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Abstract

The combined treatment with histone deacetylase inhibitors (HDACi) and retinoids has been suggested as a potential epigenetic strategy for the control of cancer. In the present study, we investigated the effects of treatment with butyrate, a dietary HDACi, combined with vitamin A on MCF-7 human breast cancer cells. Cell proliferation was evaluated by the crystal violet staining method. MCF-7 cells were plated at 5 x 10⁴ cells/mL and treated with butyrate (1 mM) alone or combined with vitamin A (10 µM) for 24 to 120 h. Cell proliferation inhibition was 34, 10 and 46% following treatment with butyrate, vitamin A and their combination, respectively, suggesting that vitamin A potentiated the inhibitory activities of butyrate. Furthermore, exposure to this short-chain fatty acid increased the level of histone H3K9 acetylation by 9.5-fold (Western blot), but not of H4K16, and increased the expression levels of p21WAF1 by 2.7-fold (Western blot) and of RARβ by 2.0-fold (quantitative real-time PCR). Our data show that RARβ may represent a molecular target for butyrate in breast cancer cells. Due to its effectiveness as a dietary HDACi, butyrate should be considered for use in combinatorial strategies with more active retinoids, especially in breast cancers in which RARβ is epigenetically altered.

Key words: Breast cancer; MCF-7 cells; Butyric acid; Vitamin A; Epigenetics

Introduction

Breast cancer is the second leading cause of death by cancer in women globally and each year approximately 1 million new cases of this disease are diagnosed (1). Thus, novel approaches to the prevention and treatment of breast cancer are imperative (2).

Deregulation of epigenetic mechanisms, such as DNA methylation and post-translational modifications of histones, has been implicated in breast carcinogenesis due to the observed silencing of critical tumor suppressor genes (3). In contrast to genetic alterations, epigenetic modifications are potentially reversible and have been identified as relevant molecular targets for cancer prevention and treatment (4). Bioactive food components exhibiting anticancer potential have been shown to modulate DNA methylation and histone acetylation (5,6) in both in vitro and in vivo systems. Reactivation of epigenetically silenced genes by dietary components could potentially have important implications for cancer control strategies (7). Among the dietary histone deacetylase inhibitors (HDACi), butyrate (BT) is of primary interest because of...
its anticancer potential (8,9) and because it has already been tested for the treatment of cancer in phase I clinical trials (10,11). This short-chain fatty acid is produced by the fermentation of non-digestible dietary carbohydrates by intestinal bacteria and is also present in honey and milk fat (12). BT represents a promising candidate for the control of breast cancer progression (13,14). Previous studies have demonstrated that BT treatment inhibits the growth of breast cancer cell lines by acting as an HDACi and by inducing the expression of several genes, including cyclin-dependent kinase inhibitor p21 (14-16).

Vitamin A (VA; retinol) is an essential nutrient that regulates several cellular processes, including growth, death and differentiation, and is known to be frequently deregulated during carcinogenesis. A majority of the functions exhibited by VA are exerted by its conversion to retinoic acid, which modulates gene expression by activating retinoic acid receptors (RARs) and retinoid X receptors. Due to these properties, VA has been considered for the treatment and prevention of breast cancer (17). An important limitation of its therapeutic application, however, involves the deregulation of retinol metabolism in breast cancer cells that lack the ability to produce retinyl esters and thus to store VA (18). At the molecular level, this aberrant metabolism has been associated with epigenetic down-regulation of the tumor suppressor genes RARβ and cellular retinol-binding protein I (CRBP-I) (19-21). CRBP-I is responsible for the intracellular transport, esterification and oxidation of retinol (22).

The combination of an HDACi and retinoids represents a potential epigenetic strategy for cancer control (23), although few studies have addressed its efficacy in the context of breast cancer. In a previous study, retinoic acid treatment potentiated the inhibitory effects of the synthetic HDACi trichostatin on breast cancer cells in vitro and in vivo (24). More specifically, reactivation of RARβ expression induced by the synthetic HDACi increased the susceptibility of these cells to the inhibitory activities of retinoic acid (24). Based on these studies, we investigated the effects of the dietary HDACi BT alone and in combination with VA on estrogen receptor-α-positive MCF-7 human breast cancer cells. To assess the efficacy of these treatments, we evaluated several parameters such as cell proliferation, histone H3K9 and H4K16 acetylation patterns, global DNA methylation patterns, RARβ and CRBP-I gene and p21 protein expression, RARβ gene promoter methylation, and intracellular retinoid levels.

Material and Methods

Cell culture and solution preparation

The MCF-7 human breast cancer cell line (HTB 22, American Type Culture Collection, USA) was grown in Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (Gibco) at 37°C in a 5% CO2/air atmosphere. VA (retinyl palmitate; BASF, Germany) was dissolved in ethanol to produce 30 mM solutions. Further dilutions were made in culture medium to a final working solution of 10 µM VA containing 0.035% (v/v) ethanol. The VA solution was prepared immediately before use for each experiment. BT (sodium butyrate; Sigma-Aldrich, USA) was dissolved in culture medium to produce stock solutions of 0.5 M. Further dilutions were made in culture medium to achieve a final concentration of 1 mM BT. To control for the residual concentration of ethanol (0.035%, v/v) in the culture medium in the 10-µM VA working solution, ethanol was added to the culture medium, at this same concentration, of both BT-treated and control cells.

Cell proliferation assays

MCF-7 cells were plated at 5 x 10^4 cells/mL onto 96-well plates and incubated for 24 h. Cells were then treated with BT (0.5, 1, 2, 3, 4, and 5 mM) or VA (1, 5, 10, 15, and 20 µM) and incubated for 24 to 120 h. Next, the combinatorial effects of BT and VA on MCF-7 cell proliferation were evaluated. Cells were treated with 1 mM BT and 10 µM VA, individually or combined, and incubated with the treatments for 24 to 120 h. Following incubation, cell proliferation was evaluated by the crystal violet staining method. Data are reported as means ± SEM.

Cell cycle analysis by cell cytometry

MCF-7 cells were plated at a density of 1 x 10^5 cells/mL, treated with 1 mM BT and 10 µM VA, individually or combined, and incubated for 24 to 120 h. Following trypsinization, the harvested cells were resuspended with 1 mL chilled phosphate-buffered saline (PBS; 137 mM NaCl; 10 mM Na2HPO4; 2.68 mM KCl; 1.76 mM KH2PO4) and permeabilized with 3 mL chilled 100% methanol overnight. Afterwards the cells were centrifuged at 160 g for 5 min and washed with 1 mL PBS. The pellet was resuspended in 200 µL PBS containing propidium iodide (10 µg/mL) and RNase (10 mg/mL) and incubated for 1 h. The DNA content of the cells was then analyzed with a FACS CANTO II flow cytometer (BD Biosciences, USA).

Western blot analysis

Acetylation of H3K9 and H4K16. MCF-7 cells were plated at a density of 1 x 10^5 cells/mL, treated with 1 mM BT and 10 µM VA, individually or combined, and incubated for 96 and 120 h. Following these incubation periods, cells were harvested and subjected to histone acid extraction as previously described by Jeong et al. (25). Total histone extracts were separated on a 15% denaturing polyacrylamide gel (SDS-PAGE). Proteins were transferred to Hybond-ECL membranes (GE Healthcare, Sweden) that were blocked with the ECL Advance Chemiluminescence kit (GE Healthcare) for 1 h at room temperature. Incubation with anti-acetyl-histone H3K9 or H4K16 primary antibody (1:5000; Upstate Biotechnology, USA) was carried out at
4°C overnight, and incubation with the secondary antibody conjugated to horseradish peroxidase (1:10,000; GE Healthcare) was carried out at room temperature for 1 h. Subsequently, membranes were incubated with primary anti-H1 antibody (1:5000; Upstate Biotechnology) for 1 h at 37°C and then incubated with the secondary antibody conjugated to horseradish peroxidase (1:10,000; GE Healthcare) at room temperature for 1 h. Membranes were developed using the ECL Advance Chemiluminescence kit (GE Healthcare), and bands were revealed with the ImageQuant 400 capture imaging system (GE Healthcare). Protein bands detected at 11, 17, and 30 kDa corresponded to the expected molecular weight of histone H4, H3, and H1, respectively. A BIO-RAD densitometer (Imaging Densitometer Model GS-700, USA) with specific software (Molecular Analyst, USA) was used to quantify band intensities. To control for unequal protein loading, expression of the proteins of interest was normalized to the H1 signal (26).

p21/WAF1 expression. MCF-7 cells were plated at a density of 1 x 10^5 cells/mL, treated with 1 mM BT and 10 µM VA, individually or combined, incubated for 96 and 120 h. Cells were harvested and subjected to protein extraction with the T-PER® Tissue Protein Extraction Reagent (Pierce, USA), according to the manufacturer protocol. Western blotting was performed as described for the H3K9 and H4K16 acetylation analyses. Proteins were incubated with primary anti-p21/WAF1 antibody (1:1000; Santa Cruz Biotechnology, USA) at 4°C overnight and then incubated with a secondary antibody conjugated to horseradish peroxidase (1:10,000; GE Healthcare) at room temperature for 1 h. Subsequently, membranes were incubated with a primary anti-β-actin antibody (1:5000; Santa Cruz) for 1 h at 37°C and then incubated with a secondary antibody conjugated to horseradish peroxidase (1:10,000; GE Healthcare) at room temperature for 1 h. The resulting bands of 21 and 40 kDa, corresponding to the expected molecular weight of p21/WAF1 and β-actin, respectively, were quantified as previously described. To control for unequal protein loading, expression of the protein of interest was normalized to the β-actin signal (14).

Dot blot analysis

Dot blot analysis was performed as described by Al-yaqoub et al. (27). MCF-7 cells were plated at a density of 1 x 10^5 cells/mL, treated with 1 mM BT and 10 µM VA, individually or combined, incubated for 96 and 120 h, and then harvested. RNA extraction was performed with the illustra RNAspin Mini RNA isolation kit (GE Healthcare, Germany), according to the manufacturer protocol. RNA quality was assessed by gel electrophoresis. Approximately 1 µg of total RNA was used for the synthesis of first-strand cDNA using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, USA) according to manufacturer instructions. Quantitative PCR analysis was performed in replicates using TaqMan chemistry and Assays on Demand probes (Applied Biosystems, USA) for RARβ (Hs00233407-m1), CRBP-I (Hs01011514-m1), GAPDH (Hs99999905-m1) and mRNA levels were quantified using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Amplification reactions were performed with a three-step thermal cycling method consisting of a 2-min step at 50°C, 10-min step at 95°C, and a final step of 50 cycles at 95°C for 15 s and 60°C for 1 min.

Methylation-specific PCR analysis

To analyze the methylation pattern of the RARβ gene promoter region, bisulfite modification of genomic DNA was assessed by methods similar to those described by Goldenberg et al. (28). The primers used for PCR were specific for the methylated and unmethylated DNA sequence. A volume of 2 µL modified DNA was used in the PCR assay with 5 µL 10X RT-PCR buffer (2.5 mM MgCl₂ final concentration), 1 µL dNTP mix (10 mM of each dNTP), 1 µL forward and reverse primers (10 mM), 1.25 U Platinum Taq DNA Polymerase and PCR grade water making up a total volume of 50 µL. The primer pairs were previously described by Esteller et al. (19): unmethylated sequence (5'-TTGGGATGTTGAGAATGTGAGTGATTT-3' sense and 5'-CTTACTCAACCAATCCAACCAACAAACAAA 3' antisense) and methylated sequence (5'-TTTCCAGGACCCGATCC-3' sense and 5'-CAACCATAACCAACCCGAAACGA-C-3' antisense). The PCR conditions were as
follows: 95°C for 5 min followed by 40 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 30 s, with a final extension of 72°C for 5 min. The PCR products were separated on 2% agarose gels, and DNA was visualized with Blue Green (LGC Biotechnology, Brazil) staining.

**HPLC analysis**

MCF-7 cells were plated at a density of 1 x 10^5 cells/mL, treated with 1 mM BT or 10 µM VA, or the combination of both compounds, incubated for 96 and 120 h, and then harvested. HPLC was carried out according to Murray et al. (29). Samples were analyzed by chromatography (Shimadzu model LC9A, Japan) using a C18 reverse-phase column (CLC-ODS; 4.6 mm x 25 cm x 0.5 mm). The chromatograph consisted of a multisolvent pump system, an auto-injector (model SIL-6B) and a photodiode array UV-VIS detector (model SPD-M6A). The substances were eluted at a flow rate of 1.5 mL/min in a mobile phase consisting of acetonitrile, dichloromethane and methanol at a 20:20:10 ratio, respectively. Retinoids (retinol and retinyl palmitate) were detected at 325 nm and were identified on the basis of comparisons of their retention times and those of authentic standards. These compounds were quantified on the basis of peak areas.

**Statistical analysis**

Data are reported as means ± SEM, and all analyses were carried out with the Statistica 8.0 software (Statsoft, USA). Depending on the type of data, either one-way analysis of variance followed by the Tukey or Duncan post hoc test or the Kruskal Wallis test was applied. For all analyses, the level of significance was set at P ≤ 0.05.

**Results**

**Cell proliferation**

Figures 1A and B depict the BT and VA dose-response effects on MCF-7 cell proliferation, respectively. Relative to controls, 5 mM BT-treated cells demonstrated significantly lower (P ≤ 0.05) cell proliferation from 48 to 120 h. The other BT concentrations (0.5-4 mM) showed lower (P ≤ 0.05) MCF-7 cell proliferation only after 120 h. Relative to control, 20 µM VA-treated cells demonstrated lower (P ≤ 0.05) cell proliferation after 120 h. The other VA concentrations (1-15 µM) did not reduce MCF-7 cell proliferation at any time.

For the evaluation of the effects of combined treatment with BT and VA on MCF-7 cells, we selected doses that yielded intermediate cell proliferation inhibition in order to allow potential interactions between the dietary HDACi and the retinoid. Figure 1C depicts the results obtained after treatment with 1 mM BT and/or 10 µM VA. After 24 h of treatment, no differences was observed between control cells and cells subjected to the different treatments. Relative to controls, BT-treated cells showed lower (P ≤ 0.05) cell proliferation only after 72 h of treatment, whereas VA-treated cells did not differ from control at any time point. Compared to control, BT+VA-treated cells showed lower (P ≤ 0.05) cell proliferation after 48, 72, 96, and 120 h of treatment. Follow-

![Figure 1. Effect of treatment with butyrate and/or vitamin A on MCF-7 cell proliferation evaluated by the crystal violet staining method (absorbance at 570 nm). A. MCF-7 cells treated with butyrate (BT; 0.5-5 mM); B. MCF-7 cells treated with vitamin A (VA; 1-20 µM); C. MCF-7 cells treated with butyrate (BT; 1 mM) and/or vitamin A (VA; 10 µM). Data are reported as means ± SEM. Three independent experiments were performed with N = 6 for each treatment; *P ≤ 0.5 for 5 mM BT treatment in A, 20 µM VA treatment in B and BT+VA treatment in C compared to respective controls; **P ≤ 0.5 for 0.5-4.0 mM BT treatments in A and BT treatment alone in C compared to respective controls (ANOVA followed by the Tukey test).](image-url)
ing 120 h of BT, VA and BT+VA treatments, cell proliferation was inhibited by 34, 10 and 46%, respectively. These data suggest that VA may have potentiated the inhibitory effects of BT on MCF-7 cell proliferation.

**Cell cycle profile**

Figure 2 illustrates the cell cycle profile of MCF-7 cells treated with 1 mM BT and/or 10 µM VA for 48 h. BT+VA treatment decreased (P ≤ 0.05) and increased (P ≤ 0.05) the percentage of cells in the G0/G1 and G2/M phases, respectively. No differences were observed between treatments during the other periods (24, 72, 96, and 120 h; data not shown).

**Acetylation of H3K9 and H4K16**

Figure 3 illustrates the results of H3K9 acetylation in MCF-7 cells treated with 1 mM BT and/or 10 µM VA for 96 and 120 h. The 96-h treatment of MCF-7 cells with BT alone or in combination with VA resulted in a 9.5- and 8.0-fold increase (P ≤ 0.05), respectively, in H3K9 acetylation relative to control. This increase was not observed when cells were treated with VA alone. No differences in H3K9 acetylation were observed between BT- and BT+VA-treated cells. The 120-h treatment with BT and/or VA did not alter H3K9 acetylation compared to control. None of the treatments assessed resulted in altered H4K16 acetylation (data not shown).

**p21WAF1 expression**

Figure 4 depicts p21WAF1 protein expression in MCF-7 cells treated with 1 mM BT and/or 10 µM VA for 96 and 120 h. Following 96 h of exposure, none of the treatment groups expressed altered p21WAF1 compared to control. The 120-h treatment with BT increased p21WAF1 expression by 3.8-fold (P ≤ 0.05) and 2.7-fold (P = 0.054) relative to VA-treated and control cells, respectively. VA and BT+VA treatments did not result in altered p21WAF1 expression compared to control.

**Global DNA methylation patterns**

Figure 5 illustrates the result of global DNA methylation pattern for MCF-7 cells treated with 1 mM BT and/or 10
Figure 3. Effect of treatment with butyrate (BT) and/or vitamin A (VA) on H3K9 acetylation. Western blot analysis of H3K9 acetylation for MCF-7 cells treated or not with BT and VA, individually or combined, for 96 (A) and 120 h (B). Quantitation of histone H3K9 acetylation in MCF-7 cells treated or not with BT and VA, individually or combined, for 96 (C) and 120 h (D). The data are representative of three independent experiments and are reported as means ± SEM. aP ≤ 0.5 compared to control; bP ≤ 0.5 compared to treatment with VA (ANOVA followed by the Tukey test).

Figure 4. Effect of treatment with butyrate (BT) and/or vitamin A (VA) on p21WAF expression. Western blot analysis of p21WAF expression was carried out in MCF-7 cells treated or not with BT and VA, individually or combined, for 96 (A) and 120 h (B). Quantification of p21WAF expression in MCF-7 cells treated or not with BT and VA, individually or combined, for 96 (C) and 120 h (D). The data are representative of four independent experiments and are reported as means ± SEM. aP ≤ 0.5 compared to control; bP ≤ 0.5 compared to treatment with VA (ANOVA followed by the Duncan test).
µM VA for 96 and 120 h. Relative to control, 96- and 120-h treatments with VA and BT+VA did not alter the global DNA methylation patterns.

**RARβ and CRBP-I expression**

Figure 6 provides RARβ expression data for MCF-7 cells treated for 96 and 120 h with 1 mM BT and/or 10 µM VA. Relative to control, BT and/or VA treatments did not alter RARβ gene expression after 96 h. Compared to treatment with VA, BT treatment resulted in a 1.6-fold increase in the expression of this gene (P ≤ 0.05). Following 120 h of exposure, treatments with BT alone or in combination with VA resulted in increased RARβ gene expression by 2.0- (P ≤ 0.05) and 1.7-fold (P ≤ 0.05), respectively, compared to control. The same increase was not observed in cells treated with VA alone. Treatment with BT resulted in increased RARβ gene expression by almost 2.0-fold (P ≤ 0.05) relative to VA-treated cells. No significant differences in RARβ gene expression were detected according to the Kruskal Wallis test (P > 0.05).
expression were observed between the BT and BT+VA treatment groups. Following 96 and 120 h of exposure, CRBP-I gene expression was not altered in any treatment group compared to control (data not shown).

Methylation of the RARβ promoter

Figure 7 depicts the methylation pattern of the RARβ promoter in MCF-7 cells treated with 1 mM BT and/or 10 µM VA for 96 h. The RARβ promoter region was predominantly methylated in control MCF-7 cells and in cells treated with BT and/or VA.

Cellular levels of retinoids

Figure 8 indicates the concentrations of retinol in MCF-7 cells treated with 1 mM BT and/or 10 µM VA. Following 96 h of exposure, no statistical differences in cellular concentrations of this retinoid were detected between treatments. After 120 h of exposure, only cells treated with VA alone demonstrated increased retinol concentrations (3.4-fold) (P ≤ 0.05) relative to control. Retinyl palmitate was not detected in control MCF-7 cells or in cells treated with BT and/or VA for 96 and 120 h.

Discussion

Despite the anticancer potential of treatment with an HDACi combined with retinoids (23), few studies have addressed the potential of such interventions, specifically for the control of breast cancer. Efficacy of the combination of retinoic acid with the synthetic HDACis trichostatin (24) and valproic acid (30) was shown in breast cancer cells. Interestingly, our data showed that although VA did not inhibit MCF-7 cell proliferation, it seemed to potentiate inhibition of cell proliferation by BT. This combinatorial effect could involve arrest of MCF-7 cells in the G2/M phase. This reinforces the potential of combining the dietary HDACi BT with retinoids in the context of breast cancer. However, the BT and VA association did not present any combinatorial action on inhibition of proliferation of ER-negative MDA-MB-231 human breast cancer cells (data not shown). This suggests that the type of breast cancer is a relevant issue that should be considered in anticancer combinatorial strategies with butyrate and retinoids.

Global DNA hypomethylation leads to genomic instability and activation of oncogenes (4). MCF-7 cells express a global hypomethylation compared to normal breast cells (MCF-10-2A) (33,34). In the present study, treatment with BT alone or combined with VA did not alter global DNA methylation patterns. Thus, BT anticancer actions do not seem to involve interference with this epigenetic process.

Aberrant VA signaling has been associated with the progressive loss of RARβ2 expression (21) through epigenetic silencing by promoter hypermethylation (24,35).
nomic deregulation is known to contribute to breast cancer development and progression (36,37). In the present study, we observed that, despite the predominantly hypermethylated state of the RARβ promoter in MCF-7 cells, treatment with BT alone or combined with VA could moderately induce the expression of this gene, suggesting that RARβ may represent a molecular target for BT in breast cancer cells. Due to its effectiveness as a dietary HDACi, BT should be considered for use in combinatorial strategies with more active retinoids, especially in breast cancers in which RARβ is epigenetically altered. The possibility of achieving more synergistic anticancer interactions with these interventions remains to be further elucidated.

We confirmed further that CRBP-I is down-regulated in MCF-7 cells (19,20,38). Contrary to what was hypothesized, however, the combination of BT with VA could not reactivate CRBP-I expression and this could provide a molecular explanation of why this intervention could not restore VA metabolism in MCF-7 cells, as evidenced by lack of storage of retinol as retinyl palmitate.

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