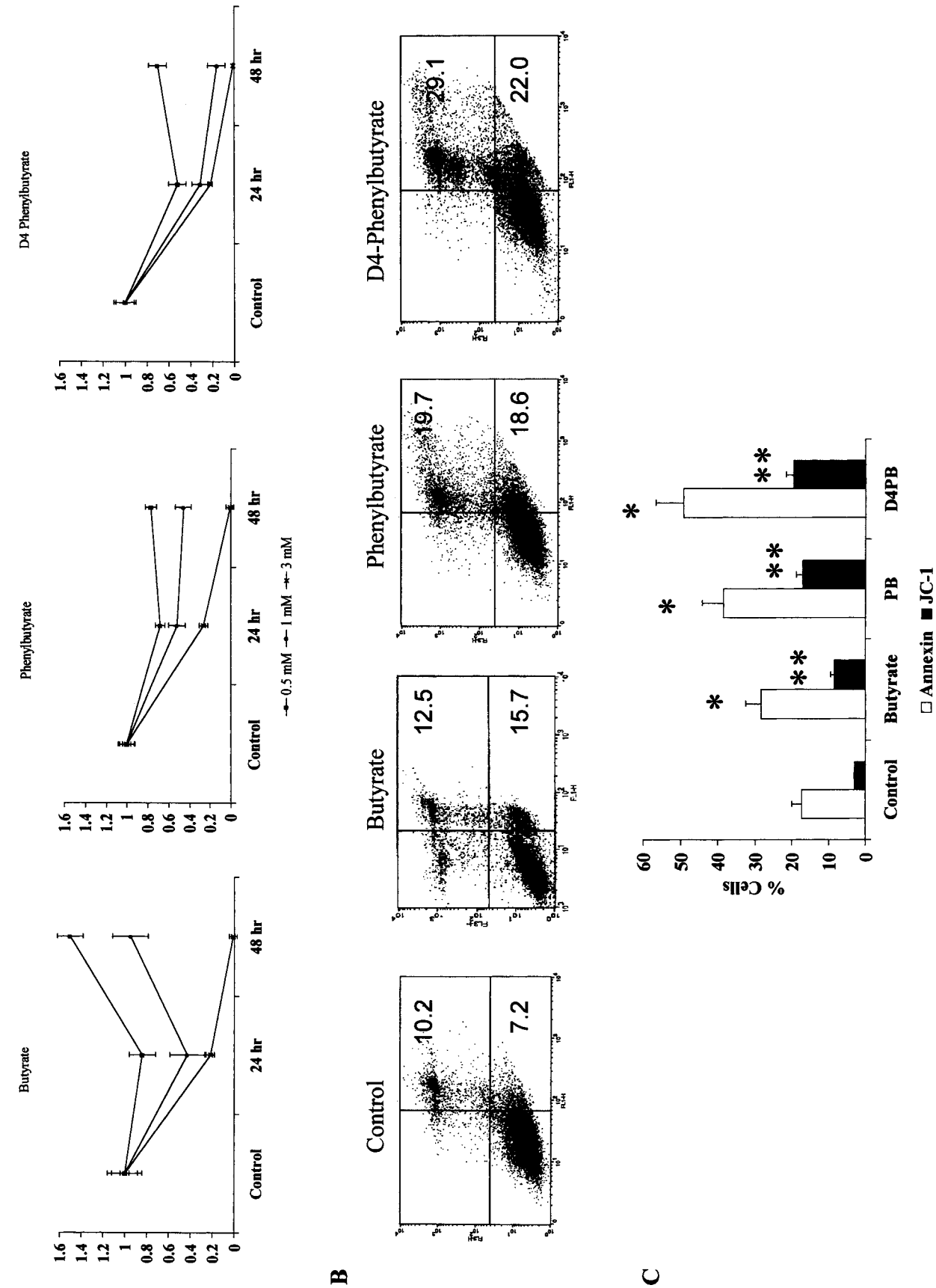
HT-29 cells were seeded in 96 well plates and treated for 24 and 48 h with butyrate, PB, and D4PB [0 (control), 0.5 mM, 1 mM, and 3 mM]. After treatment, [Methyl-^{3}H]thymidine (1.0 μCi, NEN) was added to each well and incubated for 4 h. Cells were harvested on filter paper and [^{3}H]thymidine incorporation was measured by a scintillation counter (Beckman LS 5000TD).

Flow Cytometry

For annexin V staining, FITC-conjugated annexin and propidium iodide (PI)were added to cells and fluorescence intensity was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by CellQuest software (Becton Dickinson, San Jose, CA). Mitochondrial membrane potential ($\Delta\psi_{mt}$) was determined by flow cytometry using the dye, JC-1 (5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimadozolcarbocyanine iodide, Molecular Probes, Eugene, OR). Briefly, cells were harvested, washed with PBS, and incubated with 10 μM JC-1 at 37°C for 15 min. Fluorescence intensity was determined using a FACScan flow cytometer (Becton Dickinson, San Jose, CA) and analyzed by CellQuest software (Becton Dickinson, San Jose, CA). Cell cycle distribution was determined by fixing 10^6 cells in 75% ethanol and staining with PI (10 μg/mL) in the presence of 100 U/L RNase (Boehringer-Mannheim, Indianapolis, IN). The DNA content distribution was determined using a FACScan flow cytometer (Becton Dickinson) and analyzed using ModFIT (Becton Dickinson).

Western Blot Analysis

Whole cell extracts were prepared in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 0.2 mM $NaVO_4$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 25 μg/mL leupeptin, 25 μg/mL aprotonin, 25 μg/mL pepstatin A). Cell debris was removed by centrifugation, and


A
Butyrate
Phenylbutyrate
D4 Phenylbutyrate
Control
24 hr
48 hr
0.5 mM
1 mM
3 mM
B
Control
Butyrate
Phenylbutyrate
D4-Phenylbutyrate
10.2
7.2
12.5
15.7
19.7
18.6
29.1
22.0
C
% Cells
Control
Butyrate
PB
D4PB
Annexin
JC-1

supernatants were stored at −80°C. Samples for immunoblotting were prepared by mixing aliquots of the protein extracts with 3× sodium dodecyl sulfate (SDS) sample buffer (150 mM Tris [pH 6.8], 30% glycerol, 3% SDS, bromophenol blue dye at 1.5 μg/100 mL, 100 mM DTT). Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), electrotransferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL), and incubated in 5% nonfat milk blocking buffer (Tris buffered saline, 5% dry milk and 0.05% Tween-20). The membranes were subjected to immunoblot analysis with primary antibody, and proteins were visualized by the enhanced chemiluminescence method of detection (Amersham). Equal protein loading was conformed by Ponceau red staining of all immunoblots. Densitometry was conducted using image analysis software (NIH Image, Bethesda, MD).

BrdU Pulse–Chase

Cells were pulsed with bromodeoxyuridine (BrdU, 10 μM) for 30 min and then washed with BrdU-free medium. Cells were then cultured in fresh media in the presence of 3 mM butyrate, PB, and D4PB for 0, 6, and 12 h. After harvest, cells were fixed in 75% ethanol, followed by incubation with 2 N HCl/Triton X-100 to produce single-stranded DNA. After incubation, cells were centrifuged and resuspended in 0.1 M $Na_2B_4O_7$ to neutralize the acid. Cells were then pelleted and resuspended in 100 μL of phosphate-buffered saline (PBS) containing 0.5% Tween and 1.0% serum albumin (BSA). Two microliters of anti-BrdU FITC (Becton Dickinson) were added to the suspension for 30 min at room temperature. After washing, cells were resuspended in 500 μL of PI. A FACScan flow cytometer (Becton Dickinson) was used to analyze the DNA content and the BrdU incorporation (CellQuest, Becton Dickinson).

Histone Deacetylase Assay (HDAC)

Activity of histone deacetylase was measured by the liberation of [^{3}H]acetate from labeled acetylated histones as previously described.[12] HT-29 cells were centrifuged and washed once with PBS. The pelleted cells were homogenized in TMN (10 mM Tris·HCl, pH 7.8, 3 mM magnesium chloride, 20 mM sodium chloride). Similar histone deacetylase activities were obtained using this homogenate or a crude nuclear fraction obtained by low speed centrifugation of the homogenate. Incubations at 37°C were performed in a total volume of 100 μL containing 5 μg of ^{3}H-labeled histones (3000 cpm/μg) in TMN. Incubations for 30 min were stopped by the addition of 10 μL of concentrated hydrochloric acid. Then, 1 mL of ethyl acetate was added. The mixture was mixed by vortex and centrifuged for 2 min. Finally, 0.8 mL of the top layer was taken for liquid scintillation counting.

Statistics

All experiments were run in triplicate. All data, including densitometric quantitation (NIH-Image, Scion, Frederick, MD) of protein immunoblotting are expressed as mean ± SEM. Comparison between groups is determined by ANOVA and Bonferroni post hoc analysis. A p value < 0.05 is defined as significant.

RESULTS

Antiproliferative and Pro-Apoptotic Effects of D4PB

Initial experiments were designed to evaluate the antiproliferative and pro-apoptotic effects of D4PB. HT-29 cells were treated with butyrate, PB, and D4PB for 24 and 48 h at various concentrations (0.5, 1, and 3 mM). Cell proliferation

Figure 1. D4PB induces growth inhibition and apoptosis. (A) HT-29 cells were treated with butyrate, PB, or D4PB (0.5, 1, 3 mM) for 24, 48, and 72 h. Although all three compounds demonstrated dose- and time-dependent inhibitory effects, D4PB was the most potent. (B) HT-29 cells were treated with either butyrate, PB, or D4PB (3mM) for 24 h, and apoptosis was determined by annexin V binding. Cells staining positive for annexin only (lower right panel) represent early apoptosis, whereas those cells staining positive for both annexin and PI represent cells in late apoptosis. Exposure to all three compounds resulted in an increase in the number of apoptotic cells, with D4PB being the most potent. Shown is a representative of three separate experiments. (C) Graphical representation of the total number of apoptotic cells along with decreased mitochondrial membrane potential as detected by JC-1 staining. Key: (*) $p < 0.05$ (annexin); (**) $p < 0.01$ (JC-1) compared with untreated control cells.

was measured by [^{3}H]thymidine incorporation. A significant decrease in cell proliferation was noted after 24 h of exposure of HT-29 colon cancer cells to all three drugs. By 48 h of exposure, cells proliferation resumed at the lowest dose (0.5mM; butyrate $>$PB $>$D4PB). At higher concentrations, D4PB exposure decreased cell proliferation to a greater extent than butyrate and PB (Figure 1A). To determine if this growth inhibitory effect was associated with increased apoptosis, HT-29 colon cancer cells were treated with butyrate, PB, or D4PB (3 mM for 24h) and subsequently stained and analyzed for annexin V staining by flow cytometry. Cells staining positive for annexin only (Figure 1B, lower right panel) represent early apoptosis, whereas those cells staining positive for both annexin and PI, represent cells in late apoptosis (upper right panel). Exposure to all three compounds resulted in an increase in the number of apoptotic cells. Similar to the growth inhibitory activity, D4PB proved to have the most potent pro-apoptotic response (Figure 1B,C). Because the dissipation of $\Delta\Psi_{mt}$ has been linked to the initiation of some apoptotic cascades, $\Delta\Psi_{mt}$ was measured in HT-29 cells treated with butyrate, PB, and D4PB (3 mM for 48 h). At high $\Delta\Psi_{mt}$, the JC-1 dye forms J-aggregates, which emit at 590 nm (orange range of visible light). However, at low $\Delta\Psi_{mt}$, JC-1 exists as a monomer, emitting at 527 nm (green range). The results in Figure 1C demonstrate an increase in the cell populations emitting at 527 nm (low $\Delta\Psi_{mt}$) after exposure to all three drugs. Although all agents lowered $\Delta\Psi_{mt}$ ($p < 0.05$ compared with control cells), there were significantly more cells with lower $\Delta\Psi_{mt}$ after D4PB and PB treatment than after butyrate exposure (p lt; 0.05). Bcl2, Bad, and Bax are major regulatory proteins of the apoptotic cascade. Expression of Bcl2 has anti-apoptotic properties as opposed to Bad and Bax which have pro-apoptotic properties. Western Blot analysis of control, butyrate, PB, and D4PB treated cells (3 mM for 24 h) revealed no significant effect on Bcl2 levels. In contrast, expression of Bad and Bax was significantly increased compared with control with the greatest increase noted in the PB and D4PB treated cells (Figure 2).

It is well known that butyrate and PB are potent HDAC inhibitors;[12] therefore, it was possible that the observed difference in the potency of D4PB may be related to its effect on histone deacetylation. The results in Figure 3 demonstrate that compared with untreated control, all three agents decreased *in vitro* HDAC activity ($p = 0.001$). Although butyrate treatment significantly decreased HDAC activity compared with PB or D4PB ($p = 0.04$), there was no difference in HDAC activity between PB- and D4PB-treated cells.

D4PB Treatment Retards S-phase Progression

In an attempt to explain the increased apoptotic and antiproliferative effects of D4PB compared with those of butyrate and PB, we compared their effect on the cell cycle traverse. HT-29 colon cancer cells were exposed to all three compounds, and cell cycle distribution by PI staining was performed. In contrast to a G2/M block observed after butyrate and PB exposure, D4PB treatment resulted in an increase in the percentage of cells in the S-phase compartment (Figure 4). To determine if this increase in S-phase was due to a prolongation of S-phase, we performed BrdU pulse–chase experiments to trace cell progression through the S-phase. Cells were pulsed with BrdU for 30 min and then incubated with fresh medium for 0, 6, and 12 h. To determine the rate of exit from S-phase, the proportion of BrdU-labeled cells with G_2/M DNA content was determined as a percentage of all labeled cells [% BrdU labeled cells in G2/M compartment = S3/(S1 + S2 + S3)]. At the 30-min pulse, there was no difference in the percentage of BrdU-labeled cells with G_2/M DNA content (Figures 5A and B). However, after a 6-h chase with the BrdU-free medium, D4PB-treated cells demonstrated a different pattern of S-phase cell progression than the other groups. There was an increase in the number of BrdU-labeled cells in the G2/M compartment over time in the control, butyrate, and PB groups, showing that the exit from S-phase was not impaired; however, the number of labeled cells with G2/M DNA content did not increase in the D4PB group. These results suggest that the accumulation of cells in the S-phase after D4PB exposure is due in part to a slower transit through the last 12 h of S-phase.

DISCUSSION

The salutary effects of dietary fiber as it relates to colorectal cancer prevention have been attributed in part to the production of SCFAs.[13] SCFAs are natural constituents of the colonic lumen produced during anaerobic fermentation of dietary fiber by endogenous intestinal bacteria.[14]

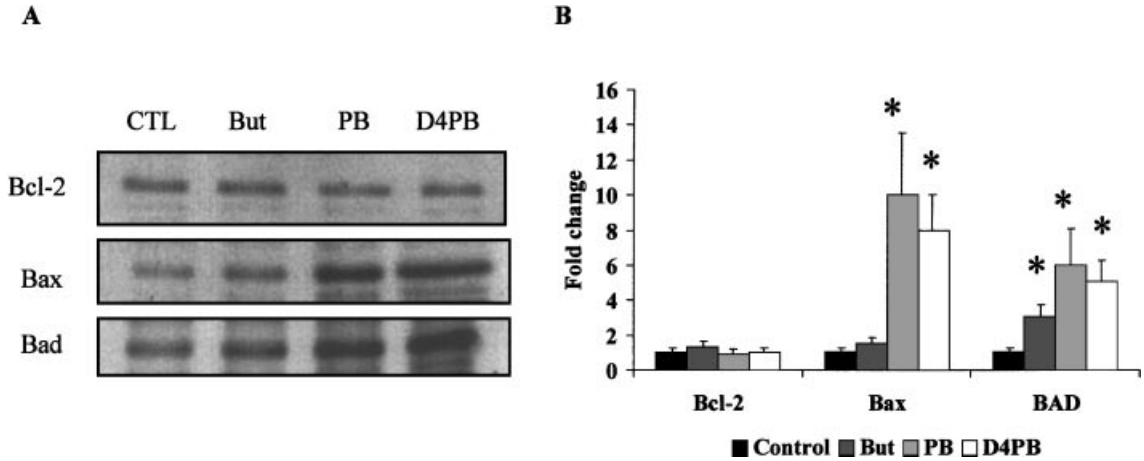


Figure 2. Exposure of HT-29 cells to D4PB results in an increase in expression of members of the Bcl2 family. (A) Exposure of HT-29 cells to butyrate, PB, and D4PB (3 mM for 24 h) did not have any significant effects of Bcl2 expression. However, there was a dramatic increase in the levels of Bax and Bad in PB- and D4PB-treated cells. Shown is a representative of three separate experiments. (B) Quantification of the immunoblots was performed by densitometry. Key: (*) $p < 0.05$ compared with untreated control cells.

Butyrate is one such SCFA, and it has been reported to inhibit proliferation and stimulate differentiation in multiple cancer cell lines.[1,2] Specifically, exposure of colon carcinoma cells to butyrate results in growth arrest and cellular differentiation.[3,15,16] Furthermore, increased colonic butyrate levels correlate with reduced colon cancer cell proliferation.[17] The use of butyrate, although demonstrated to have pro-apoptotic effects and differentiating ability, is limited because of its pharmacokinetic profile. As a result, there has been an impetus to develop fatty acid analogues that demonstrate an improved spectrum of activity with a longer half-life. PA has been identified as one potential differentiation agent, but its intense odor limits its use as an oral drug.[18,19] PB, originally considered as an oral prodrug for PA, has subsequently been shown to provide an increased antitumor effect in multiple cell lines compared with PA.[20,21] In addition, experimental and clinical trials suggest that PB may represent a clinically relevant differentiation agent with therapeutic effects independent of PA.[22] Although promising, sustained efficacy of PB requires prolonged millimolar serum concentrations.

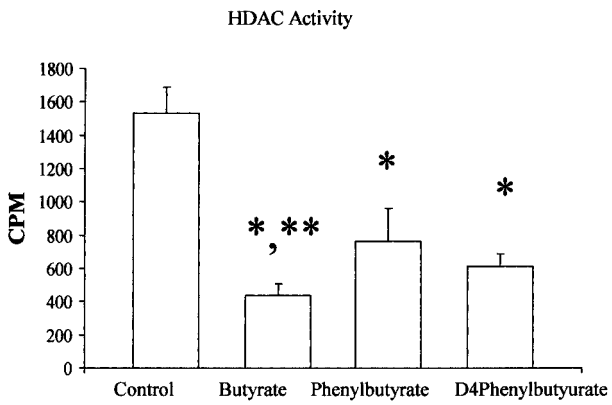


Figure 3. Inhibition of histone deacetylase activity of HT-29 cells to D4PB. Incubation of homogenates of HT-29 cells with 3 mM butyrate, PB, and D4PB resulted in the inhibition of histone deacetylase activity compared with untreated control cells ($p = 0.001$). Whereas butyrate treatment significantly decreased HDAC activity compared with PB or D4PB treatment, there was no difference in HDAC activity between PB- and D4PB-treated cells. Key: (*) $p = 0.001$ compared with untreated control cells; (**) $p = 0.04$ compared with PB and D4PB.

If the therapeutic role of PB is to act as a prodrug, an analog that would slow the conversion of PB to PA would allow for a longer plasma half-life of PB with the potential to administer a longer acting drug at lower concentrations. Based on this rationale as well as the understanding that the metabolism of PB to PA occurs through β-oxidation, D4PB was synthesized. Deuterium atoms at the α and β positions would be expected to exert primary kinetic isotope effects on at least two steps of the β-oxidation pathway, the initial

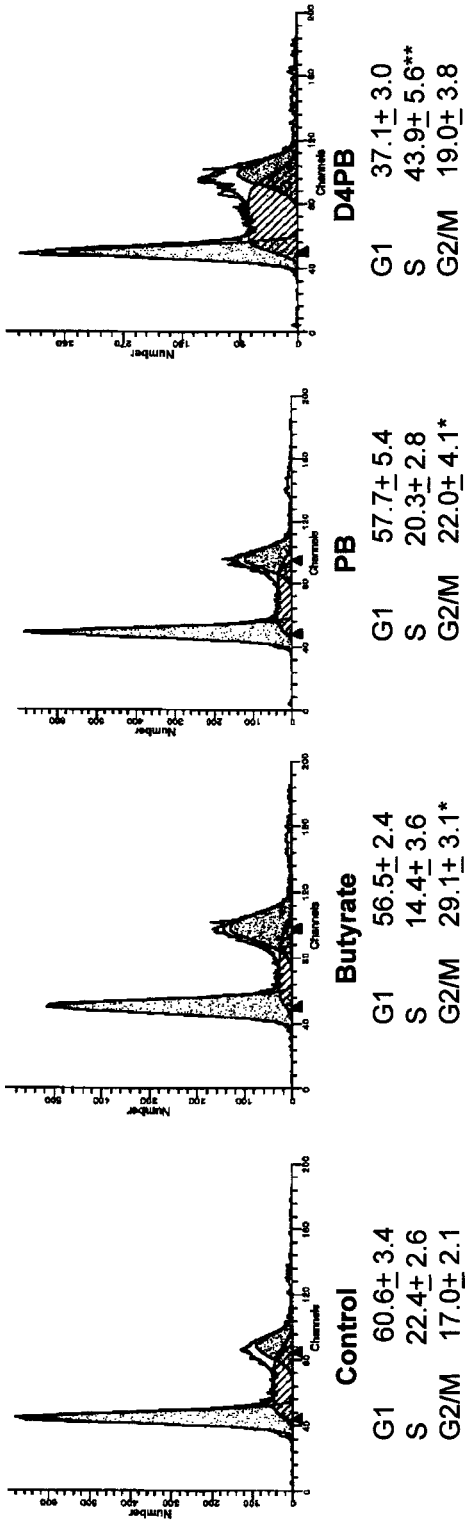


Figure 4. Exposure of HT-29 cells to butyrate and analogs results in cell cycle block.HT-29 cells exposed to butyrate and PB (3 mM for 24 h) demonstrate a G2/M cell cycle block compared with untreated control cells. In contrast, the D4PB-treated cells revealed a dramatic increase in the S-phase fraction. Key: (*) $p = 0.05$; (**), $p = 0.003$.

dehydrogenation and the subsequent formation of the β carbonyl. Initial studies comparing the phenotypic effects of D4PB to its analogues, butyrate or PB, were performed, and the results are consistent with our hypothesis that D4PB treatment resulted in a greater decrease in cell proliferation ([^{3}H]thymidine incorporation) and increased apoptosis (annexin staining) compared with butyrate and PB.

Differences in the apoptotic rate between PB and D4PB could not be explained by changes in the expression of three major regulatory proteins of the apoptotic pathway. Treatment with butyrate, PB, or D4PB did not result in any effect on Bcl2 expression. Protein levels of Bax and Bad, two members of the Bcl2 family that function to promote the apoptotic cascade, were increased in all treated cells compared with control. Compared with butyrate-treated cells, PB and D4PB exposure resulted in a higher expression, but no difference in this increased expression was detected between PB and D4PB treated cells. Agents that inhibit HDAC have been studied as anticancer agents by allowing re-expression of silenced regulatory genes in transformed cells, which may reverse the malignant phenotype.[6] Butyrate and its derivatives have been shown to inhibit histone deacetylase. Because butyrate and PB are known effective inhibitors of histone deacetylation,[12] we studied the effect of the three fatty acids on HDAC activity in an attempt to rationalize the effect of D4PB on cell growth and apoptosis. Exposure to all three drugs resulted in a decrease in HDAC activity, with butyrate being the most effective, followed by D4PB and PB. Based on the fact that D4PB demonstrates no additional HDAC inhibition compared with butyrate or PB, while providing increased cytotoxicity, we do not believe that this mechanism can explain its increased potency. We concluded that the difference in the inhibition of HDAC activity caused by PB and D4PB was not of sufficient magnitude to explain the differences in potency observed with treatment. The induction of apoptosis has been linked in part to cell cycle regulation, and fatty acids have been shown to induce a G1/S and/or G2/M cell cycle block, depending on the cell type, conditions, and agent.[3,18,23] In this study we demonstrated that butyrate and PB induced a G2/M cell cycle block in HT-29 cells. In contrast, D4PB treatment resulted in an increase in the percent of cells in S-phase in the absence of either a G1/S or G2/M block. Based on the [^{3}H]thymidine incorporation data, this increase was not due to

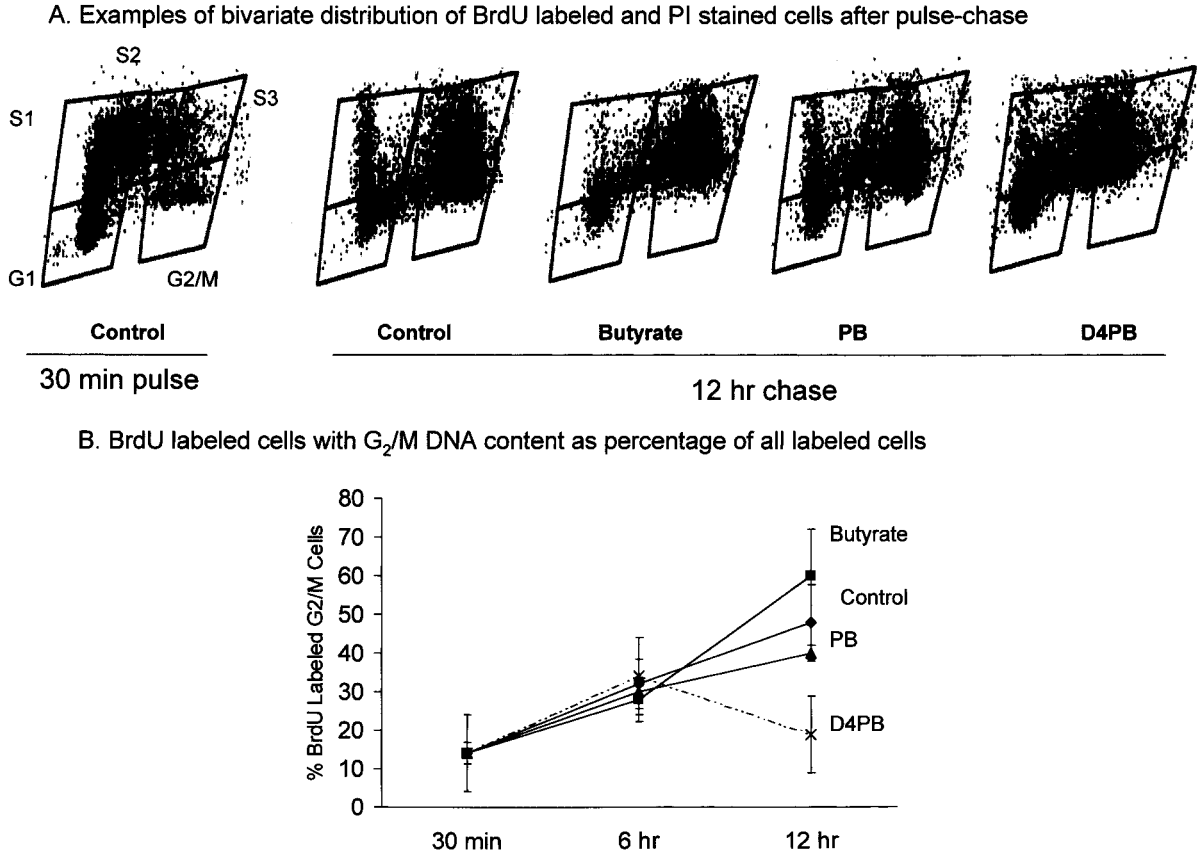


Figure 5. BrdU incorporation in HT-29 cells demonstrates S-phase slowing in D4PB-treated group. HT-29 cells were initially pulsed with 30 μM BrdU for 30 min. Cells were then washed and fresh media was added with the appropriate fatty acid. After 6 and 12 h, BrdU incorporation was analyzed by double staining (anti-BrdU FITC and PI staining). The percentage of BrdU-positive cells with G2/M DNA content was calculated following the 6- and 12-h chase to determine the exit from S-phase [% BrdU-labeled cells with G2/M DNA content = S3/(S1 + S2 + S3)]. After a 6-h chase, there was no difference in the % BrdU-labeled cells in the G2/M compartment in any of the groups. After a 12-h chase, there was an increase in the percentage of BrdU-labeled cells in the G2/M compartment (compared with the 6-h chase) in the control and butyrate- and PB-treated groups. However, in the D4PB-treated group, the percentage of labeled cells did not increase, suggesting a slower transit through S-phase. (A) Examples of the bivariate distribution of BrdU-labeled cells after pulse–chase are shown in panel. (B). Quantitation of the percentage of BrdU-labeled cells with G2/M DNA content as a fraction of all labeled cells at 6 and 12 h.

increased cell proliferation (Figure 1) but the result of slowing of transit cells through the S-phase. BrdU pulse–chase experiments confirmed this slower exit from the S-phase after D4PB treatment compared with control and butyrate- and PB-treated cells (Figure 5), suggesting that in contrast to butyrate and PB, D4PB may exert its effect through interfering with S-phase progression. This difference in cell cycle effect may offer insight into the relative potency of D4PB compared with PB. If the only mechanism of action of isotopic modification was slowing of PB degradation, then one would predict accentuation of the G2/M cell cycle block seen with PB exposure. The fact that retardation of S-phase transit was seen only with D4PB suggests deuteration of PB has other effects as well. In addition to a slowing of β oxidation, other possible mechanisms include steric changes to PB by the isotopic modifications, as well as changes in

solubility characteristics. With respect to solubility, a 1 mM solution of PB in PBS at pH 7.4 was readily obtained. In contrast, D4PB was insoluble at pH 7.4 in the absence of a cosolvent such as DMSO. To prevent precipitation of compound, D4PB was dissolved to a maximum concentration of 0.1 M in DMSO, and this solution was then diluted with buffer. This rather dramatic change in solubility on isotopic substitution was unexpected and warrants investigation, perhaps through *ab initio* calculations of structural/conformational effects. Further studies are needed to elucidate the underlying mechanisms responsible for this apparent slowing of the cell cycle.

In summary, this is the first report of the synthesis of deuterated PB, as well as the phenotypic effects observed after exposure of HT-29 cells to D4PB. The data presented indicate that D4PB, a deuterated analogue of PB, inhibits proliferation and induces apoptosis more effectively than butyrate and PB in HT-29 colon cancer cells. This differential effect may be related to the effect of D4PB on the cell cycle. D4PB may represent an improvement in PB as a treatment option for advanced colorectal cancer.

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