Am J Cancer Res 2022;12(7):3422-3436
www.ajcr.us /ISSN:2156-6976/ajcr0133389

Original Article

Short chain fatty acids exhibit selective estrogen receptor downregulator (SERD) activity in breast cancer

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Received March 22, 2021; Accepted July 23, 2021; Epub July 15, 2022; Published July 30, 2022

Abstract: Early stage estrogen receptor α (ERα, ESR1)-positive breast cancer patients can develop more aggressive endocrine-resistant tumors that express constitutively active mutant forms of ERα including ERα-Y537S and ERα-D538G. These patients are treated with selective ER down regulators (SERDs) such as the ERα antagonist fulvestrant. Previous studies show that histone deacetylase (HDAC) inhibitors downregulate ERα and since some dietary derived short chain fatty acids (butyrate, propionate and acetate) exhibit HDAC inhibitory activity we investigated their effects as SERDs in MCF-7 and T47D cells expressing wild-type and mutant ERα-D538G and ERα-Y537S. The SCFAs exhibited SERD-like activity in both cell lines expressing wild-type and mutant ERα. The results for propionate and butyrate correlated with parallel induction of histone acetylation and this was also observed for the HDAC inhibitors Panobinostat, Vorinostat and Entinostat which also downregulated wild-type and mutant ERα and induced histone acetylation. Although acetate induced ERα degradation the mechanisms may be independent of the HDAC inhibitory activity of this compound. These results suggest that high fibre diets that induce formation of SCFAs may have some clinical efficacy for treating ER-positive endocrine resistant breast cancer patients and this is currently being investigated.

Keywords: SCFAs, HDAC, inhibitors, ERα, downregulation

Introduction

Approximately 70% of all diagnosed breast cancer cases express estrogen receptor α (ERα, ESR1) and 17β-estradiol-mediated activation of ERα induces patterns of gene expression that are important for breast tumor growth and survival [1, 2]. Established therapies for ERα-expressing tumors include antiestrogens such as tamoxifen that block ERα-mediated responses and aromatase inhibitors that decrease estrogen synthesis and the combination of antiestrogen plus aromatase inhibitors are highly effective for treating patients with ERα-positive tumors [3, 4]. Despite the success of endocrine therapies some patients develop resistance to this therapeutic regimen and this is due in part to expression of constitutively active ERα mutants [6-9]. Most of the mutations are observed in amino acids in the ligand binding domain of ERα and the most frequent mutants are D538G and Y537S which are constitutively active [10]. Endocrine-resistant ER-positive breast cancer patients have a poor prognosis and are treated with the antiestrogen Fulvestrant which also induces degradation of wild-type and mutant ER. Fulvestrant is a prototypical selective ER degrader or downregulator (SERD), however, the clinical effectiveness of this compound is limited due to poor oral bioavailability and toxic side effects [11-14]. There is considerable ongoing research and clinical evaluation of novel SERDs that target ER degradation. For example, AZD9496 is non-steroidal ER antagonist and SERD being developed for treatment ER+ advanced breast cancer and this agent shows promising preclinical and clinical results [15-17].

SERDs such as Fulvestrant interact directly with ER and activate proteasome dependent degradation of the receptor, however, there are many other examples of pathways resulting in decreased expression of ERα [18]. For example, the potent AhR ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induces inhibitory AhR-

ERα crosstalk which results AhR-dependent downregulation of ER in breast cancer cells and this is also accompanied by AhR degradation [19, 20]. This pathway is unidirectional since E2 does not induce AhR degradation but activates proteasome-dependent degradation of ERα in breast cancer cells. In addition, there is also a report showing that ligand activated PPARγ induces ERα degradation [21]. Histone deacetylase (HDAC) inhibitors are being developed for treatment of breast and other cancers [22] and there is evidence that structurally-diverse HDAC inhibitors induce ERα degradation in breast cancer cells [23-28]. Short chain fatty acids (SCFAs) such as butyrate, propionate and acetate are produced in the gut by microbial degradation of high fibre diets and SCFAs also exhibit activity as HDAC inhibitors [29-31]. We hypothesized that SCFAs, like other HDAC inhibitors would also downregulate ERα and thereby act as SERDs and be effective for treating endocrine-resistant ERα positive breast cancers. This hypothesis was confirmed and this study shows that SCFAs induce degradation of wild-type and mutant ERα in MCF-7 and T47D breast cancer cells. Moreover, in an *in vivo* athymic nude mouse orthotopic model bearing MCF-7-ERα-Y537S cells butyrate inhibits tumor growth and downregulates mutant ERα in the tumors. These results support future studies on development of high fibre diets that are converted by intestinal microorganisms into SCFAs as a novel dietary approach for delivering SERD activity to enhance effectiveness of current chemotherapies for endocrine-resistant breast cancer.

Materials and methods

Cell lines, antibodies, and reagents

Breast cancer cell lines, MCF-7 and T-47D cells expressing wild-type and mutant ERα were kindly provided by Dr. Steffie Oesterreich, University of Pittsburgh, Department of Pharmacology and Chemical Biology and by Dr. Ben Ho Park of Vanderbilt University School of Medicine (Nashville, TN). Cells were grown and maintained at 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) or RPMI-1640 Medium with 10% FBS. DMEM, FBS, and trypsin were purchased from Gibco. Estrogen Receptor α (D6R2W), HDAC6 (D2E5), Acetyl-Histone H3 (Lys9/Lys14), Acetyl-Histone H3 (Lys27), Acetyl-Histone H4 (Lys8) antibodies were purchased from Cell Signaling (Boston, MA); HDAC1 (10E2) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA); β-Actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Sodium butyrate, sodium propionate, and sodium acetate were purchased from Sigma Aldrich. HDAC inhibitors Entinostat, Vorinostat, and Panobinostat were purchased from LC Laboratories (Woburn, MA).

Cell viability assay

Cells were plated in a 12 well plate at a density of 100,000 per well with DMEM containing 10% FBS. After 24 hours, cells were treated with DMSO and containing different concentrations of butyrate, propionate, or acetate with DMEM containing 2.5% FBS for 24 hours. After treatment with SCFAs, cells were washed with 100 µL of Hank's balanced salt solution (HBSS) and trypsinized with 100 µL. Once cells detached, 900 µL of DMEM containing 2.5% FBS was added. Cells were counted using a hemactyometer.

Measurement of apoptosis (annexin V staining)

Cells were seeded in a 6 well plate at a density of 200,000 per well with DMEM containing 10% FBS. After 24 hours, cells were treated with either DMSO or butyrate, propionate, or acetate for 24 hours. Cells were stained and analyzed by flow cytometry using Annexin V staining kit kit according to the manufacturer's protocol by Invitrogen (Grand Island, NY).

Real-time polymerase chain reaction (RT-PCR)

Expression of ERα-WT, ERα-D538G, and ERα-Y537S were measured after treatment with butyrate, propionate, or acetate using RT-PCR. MCF-7 cells were seeded in 6 well plates at a density of 200,000 cells per well. Cells were treated with butyrate, propionate, or acetate for 24 hours. Total RNA was extracted from cells using RNA mini prep kit from Zymo Research (Irvine, CA) according to the manufacturer's protocol and contaminated chromosomal DNA was removed by treatment of DNase I (Zymo Research). cDNA was prepared from total RNA of cells using amfiSure qGreen Q-PCR Master Mix (2X), Fluorescein, Q5601 from GenDEPOT. PCR was done in triplicate using iTaq universal SYBR Green mix (Bio-Rad) and

Bio-Rad CFX384 (C1000) real-time PCR System. TATA-binding protein (TBP) mRNA was used to normalize the expression levels. Primers were purchased from IDT Technologies (North Carolina, USA). The sequence of primers used for real-time PCR are: ESR1 WT #1 forward 5'-TGATGAAAGGTGGGATACGA-3' and reverse 5'-AAGGTTGGCAGCTCTCATGT-3' (https://www.sciencedirect.com/science/article/pii/S0945053X15000451); ESR1 WT #2 forward 5'-CCACCAACCAGTGCACCATT-3' and reverse 5'-GGTCTTTTCGTATCCCACCTTTC-3' (https://academic.oup.com/endo/article/151/6/2462/2456777). ESR1 Y537S forward 5'-CAGCATGAAGTGCAAGAACGT-3' and reverse 5'-TGGGCGTCCAGCATCTC-3'; ESR1 D538G forward 5'-GCATGAAGTGCAAGAACGTG-3' and reverse 5'-AAGTGGCTTTGGTCCGTCT-3' (https://clincancerres.aacrjournals.org/content/suppl/2015/11/24/1078-0432.CCR-15-1534.DC1).

Western blot analysis

MCF-7 and T47-D cells were seeded at a density of 200,000-300,000 in 6 well plates and allowed to attach for 24 hours. Cells were treated with either DMSO or various concentrations of butyrate, propionate, and acetate. Whole cell proteins were extracted using RIPA lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 (w/v), 0.5% sodium deoxycholate, and 0.1% SDS with protease and phosphatase inhibitor cocktail from GenDEPOT (Baker, TX). Protein concentrations were measured using Bradford assay and equal amounts of protein were separated in either 8%, 10%, or 15% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. Membranes were blocked for 1 hour with either 5% BSA or 5% skim milk. Membranes were incubated with primary antibodies overnight at 4°C and incubated with corresponding HRP-conjugated secondary IgG antibodies for 3 hours or overnight in 5% skim milk. Cell signaling antibodies were used at a ratio of 1:1000 in 5% BSA, SCBT antibodies were used at a ratio of 1:500 in 5% BSA; Sigma Aldrich antibodies were used at a ratio of 1:50,000 in 5% skim milk. Immune-reacted proteins were detected with chemiluminescence reagent.

Small interfering RNA interference assay

Cells were seeded in 6 well plates at a density of 50,000 cells per well in DMEM supplemented with 10% FBS. After 24 hours, cells were transfected with 100 nM of each siRNA for 6 hours using OptiMEM I Reduced Serum Medium (Gibco) and Lipofecatamine 2000 reagent (Invitrogen) following the manufacturer's protocol. Protein was extracted using RIPA lysis buffer after 72 hours post transfection incubation and western blot analysis was performed. The siRNA used to perform this assay were purchased from Sigma Aldrich and are: SASI_Hs01_00079968 (HDAC1 #1), SASI_Hs01_00079964 (HDAC1 #2), SASI_Hs01_00048982 (HDAC6 #1), SASI_Hs02_00340796 (HDAC6 #2).

Xenograft study

Female athymic nu/nu mice (4-6 weeks old) were purchased from Charles River Laboratory (Wilmington, MA). MCF-7 Y537S cells (5×10^6) were harvested in 100 µL of DMEM and suspended in ice-cold Matrigel (1:1 ratio) and orthotopically injected into the mammary fat pad of the mice. After two weeks of tumor cell inoculation, mice were divided in to two groups of 7 animals each. The first group received 100 µL of vehicle (corn oil), and the second group of mice received an oral gavage of 200 mg/kg/day of sodium butyrate in corn oil for three weeks. All mice were weighed once a week over the course of treatment to monitor changes in body weight and tumor volume was measured. After three weeks of treatment, mice were sacrificed, and tumor weights were determined. All animal studies were carried out according to the procedures approved by the Texas A&M University Institutional Animal Care and Use Committee.

Statistical analysis

All of the experiments were repeated a minimum of three times. The data are expressed as the mean ± standard deviation (SD) and significant differences between treatment groups compared to the untreated control were determined by students' t-test. Data with *P*-values less than 0.05 were considered statistically significant.

Results

SCFAs: inhibition of cell growth in cells expressing wild-type and mutant ERα

MCF-7 cells expressing wild-type ERα and mutant Y537S and D538G were generated using

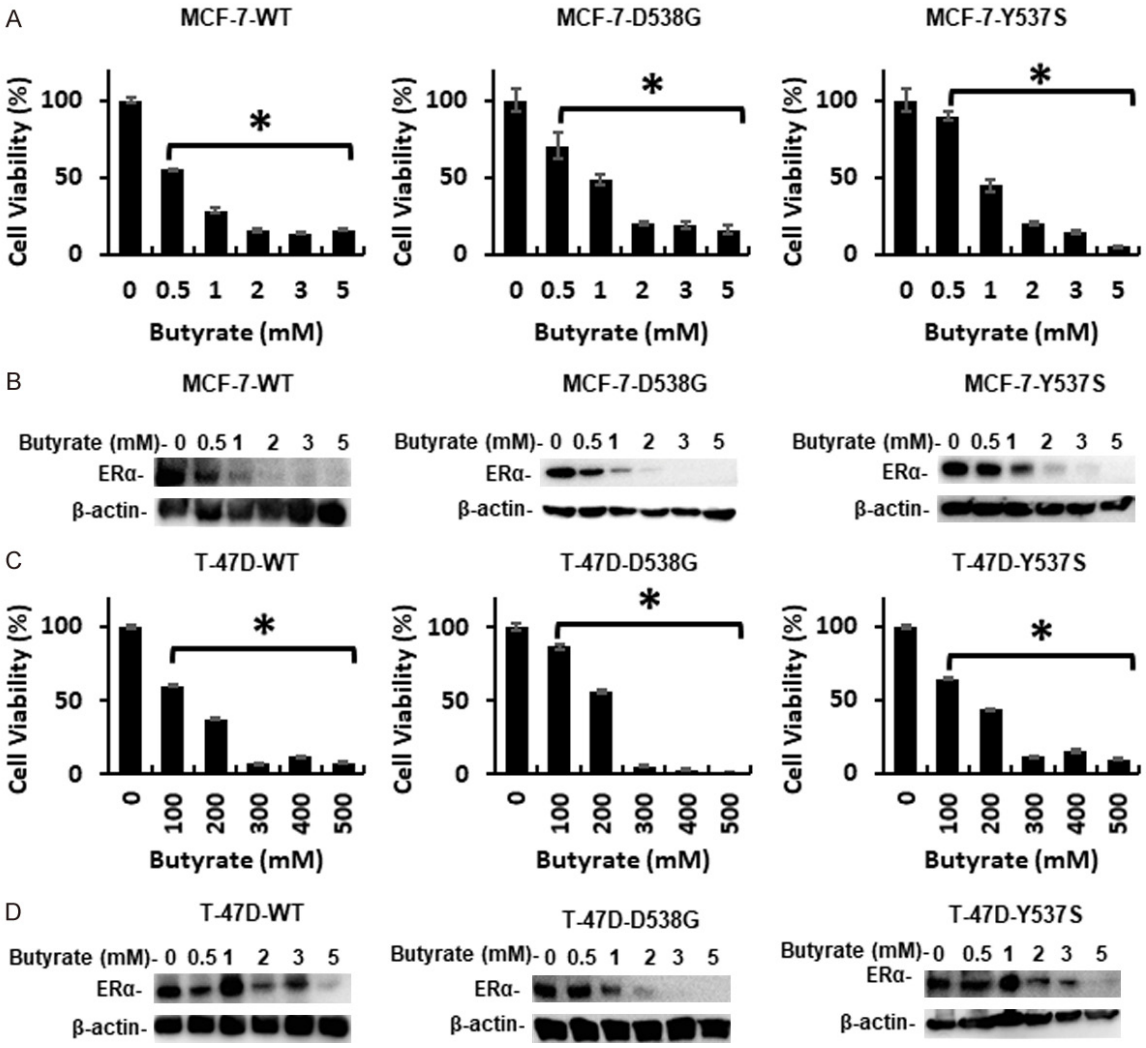

Figure 1. Effects of butyrate on breast cancer cell growth and ER downregulation. MCF-7 cells expressing wild-type and mutant ERα were treated with different concentrations of butyrate and effects on cell proliferation (A) and ERα downregulation (B) were determined as outlined in the Methods. T47D cell expressing wild-type and mutant ERα were treated with different concentrations of butyrate and effects on cell proliferation (C) and ERα downregulation (D) were determined as outlined in the Methods. Results (B and D) are means ± SD for at least 3 determinations and significant ($P<0.05$) inhibition is indicated (*).

recombinant adeno-associated virus (AA) technology using AAV vectors for both ERα mutants as described [32]. T47D breast cancer cells expressing wild-type and mutant ERα were generated by Bahreini and coworker using CRISPR-Cas9 genome editing [32]. The functional activities of butyrate, propionate and acetate were initially investigated for their effects on proliferation of MCF-7 cells expressing wild-type and mutant ERα. **Figure 1A** shows that 0.5-5.0 mM butyrate inhibits growth of MCF-7 cells expressing wild-type and mutant ERα (D538G and Y537S). Results illustrated in **Figure 1B** show that butyrate also downregulates ERα, ERα-D538G and ERα-Y537S proteins (Figure S1A) and there was a similar concentration-dependent decrease in cell proliferation and ERα downregulation in MCF-7 cells expressing wild-type and mutant ERα. Preliminary studies in T47D cells expressing ERα, ERα-D538G and ERα-Y537S indicated that butyrate concentrations ≤80 mM did not affect cell growth, however, significant growth inhibition was observed for 100 mM butyrate and higher concentrations (**Figure 1C**). In contrast, lower concentrations of butyrate (≥2 mM) downregulated wild-type and mutant ERα in T47D cells (**Figure 1D**). Propionate (≥10 mM) also decreased growth of

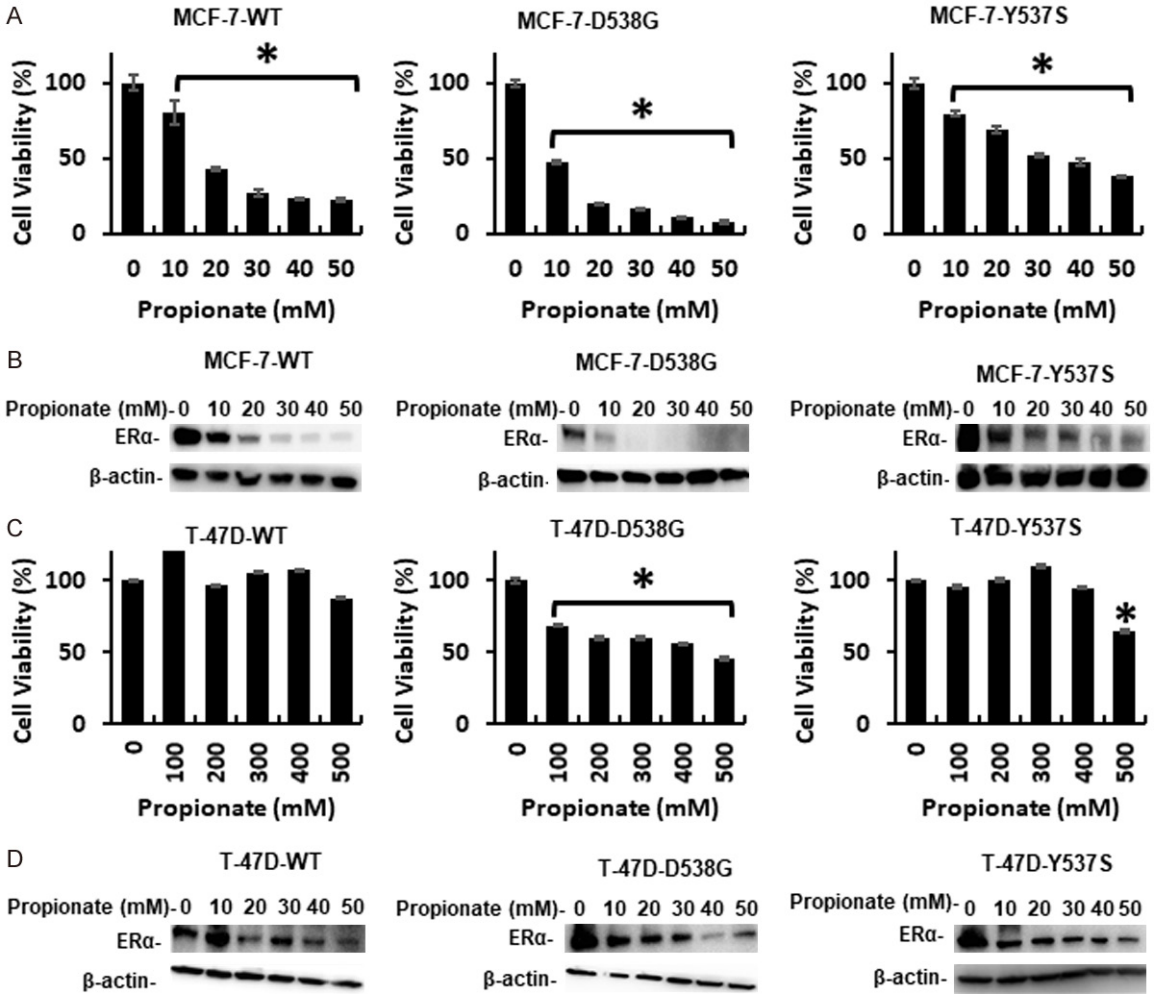

Figure 2. Effects of propionate on breast cancer cell growth and ER downregulation. MCF-7 cells expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (A) and ERα downregulation (B) were determined as outlined in the Methods. T47D cell expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (C) and ERα downregulation (D) were determined as outlined in the Methods. Results (B and D) are means ± SD for at least 3 determinations and significant ($P<0.05$) inhibition is indicated (*).

MCF-7 cells expressing wild-type or mutant ER (**Figure 2A**) and this was paralleled by downregulation of wild-type and mutant ERα at similar concentrations of propionate (**Figures 2B** and S1B). In contrast, up to 500 mM propionate did not inhibit growth of T47D cells expressing wild-type ERα or ERα-Y537S and 100 mM propionate decreased growth of T47D cells expressing ERα-D538G and minimal effects were observed at lower concentration (**Figure 2C**). Variable concentrations of propionate (<40-50 mM) downregulated wild-type and mutant ERα expressed in T47D cells (**Figure 2D**) and these concentrations were considerably lower than those required for growth inhibition. Acetate (>30-50 mM) inhibited growth of MCF-7 cells expressing wild-type and mutant ERα (**Figure 3A**) and downregulation of ERα by acetate was not observed in cells expressing wild-type ERα; acetate decreased ERα protein in cells expressing ERα-D538G (not significant) and ERα-Y537S (significant) (**Figures 3B** and S1C). Acetate (≤80 mM) did not inhibit growth of T47D cells (data not shown) whereas concentrations of acetate ≥100 mM inhibited growth of cells expressing wild-type or mutant ERα (**Figure 3C**). Although lower concentrations of acetate downregulated wild-type ER (≥50 mM), ERα-D538G (≥100 mM), ERα-Y537S (≥100 mM) expressed in T47D cells, the differential

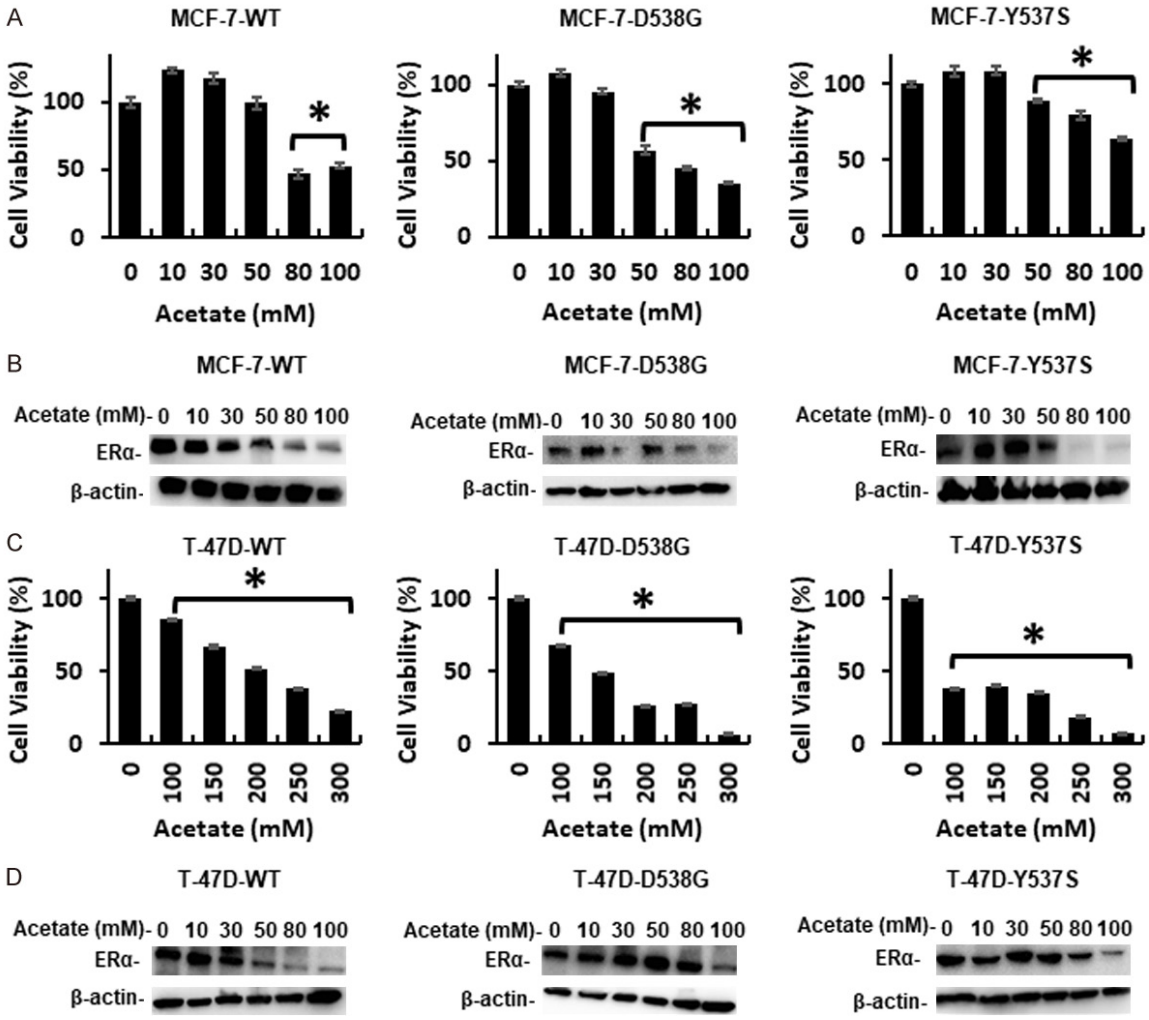

Figure 3. Effects of acetate on breast cancer cell growth and ER downregulation. MCF-7 cells expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (A) and ERα downregulation (B) were determined as outlined in the Methods. T47D cell expressing wild-type and mutant ERα were treated with different concentrations of propionate and effects on cell proliferation (C) and ERα downregulation (D) were determined as outlined in the Methods. Results (B and D) are means ± SD for at least 3 determinations and significant ($P<0.05$) inhibition is indicated (*).

concentration gradient required for growth inhibition (higher) and ERα downregulation (lower) observed for butyrate and propionate (**Figures 1** and **2**) was not observed for acetate (**Figure 3D**). However, the results obtained for butyrate and propionate indicate that downregulation of wild-type or mutant ERα by these SCFAs is observed at concentrations that do not affect cell growth in T47D cells.

SCFA induction of annexin V staining

We used MCF-7 cells as models to further characterize SCFA-induced functional effects in cells expressing wild-type and mutant ERα. Both acetate (100 mM) and propionate (50 mM) but not butyrate induced Annexin V staining in cells expressing wild-type ERα (**Figures 4A** and S2A). In contrast, 5 mM butyrate induced Annexin V staining in cells expressing ERα-D538G whereas acetate and propionate did not induce Annexin V staining (**Figures 4B** and S2B). Butyrate and propionate but not acetate induced Annexin V staining in cells expressing ERα-Y537S (**Figures 4C** and S2C). Thus, the pattern of induction of Annexin V staining in MCF-7 cells expressing wild-type and mutant ERα was compound and cell context-dependent and different from the SCFA-induced downregulation of ERα and growth inhibition (**Figures 1-3**).

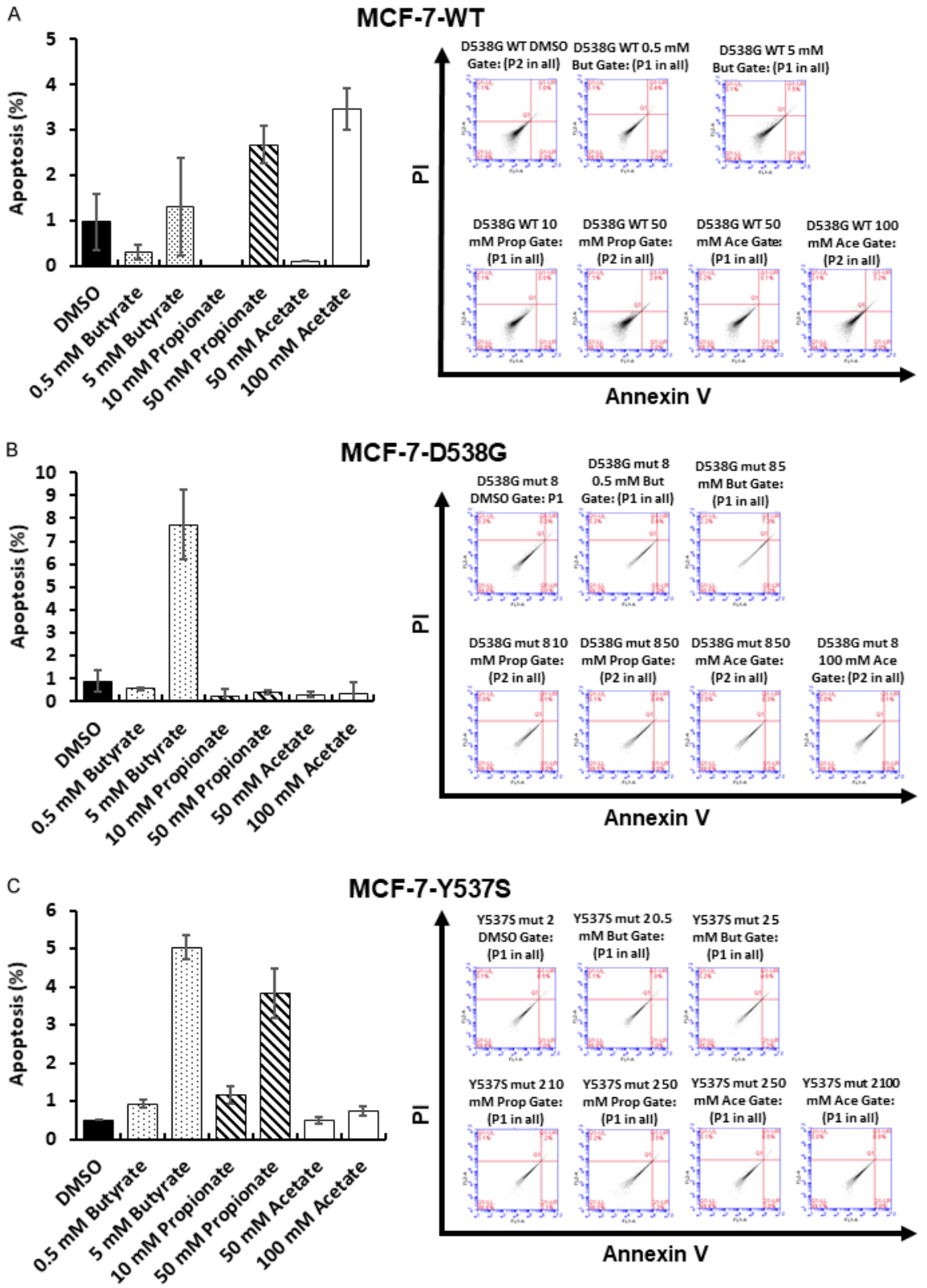

Figure 4. SCFAs induced Annexin V staining MCF-7 cells expressing wild-type and mutant ERα. MCF-7 cells expressing wild-type ERα (A), ERα-D538G (B) and ERα-Y537S (C) were treated with different concentrations of SCFAs and Annexin V staining was determined as outlined in the Methods. Results are expressed as means ± SD for at least 3 determinations per treatment group and significant ($P<0.05$) induction is indicated (*).

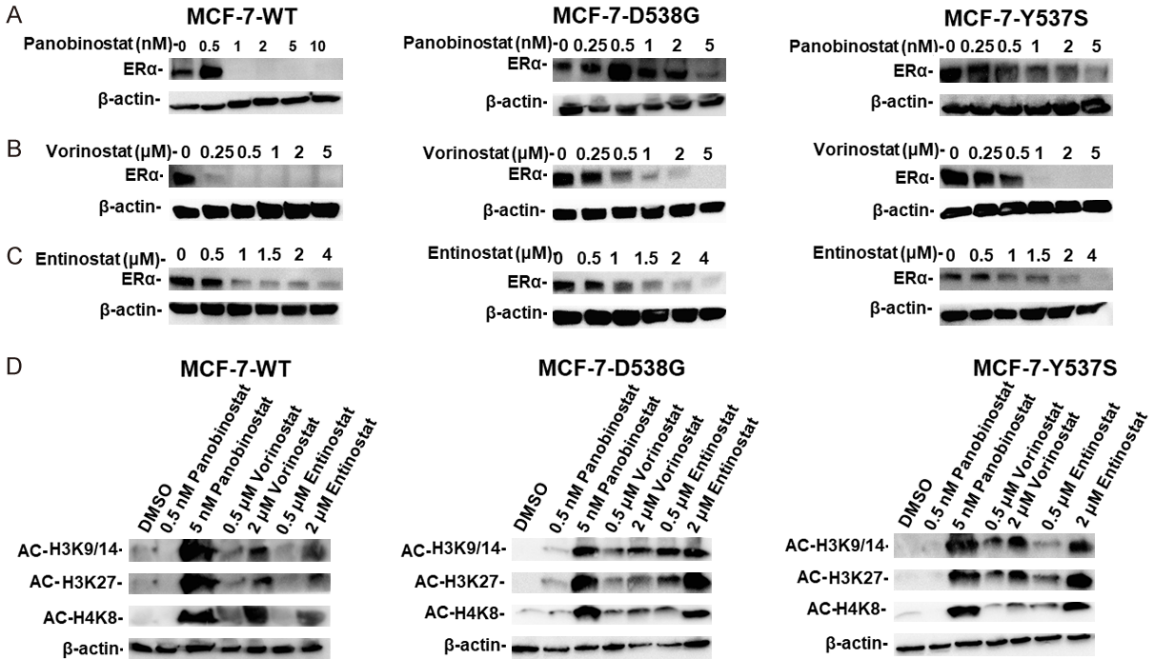

Figure 5. HDAC inhibitors downregulate wild-type and mutant ERα. MCF- cells expressing wild-type and mutant ERα were treated with Panobinostat (A), Vorinostat (B) and Entinostat (C) for 24 hours and whole cell lysates were analyzed by western blots. (D) Treatment with the HDAC inhibitors and effects on histone acetylation were also determined is outlined in (A-C) and in the Methods.

Thus, SCFA-induced growth inhibition of MCF-7 cells is primarily due to diverse ERα (wild-type and mutant) regulated pathways.

HDAC inhibitors downregulate wild-type and mutant ERα and enhance histone acetylation

We also examined the effects of well characterized HDAC inhibitors Panobinostat, Vorinostat and Entinostat of an ERα downregulation and histone acetylation in MCF-7 cells. All three HDAC inhibitors downregulated wild-type and mutant ERα expressed in MCF-7 cells (**Figure 5A-C**). Panobinostat decreased wild-type ERα at concentrations from 0.5-1.0 nM whereas higher concentration (up to 5 nM) was required to decrease D538G and Y537S. Both Vorinostat and Entinostat decreased wild-type and mutant ERα in the low μM range. All three HDAC inhibitors increased H3K9/14, H3K27 and H4K8 acetylation in MCF-7 cells expressing wild-type ERα, and mutant D538G and Y537S with some differences in the intensities of the acetylated histone bands in the gels (**Figure 5D**).

SCFAs inhibit HDAC activity and role of HDACs in downregulating ERα

Previous studies show that SCFAs inhibit HDAC activity in colon cancer cells (31) and therefore we examined the effects of butyrate, propionate and acetate and their effects on acetylation of H3K9/14, H3K27 and H4K8 in expressing wild-type and mutant ERα MCF-7 cells. In cells expressing wild-type ERα butyrate and propionate enhanced acetylation of all three histones whereas acetylated histone bands were less intense after treatment with up to 80 mM acetate (**Figure 6A**), a concentration that induced degradation of wild-type and mutant ERα (**Figure 3C**). Both butyrate and propionate induced acetylation of the 3 histones in MCF-7 cells expressing ERα-D538G (**Figure 6B**) with minimal increased acetylation after treatment with acetate. In cells expressing ERα-Y537S butyrate increased acetylation of H3K9/14, H3K27 and H4K8 whereas minimal effects were observed for propionate and acetate (**Figure 6C**). These results show that butyrate enhances histone acetylation in MCF-7 cells expressing wild-type and mutant ERα. However, that pattern of propionate/acetate-induced histone acetylation is cell context-dependent suggesting that SCFA-induced ERα downregulation is dependent on the individual SCFA and cell context.

It has previously been reported that downregulation of individual HDACs can be accompanied

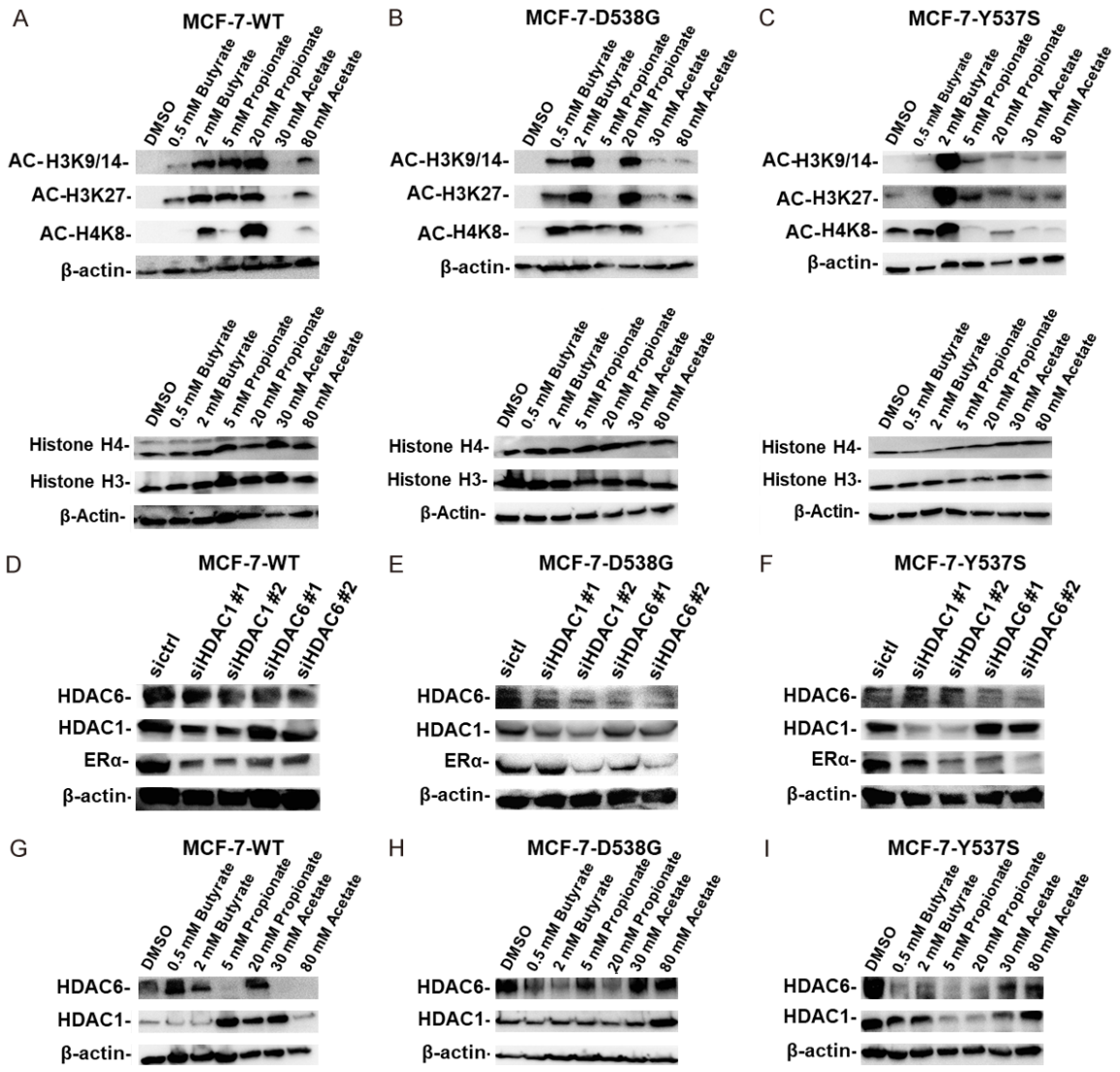

Figure 6. Effects of SCFAs on HDAC1 and HDAC6 expression. MCF-7 cells expressing wild-type ERα (A), ERα-D538G (B) and ERα-Y537S (C) were treated with SCFAs for 24 hours and whole cell lysates were analyzed by western blots for changes in histone acetylation and effects on total H3 and H4 are also given. MCF-7 cells expressing wild-type ERα (D), ERα-D538G (E) and ERα-Y537S (F) were transfected with oligonucleotides targeted against HDAC6 or HDAC1 and after 72 hours whole cell lysates were analyzed by western blots. MCF-7 cells expressing wild-type ERα (G), ERα-D538G (H) and ERα-Y537S (I) were treated with SCFAs by western blots as outlined above and in the Methods.

by decreased expression of ERα [24, 26] and we therefore investigated the possible linkage between SCFAs, HDAC downregulation and ERα downregulation. Results in **Figure 6D-F** summarize studies on the effects of HDAC1 and HDAC6 knockdown on expression of wild-type and mutant ERα. The individual HDACs selected were highly expressed in the breast cancer cell lines. In MCF-7 cells expressing wild-type ERα knockdown of HDAC1 or HDAC6 by RNA interference (RNAi) decrease ERα (**Figure 6D**) and similar results were observed in cells expressing ERα-D538G (**Figure 6E**) and ERα-Y537S (**Figure 6F**). Quantitation of the data (**Figure 6D-F**) showed highly variable results for knockdown of the individual HDACs, however, siHDACi and siHDAC6 oligonucleotides significantly decreased ERα and ERα-Y537S (Figure S2). Since loss of HDAC1 or HDAC6 by RNAi decreased ERα we investigated the effects of individual SCFAs on expression of HDAC1 and HDAC6. In MCF-7 cells expressing wild-type ERα (**Figure 6G**) only 80 mM acetate significantly decreased expression of HDAC6 (Figure

S3A), and 5 mM butyrate and 20 mM propionate decreased HDAC6 (**Figures 6H** and S3) in cells expressing ERα-D538G. Acetate had no effect on HDAC6 expression in this cell line and HDAC1 was not affected by the SCFAs. In MCF-7 cells expressing ERα-Y537S, one or more concentrations of butyrate and propionate decreased HDAC1 and HDAC6 and acetate did not significantly modulate HDAC expression (Figure S3). Thus, the SERD-like activity of SCFAs may be associated with their downregulation of HDAC6 and HDAC1 by butyrate and propionate in cells expressing ERα-Y537S whereas the results in the other two cell lines are highly variable.

Butyrate inhibits tumor growth in vivo

The *in vivo* effects of SCFAs were investigated using butyrate as a model and it was administered in the drinking water (150 mM) as previously described [33]. Over the 21 days of the experiment butyrate significantly inhibited an increase in tumor volume in athymic nude mice bearing MCF-7 cells expressing ERα-Y537S (**Figure 7A** and **7B**). Tumor weights were also decreased in the butyrate treated mice (**Figure 7C**) whereas butyrate did not affect body weight over the duration of the study (**Figure 7D**). Western blot analysis of tumor lysates demonstrated that treatment with butyrate decreased ERα-Y537S, HDAC1 and HDAC6 and increased expression of acetylated H3K9/14 and H4K8 (**Figure 7E**). These in vivo results were comparable to those observed in cell culture studies and confirm that butyrate and SCFAs are SERDs with potential for clinical applications in treating patients with endocrine-resistant ER-positive tumors.

Discussion

Fiber-enriched diets enhance microbial-induced formation of short chain fatty acids such as butyrate, acetate and propionate which play a protective role in maintaining gut health. SCFAs also exhibit anticancer activities in both ER-positive and ER-negative breast cancer cells and this includes inhibition of cell proliferation, survival and migration/invasion [34-41]. Several studies have reported that butyrate and propionate exhibit activity as HDAC inhibitors and recent studies in this laboratory show that not only butyrate and propionate but also acetate is HDAC inhibitors in colon cancer cells (31). Several previous studies show that HDAC inhibitors induce ER downregulation in breast cancer cells (23-28) and in this study we have also observed these effects in MCF-7 cells expressing wild-type ER and mutant ERα-D538G and ERα-Y537S (**Figure 5**). Based on the reported SERD-like activity of HDAC inhibitors we hypothesized that butyrate, propionate and acetate may also exhibit SERD-like activity and have some clinical utility for treating highly aggressive endocrine-resistant ER-positive breast cancers.

Previous studies generated and characterized MCF-7 and T47D cells expressing wild-type ERα and the constitutively-active ERα-D538G and ERα-Y537S mutants (32) and these cell lines were used as models to evaluate SCFAs as SERDs. Treatment of the MCF-7 and T47D cells with butyrate, and propionate decreased wild-type and mutant ERα protein levels in both cell lines and MCF-7 cells were more responsive than T47D cells to the SERD-like activity of SCFAs. The effects of acetate in MCF-7 cells expressing wild-type and mutant ER were variable (**Figures 4**, S1C and S2) and cells expressing ERα or ERα-D538G were growth inhibited by acetate at concentrations that did not degrade ERα. Result of SCFA-dependent inhibition of cell proliferation demonstrated that the range of propionate and butyrate concentrations that downregulated wild-type and mutant ERα in MCF-7 cells also inhibited cell growth whereas this was not observed in T47D cells (**Figures 1-3**). Mutant ERα-D538G and ERα-Y537S are constitutively active and important for cell growth and survival in cells expressing the mutant ERα variants (32), however, in T47D cells the concentrations of propionate and butyrate required for downregulation of mutant ERα did not inhibit growth suggesting involvement of other factors. Therefore, our subsequent studies focused on MCF-7 cells expressing wild-type and mutant ERα as a model for further investigating SCFAs as SERDs.

It has previously been reported that HDAC inhibitors downregulate ERα (23-28) and we confirmed that like SCFAs the HDAC inhibitors Panobinostat, Vorinostat and Entinostat decrease not only wild-type ERα but also ERα-D538G and ERα-Y537S (**Figure 5D-F**). Not surprisingly, SCFAs and HDAC inhibitors enhanced histone acetylation in MCF-7 cells expressing wild-type and mutant ERα (**Figures 5** and **6**),

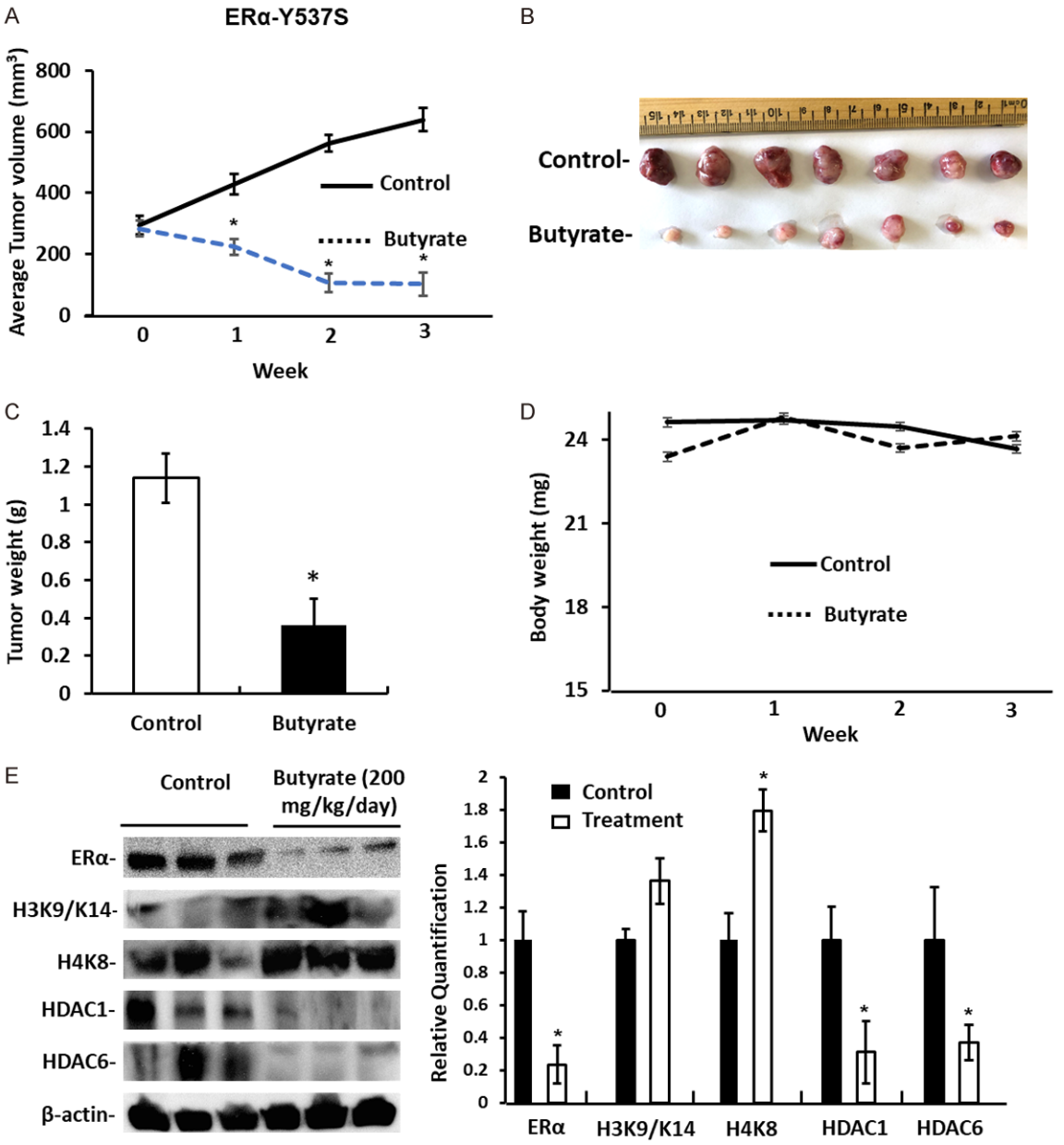

Figure 7. In vivo studies using MCF-7 (ERα-Y537S) cells and in an orthotopic model effects of oral butyrate. Athymic nude mice bearing MCF-7 (ERα-Y537S) cells orthotopically were administered butyrate in the drinking water for 3 weeks and effects of butyrate on tumor volumes (A, B), tumor weight (C) and whole-body weight (D) compared to controls were determined. For select tumors, lysates were obtained and analyzed by western blots (E) and effects on expression of selected proteins was determined by western blots as outlined in the Methods. Protein levels were quantitated relative to β-actin and levels in the control group were set at 1.0. Significant ($P<0.05$) decreases are indicated.

however, the patterns of histone acetylation were highly variable and dependent on the compound and cell context. Moreover, in at least one cell line (ERα-Y537S) acetate did not enhance acetylation of H3K9/14, H3K27 or H4K8 (**Figure 6C**) even though downregulation of mutant ERα was observed (**Figure 3B**). These results suggest that pathways other than histone acetylation may be contributing to the SERD-like activity of SCFAs and HDAC inhibitors. It has previously been reported that butyrate downregulates HDAC expression in lung cancer cells [42] and mouse neural cells in culture [43] and the HDAC inhibitor LAQ824

decreases both ERα and HDAC6 in MCF-7 cells (43). Moreover, it has also been reported that HDACs stabilize ERα and knockdown of HDAC6 by RNA interference decreased levels of ERα protein in MCF-7 cells (24). **Figure 6D-F** show that HDAC1 and HDAC6 are expressed in MCF-7 cells expressing wild-type and mutant ERα and knockdown by RNAi decreased expression of wild-type and ERα-Y537S but not ERα-D538G. However, the effects of SCFAs on expression of HDAC1 and HDAC6 gave variable results that were dependent on the individual SCFA and cell line. The only consistent results were observed in MCF-7 cells expressing ERα-Y537S where both butyrate and propionate and to a lesser extent acetate downregulated HDAC6 and HDAC1 (only butyrate and propionate). These results suggest a possible role for SCFA-induced down regulation of HDACs in mediating SERD-like activity targeting mutant ERα-Y537S and further mechanism studies are ongoing. *In vivo* studies using mouse xenografts bearing MCF-7 cells expressing ERα-Y537S show that butyrate in the diet decreased tumor growth and also decreased ERα-Y537S, HDAC1 and HDAC6 proteins (**Figure 7**) and this paralleled *in vitro* studies with butyrate in this cell line.

In summary, SCFAs butyrate, propionate and acetate inhibit growth of MCF-7 cells expressing wild-type and mutant ERα and also downregulated ERα thus exhibiting SERD-like activity. Similar results were observed for the HDAC inhibitors Entinostat, Panobinostat and Vorinostat. The mechanisms of downregulation of wild-type and mutant ERα may be linked, in part to the activity of SCFAs as HDAC inhibitors, and SCFA-induced HDAC1 and HDAC6 downregulation may also contribute to their SERD-like activity in MCF-7 cells expressing ERα-Y537S. The observations that SCFAs exhibit SERD-like activity may have potential clinical applications and we are currently investigating effects of high fiber diets which enhance microbial production of SCFAs as a potential dietary modification that can be used in combination with SERDs for treating patients with endocrine resistant ER-positive breast cancer.

Acknowledgements

This work was supported by the National Institutes of Health P30-ES029067 (SS), Texas A&M AgriLife Research (SS), and the Sid Kyle Chair Endowment (SS). The generous gift of the cell lines from Drs Steffie Oesterreich and Ben Ho Park made this study possible. Financial support was provided by the National Institutes of Health P30-ES029067 (SS) and R35-CA-197707 (RSC).

Disclosure of conflict of interest

None.

Abbreviations

ERα, estrogen receptor α; ESR1, estrogen receptor 1; SERDs, selective estrogen receptor downregulators; HDAC, histone deacetylase; FBS, fetal bovine serum; TCDD, tetrachlorodibenzo-p-dioxin; PVDF, polyvinylidene difluoride; SCFA, short chain fatty acid; TBP, TATA-binding protein; RT-PCR, Real-time polymerase chain reaction; HBSS, Hank's balanced salt solution.

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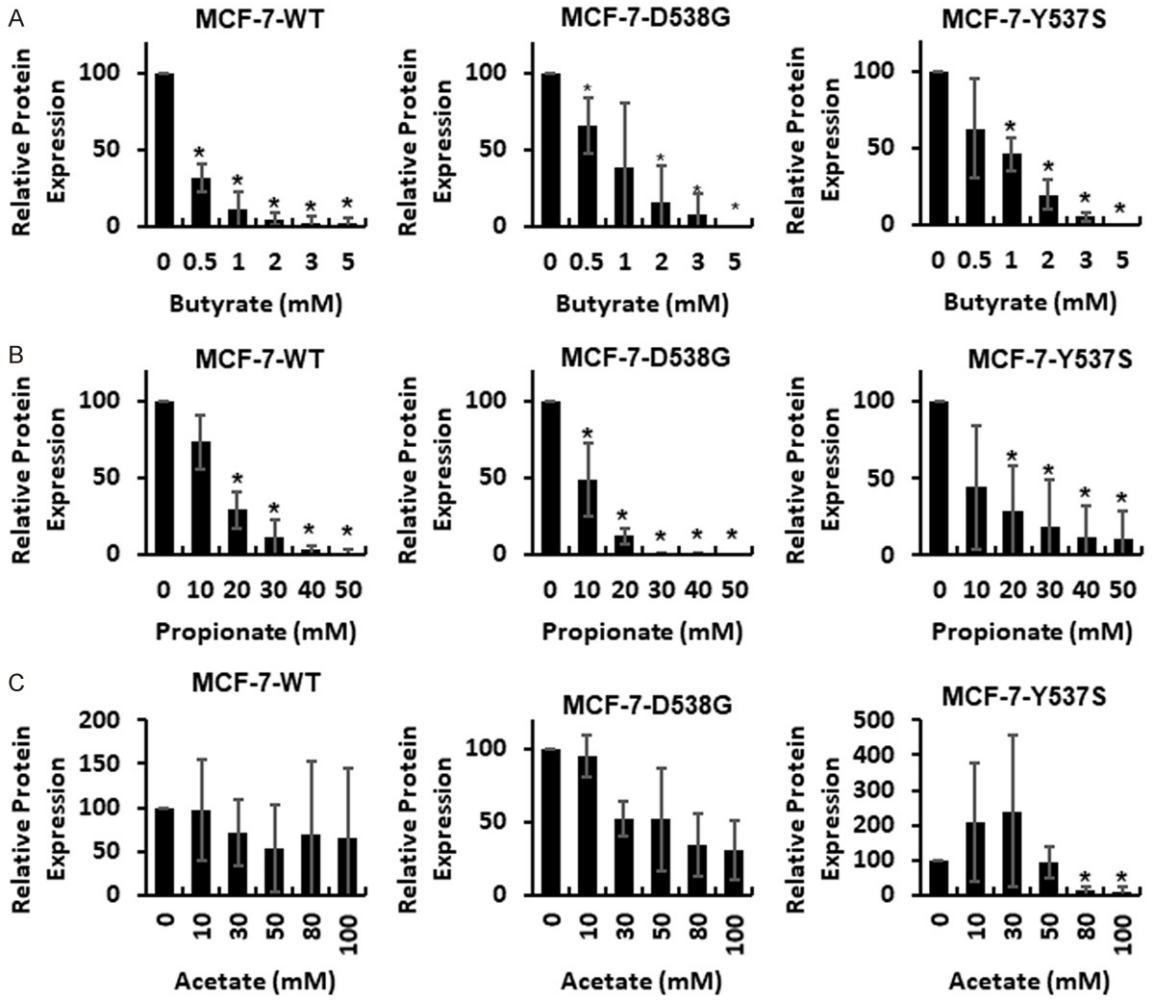

Figure S1. Quantitation of SCFA-induced ERα downregulation. MCF-7 cells expressing ERα, ERα-D538G and ERα-Y537S were treated with butyrate (A), propionate (B) and acetate (C) and levels of ERα protein were quantitated relative to control (solvent) treatment and normalized to β-actin as the loading control (**Figures 1-3**). Results are expressed as means ± SD for 3 replicate determinations for each treatment group and significantly ($P<0.05$) decreased ERα protein is indicated (*).

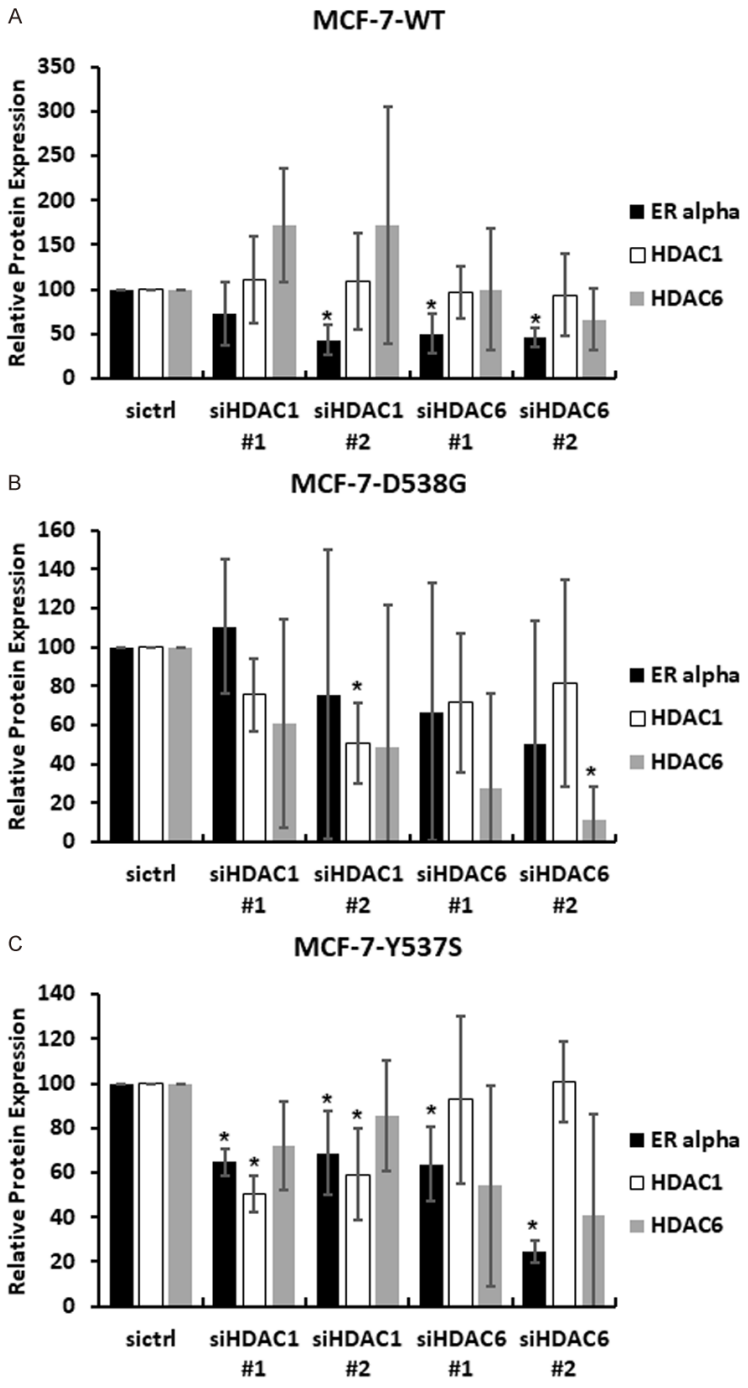

Figure S2. Quantitation of ERα in MCF-7 cells after knockdown of HDAC1 or HDAC6. MCF-7 cells expressing ERα (A), ERα-D538G (B) or ERα-Y537S (C) were transfected with oligonucleotides (2) targeting HDAC1 and HDAC6 (**Figure 6D-F**) and levels of ERα, HDAC1 and HDAC6 were quantitated relative to control (solvent) treatment and normalized to β-actin as the loading control. Results are expressed as means ± SD for 3 replicate determinations for each treatment group and significantly ($P<0.05$) decreased ERα, HDAC1 and HDAC6 proteins are indicated (*).

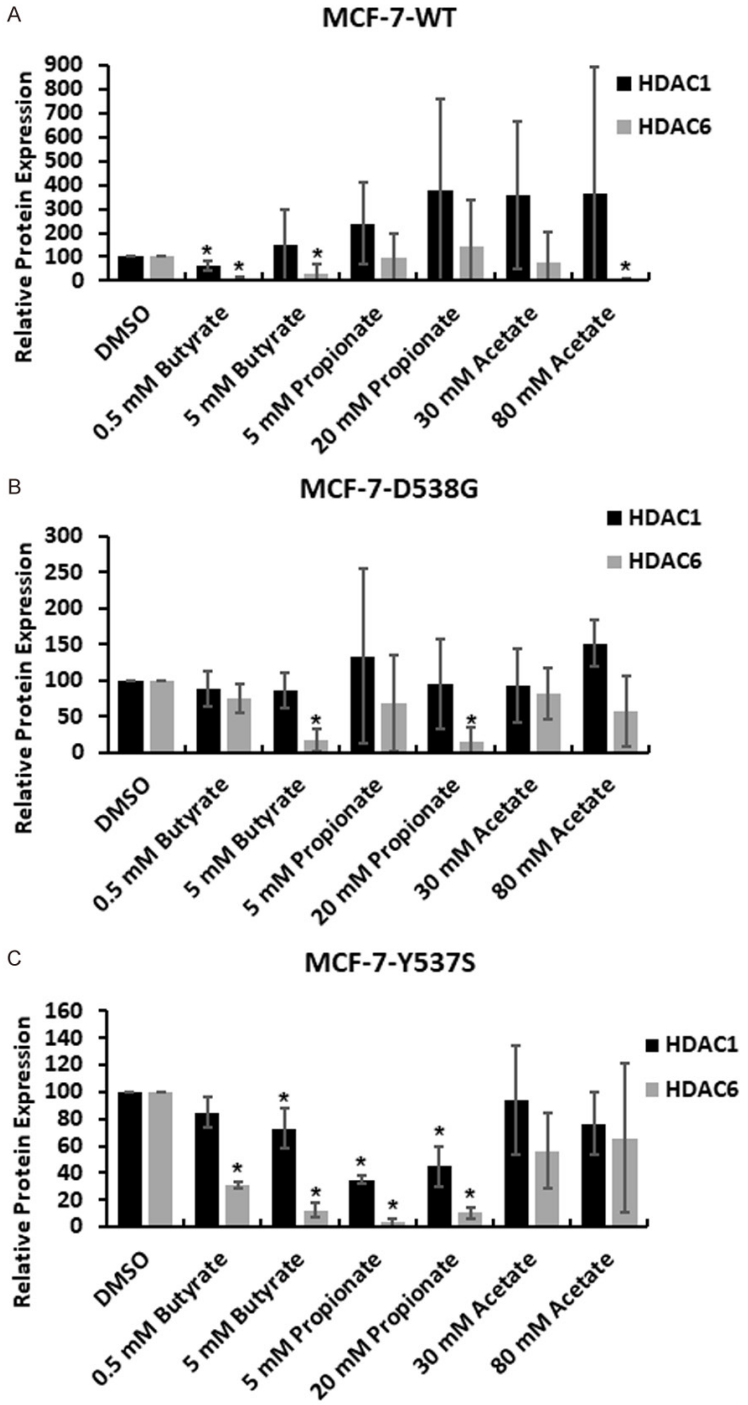

Figure S3. Quantitation of ERα in MCF-7 cells after treatment with SCFAs. MCF-7 cells expressing ERα (A), ERα-D538G (B) or ERα-Y537S (C) were transfected SCFAs (**Figure 6G-I**) and levels of HDAC1 and HDAC6 were quantitated relative to control (solvent) treatment and normalized to β-actin as the loading control. Results are expressed as means ± SD for 3 replicate determinations for each treatment group and significantly ($P<0.05$) decreased HDAC1 and HDAC6 proteins are indicated (*).