Valproic acid, in combination with all-*trans* retinoic acid and 5-aza-2′-deoxycytidine, restores expression of silenced *RARβ2* in breast cancer cells

Nigel P. Mongan and Lorraine J. Gudas

Department of Pharmacology, Weill Medical College, Cornell University, New York, New York

Abstract

Epigenetic silencing of tumor suppressor genes has been established as an important process of carcinogenesis. The retinoic acid (RA) receptor β2 (*RARβ2*) gene is one such tumor suppressor gene often silenced during carcinogenesis. The combined use of histone deacetylase and DNA methyltransferase inhibitors has been shown to reverse the epigenetic silencing of numerous growth regulatory genes. Valproic acid (VPA), which has long been used in the treatment of epilepsy, was shown recently to be an effective histone deacetylase inhibitor that can induce differentiation of neoplastically transformed cells. In this study, we show for the first time that VPA, in combination with RA and the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (Aza-dC), can overcome the epigenetic barriers to transcription of a prototypical silenced tumor suppressor gene, *RARβ2*, in human breast cancer cells. Chromatin immunoprecipitation assays show that the combination of VPA, RA, and Aza-dC increases histone acetylation at the silenced *RARβ2* promoter of MCF-7 breast cancer cells. Furthermore, reverse transcription-PCR analyses reveal cell type–specific effects in the actions of VPA on *RARβ2* expression in cultured human breast cancer cells. Finally, we show that VPA, in combination with RA and Aza-dC, inhibits the proliferation of both estrogen receptor α-positive (MCF-7) and estrogen receptor α-negative (MDA-MB-231) breast cancer cell lines. These data suggest that VPA may ultimately be useful in combination therapies in the treatment of human breast cancers. [Mol Cancer Ther 2005;4(3):477–86]

Introduction

Histone deacetylases (HDAC; ref. 1) and DNA methyltransferases (DNMT; ref. 2) play essential roles in the developmental and temporal regulation of gene expression. However, epigenetic silencing of tumor suppressor genes has emerged as an important process of carcinogenesis (3). It has been shown that HDAC inhibitors, including trichostatin A, suberoylanilide hydroxamic acid, and the butyrates, can induce differentiation and inhibit proliferation of many cancer cell types *in vitro* (4). The anticancer actions of HDAC inhibitors are attributable at least in part to the restoration and up-regulation of multiple functionally distinct growth inhibitory pathways (5–8). Indeed, the use of HDAC and DNMT inhibitors has been shown to restore the expression of silenced estrogen receptor α (*ERα*; ref. 9), retinoic acid (RA) receptor β (*RARβ*; refs. 10–12), and numerous other growth regulatory genes (5, 6, 13, 14). For these reasons, HDAC and DNMT inhibitors have emerged as promising candidates for differentiation therapy of many cancer types (4, 15–18).

RA, the primary physiologic metabolite of vitamin A, plays essential roles in normal development, growth, and cellular differentiation (19). Clinical trials are now under way to investigate the use of HDAC inhibitors, alone and in combination with RA and/or DNMT inhibitors, for the treatment of solid tumors (4), as both RA (20, 21) and HDAC inhibitors have shown promising results for the treatment of acute promyelocytic leukemia (17). The signaling actions of RA are mediated by RA receptors, RARs and retinoid X receptors, which are members of the nuclear receptor superfamily of ligand-dependent transcription factors (19). There is evidence that *RARα* (14), *RARβ2* (22–25), and *RARγ* (26) can each act as tumor suppressors, and their expression is reduced in breast cancer cells (27). However, the antineoplastic pathways induced by RA are regulated predominantly by *RARβ2* (24, 25). However, expression of *RARβ2* is frequently decreased or lost in many cancer types (28–30), including breast cancer (12, 23, 27, 31). The *RARβ2* P2 promoter possesses a RA response element (RARE) normally transactivated by a retinoid X receptor-RAR heterodimer in the presence of RA (32). Recent studies have shown that the *RARβ2*-RARE is epigenetically silenced in breast (10–12) and other cancer types (28, 30, 33). This combination of local histone deacetylation and CpG island methylation results in strong epigenetic repression of *RARβ2* and resistance to the growth inhibitory effects of RA (27). However, the combined use of RA with HDAC and/or DNMT inhibitors has shown synergistic effects in restoring the transcription and growth regulatory effects of silenced *RARβ2* (10, 11, 23). Furthermore, restoration of *RARβ2* expression in MCF-7 (23) and MDA-MB-231 (34) breast cancer cells has been shown to restore the growth inhibitory and proapoptotic actions of RA.

Valproic acid (2-propylpentanoic acid; VPA) is a short-chain fatty acid that is structurally related to the butyrate

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Requests for reprints: Lorraine J. Gudas, Department of Pharmacology, Weill Medical College, Cornell University, 1300 York Avenue, New York, NY 10021. Phone: 212-746-6250; Fax: 212-746-8858.
E-mail: ljgudas@med.cornell.edu

class of HDAC inhibitors (4) and was shown recently to be an effective HDAC inhibitor at concentrations currently in use for the treatment of epilepsy (35–37). VPA is also known to stimulate differentiation and/or inhibit cell growth of cultured leukemic (8, 37), endometrial (38), and glioma cells (39) and diminishes adenoma formation in the APC^{min} colon cancer mouse model (40). VPA is a particularly attractive candidate HDAC inhibitor for use in the treatment of neoplastic disease, as it is generally well tolerated by patients (41). Although clinical trials with VPA for cancer are under way (4, 42), the effects of VPA on the proliferation and tumor suppressor gene expression have not yet been reported for breast cancer cells. In this study, we have investigated the HDAC inhibitory activity of VPA in the context of a prototypical epigenetically silenced tumor suppressor gene, *RARβ2*, in human breast cancer cells. Of particular significance to future mouse xenograft and clinical studies, we investigated the efficacy of combinations of low concentrations of VPA with RA and/or the DNMT inhibitor 5-aza-2′-deoxycytidine (Aza-dC/Decitabine), which has been shown in previous studies to enhance reactivation of silenced *RARβ2* in cancer cells (10, 23, 28, 43, 44). We show that the combination of VPA, RA, and Aza-dC increases histone H3 acetylation at the silenced *RARβ2*-RARE in MCF-7 breast cancer cells. Consistent with this, expression of *RARβ2* is restored or enhanced in several human breast cancer cell types treated with VPA, RA, and Aza-dC. Furthermore, we show that VPA, in combination with RA and Aza-dC, inhibits proliferation of both ERα-positive and ERα-negative breast cancer cell lines. These data suggest that VPA may be useful in the treatment of breast cancer.

Materials and Methods

Cell Culture, Drug Treatments, and Growth Analyses

Human breast cancer cell lines MCF-7, MDA-MB-231, SK-BR3, MDA-MB-453, and HS578T were maintained as directed by the American Type Culture Collection (Manassas, VA). The MDA-MB-231 clone employed in this study expresses *RARβ2* following RA treatment as reported previously (11, 45). Cells were placed in six-well plates at a density of 1×10^5 cells per well 24 hours before drug treatment to allow cell attachment. All-*trans* RA (Sigma, St. Louis, MO) was dissolved in ethanol and used at a final concentration of 1 μmol/L. RA is essential to induce expression of *RARβ2* via the RARE (32). Aza-dC (Sigma) was dissolved in PBS (pH 6.8) and used at a final concentration of 2 μmol/L, a concentration within the range employed previously (1-10 μmol/L; refs. 28, 43). VPA was purchased from Sigma and dissolved in sterile water and used at a final concentration of 250 μmol/L or as indicated in the figure legend (Fig. 5). Cells were treated with fresh medium and drug combinations twice (at 0 and 48 hours) during a 96-hour period. Under these conditions, dividing cells remain subconfluent, thereby permitting incorporation of Aza-dC throughout the treatment time course. Control cells were treated with ethanol. For growth analyses, cells were counted every 24 hours using a Coulter counter (Fullerton, CA). Percentage growth inhibition was determined as TC/CC × 100, where TC represents treated cells and CC represents control cell numbers. The growth experiments were done at least four times.

Epigenetic Status of the *RARβ2*-RARE P2

Chromatin immunoprecipitation (ChIP) assays were done as described previously (23, 46) using ChIP-grade antibodies to acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY). Cells were placed in six-well plates at a density of 1×10^5 cells per well 24 hours before drug treatment to allow cell attachment. Chromatin was isolated from cells treated twice with fresh medium and drug combinations (at 0 and 48 hours) during a 96-hour period. At the conclusion of the drug treatment, RA (1 μmol/L) was added to all cells for 30 minutes immediately before the ChIP assay. This ensures that all cells are exposed to equivalent concentrations of RA for the ChIP assays and compensates for defects in retinoid metabolism, which render MCF-7 cells incapable of accumulating retinoids (47, 48). During this 30-minute period, β-estradiol (100 nmol/L) was also added to the cells to enhance transcription at the PS2 estrogen response element and acts as a positive control for nuclear receptor–mediated transcription. Cells were then cross-linked with formaldehyde (1%) for 10 minutes at 37°C. Cross-linked samples were sonicated thrice for 10 seconds each at output level 3 using a Branson sonifier model 150, to yield an average DNA size of 200 to 800 bp, as confirmed by electrophoresis on 2% TAE-agarose gels. Soluble chromatin was immunoprecipitated overnight with antibodies specific for acetylated histone H3. The immunoprecipitated chromatin complex was harvested using protein A-agarose beads (Upstate Biotechnology). DNA was extracted using the QIAquick extraction system (Qiagen, Valencia, CA) and eluted in a volume of 50 μL ultrapure water. PCR was carried out as described below, with the number of cycles determined by that required to yield amplification within the linear range. Primers specific for the *RARβ2*-RARE P2 promoter (192-bp product) were forward 5′-CTCTGGCTGTCTGCTTTTGC-3′ and reverse 5′-CAGCTCACTTCCTACTACTTC-3′. Primers specific for the estrogen-responsive ER response element of the *PS2* promoter (323-bp product; ref. 46) and *GAPDH* (265-bp product; ref. 23) were used as internal positive controls as described previously. Negative control PCRs using water in place of template were done for each experiment.

RNA Isolation, Reverse Transcription-PCR, and Real-time PCR Quantification

Total RNA was isolated from cells using Trizol (Invitrogen, Carlsbad, CA) and first-strand cDNA synthesized from 2 to 5 μg total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) as directed by the manufacturer. The synthesized cDNA was diluted to 100 μL with ultrapure water. Primers specific for the *β-actin* cDNA (Genbank accession no. NM001101) were used to confirm cDNA integrity and oligonucleotide primers were designed to amplify the

RARα (accession no. BC008727), *RARβ2* (accession nos. M96016-M96023), and *RARγ* (M24857) cDNAs. Primer pairs used in reverse transcription-PCR (RT-PCR) were as shown in Table 1. Primers were designed to span a least one intron-exon boundary and thereby prevent amplification of any contaminating genomic DNA. The RT-PCR products of each primer pair were sequenced to confirm the identity of the amplified fragment and the specificity of amplification. Each PCR contained 4 ng of each oligonucleotide, 1 μL cDNA, 0.5 unit Taq DNA polymerase and accompanying 1× buffer (Invitrogen), 1.5 mmol/L $MgCl_2$, and 0.2 mmol/L deoxynucleotide triphosphates. Thermal cycling was done as follows: 95°C for 5 minutes followed by 33 to 40 cycles of 94°C for 30 seconds (template denaturation), 55°C for 30 seconds (oligonucleotide annealing), and 72°C for 30 seconds (product extension). PCR products were analyzed by electrophoresis through 1.5% TAE-agarose gels.

Real-time PCR analysis was done using a DNA Engine Opticon System (MJ Research, Boston, MA) and SYBR Green I Quantitect kit (Qiagen). The real-time PCR technique employed enables the detection and quantification of very low levels of *RARβ2* expression in breast cancer cells not possible with end point RT-PCR. The threshold cycle (C_T) was set at 10 times the SD above the mean baseline emission for cycles 3 to 7. Expression of *β-actin* was unaffected by drug treatments and therefore used as an internal reference. Primer pairs for *RARβ2* and *β-actin* were as described above. Amplification efficiencies of these primer pairs were compared using template dilutions and found to have similar ΔC_T values ($\Delta C_T = C_T$ *RARβ2* $- C_T$ *β-actin*). Reaction conditions were 95°C for 10 minutes to activate the polymerase followed by 50 cycles of 94°C for 30 seconds, primer annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds; fluorescence was read after each cycle at 82°C. The relative levels of *RARβ2* expression for each cell type and treatment condition were determined with reference to the internal *β-actin* control using the $2^{-\Delta\Delta CT}$ method (49, 50). With the exception of MDA-MB-453, expression of *RARβ2* was detected in all cell lines treated with RA; hence, this sample was chosen as the calibrator sample with an arbitrary normalized value of 1. In MDA-MB-453, the calibrator sample was derived from RA- and Aza-dC-treated cells. Real-time analysis was done in triplicate for each cDNA sample. Negative control PCRs using water in place of template were done for each experiment.

Results

VPA, in Combination with RA and Aza-dC, Restores Expression of *RARβ2* in MCF-7 Breast Cancer Cells

RT-PCR was used to monitor *RARβ2* expression in MCF-7 breast cancer cells. Expression of *RARβ2* was not detected in untreated control cells (Fig. 1A, *lane 1*) and not induced in MCF-7 cells following treatment with RA (1 μmol/L) alone or in combination with VPA (250 μmol/L) or Aza-dC (2 μmol/L) for 96 hours (Fig. 1A, *lanes 2-6*). Indeed, expression of *RARβ2* mRNA was detected only in MCF-7 cells treated simultaneously with RA, Aza-dC, and VPA (Fig. 1A, *lane 7*).

Histone H3 deacetylation is believed to be an important marker of the epigenetic silencing of the *RARβ2*-RARE promoter (28). ChIP assays were therefore employed to examine the effects of VPA in combination with RA and Aza-dC on histone H3 acetylation associated with the *RARβ2*-RARE of MCF-7 breast cancer cells. Consistent with the RT-PCR analysis (Fig. 1A), treatment of MCF-7 cells with RA or Aza-dC alone failed to increase histone H3 acetylation at the *RARβ2*-RARE (Fig. 1B). This absence of histone H3 acetylation in MCF-7 cells following RA treatment provided further evidence of the epigenetic silencing of the *RARβ2*-RARE P2 in MCF-7 cells (10). The estrogen-responsive *PS2*-ER response element acted as a positive control for nuclear receptor–stimulated histone H3 acetylation and *GAPDH* acted as a control for constitutively active chromatin (Fig. 1B). As expression of *RARβ2* was detected only in MCF-7 cells treated simultaneously with VPA, RA, and Aza-dC (Fig. 1A), we next examined the effects of RA and Aza-dC in combination with increasing VPA concentrations (Fig. 1C) on *RARβ2*-RARE-associated histone H3 acetylation. VPA concentrations examined ranged from 5 mmol/L VPA, which was examined in early biochemical (35) and cellular (38) studies, to 250 μmol/L VPA, a concentration of more pharmacologic relevance (51, 52). At the conclusion of the 96-hour drug treatment, RA (1 μmol/L) and β-estradiol (100 nmol/L) were added to the cells for 30 minutes and the effects on histone H3 acetylation at the *RARβ2*-RARE and PS2-ER response element were examined using ChIP assays. The estrogen-responsive *PS2*-ER response element and *GAPDH* acted as a positive controls for active chromatin. Whereas all concentrations of VPA, in combination with RA and Aza-dC, could increase histone H3 acetylation associated with the *RARβ2*-RARE in MCF-7 cells, lower VPA concentrations (250 μmol/L-3 mmol/L) were more effective in inducing

Table 1. Sequences of primer pairs used to assess mRNA expression using RT-PCR

Gene	Sense primer (5′-3′)	Antisense primer (5′-3′)	Product (bp)
RARα	GTCTGTCAGGACAAGTCCTCAGG	GCTTTGCGCACCTTCTCAATGAG	314
RARβ2	GACTGTATGGATGTTCTGTCAG	ATTTGTCCTGGCAGACGAAGC	255
RARγ	AATGACAAGTCCTCTGGCTACCAC	CAGATCCAGCTGCACGCGGTGGTC	394
β-actin	GCTCGTCGTCGACAACGGCTC	GTACATGGCTGGGGTGTTGAAGG	379

NOTE: The identity of the product of each primer pair was confirmed by automated DNA sequencing.

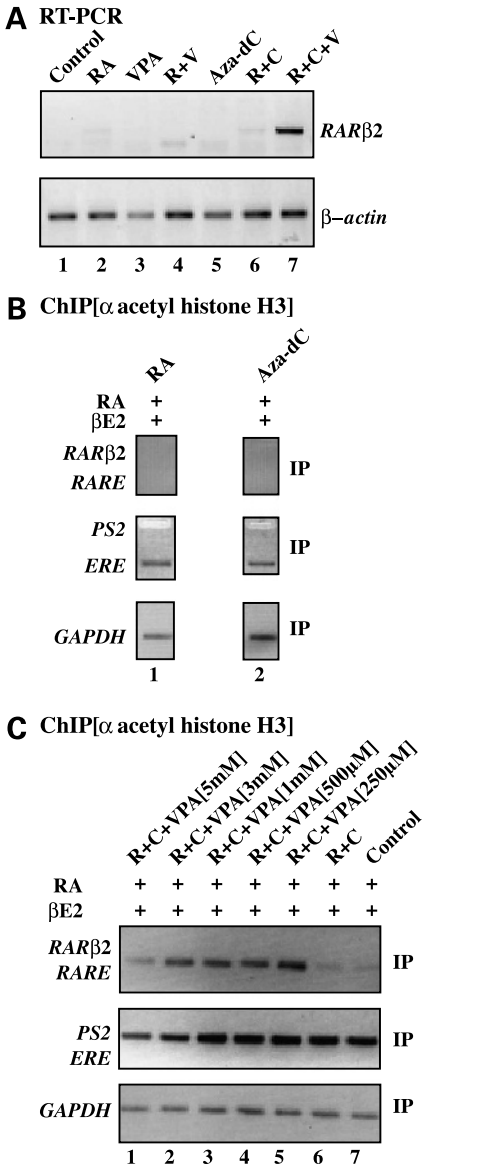

Figure 1. **A,** cells were treated twice over a 96-h period with ethanol (*lane 1*), RA (1 μmol/L; *lane 2*), VPA (250 μmol/L; *lane 3*), RA + VPA (*lane 4*), Aza-dC (2 μmol/L; *lane 5*), RA + Aza-dC (*lane 6*), and RA + Aza-dC + VPA (*lane 7*) and RT-PCR was done as described in Materials and Methods. PCR products were analyzed by electrophoreses on 1.5% TAE-agarose gel and stained with ethidium bromide. **B** and **C,** ChIP assays using an antibody to acetylated histone H3 were used to assess the ability of 1 μmol/L RA (**B**, *lane 1*) or 2 μmol/L Aza-dC (**B**, *lane 2*) in combination with differing VPA concentrations (**C**, *lanes 1–5*) to restore histone H3 acetylation to the *RARβ2* P2 promoter. Cells were treated twice over a 96-h period with RA and Aza-dC in combination with 5 mmol/L VPA (**C**, *lane 1*), 3 mmol/L VPA (**C**, *lane 2*), 1 mmol/L VPA (**C**, *lane 3*), 500 μmol/L VPA (**C**, *lane 4*), 250 μmol/L VPA (**C**, *lane 5*), RA + Aza-dC (**C**, *lane 6*), and vehicle control (**C**, *lane 7*). Immediately before the ChIP assay, RA (1 μmol/L) and β-estradiol (100 nmol/L) were added to the cells for 30 min before formaldehyde cross-linking and histone H3 acetylation assessed at the *RARβ2*-RARE. The promoter regions of *PS2* and *GAPDH* act as internal positive controls. Thirty-two (**B**) and 33 (**C**) cycles of PCR were used in the ChIP experiments. PCRs were analyzed by electrophoresis on a 2% TAE-agarose gel and repeated yielding similar results. *βE2,* β-estradiol; *C,* Aza-dC; *IP,* immunoprecipitation with anti–histone H3 antibody; *R,* RA; *V,* VPA.

histone acetylation at the MCF-7 *RARβ2*-RARE than the drug combination employing 5 mmol/L VPA (Fig. 1C). It is possible that 5 mmol/L VPA may directly inhibit proliferation of MCF-7 cells and this is reflected in decreased H3 acetylation (Fig. 1C, *lane 1*) relative to that detected in cells treated with lower VPA concentrations (Fig. 1C, *lanes 2-5*). Indeed, VPA is known to induce apoptosis of cultured endometrial cancer cells in a dose-dependent manner (38). For these reasons, this lower VPA concentration (250 μmol/L), which is within the range of mean serum levels (260-500 μmol/L), achieved in epilepsy patients treated with VPA (51, 52), was employed in RT-PCR experiments (Figs. 1A, 2, and 3) and growth analyses (Fig.4). Although previous studies have investigated the antitumor effects of VPA in neuroblastoma (53) and endometrial mouse xenograft (38) studies, these data suggest that future breast cancer xenograft studies may wish to examine VPA alone and in combination with RA and DNMT inhibitors.

VPA, RA, and Aza-dC Restore and Enhance *RARβ2* Expression in Breast Cancer Cells

RT-PCR was used to detect retinoid receptor transcripts in several breast cancer cell types treated with VPA (250 μmol/L), RA (1 μmol/L), and Aza-dC (2 μmol/L; Fig. 2). Constitutive expression of *RARα* and *RARγ* was detected in all breast cancer cell lines examined using RT-PCR (Fig. 2). However, cell type–specific differences in *RARβ2* expression were observed. As described above, expression of *RARβ2* was only detected in MCF-7 cells following treatment with RA, VPA, and Aza-dC (Fig. 1A). In MDA-MB-231, *RARβ2* expression was detected in cells treated with RA alone (11) and in combination with either VPA and/or Aza-dC (Fig. 2B). Expression of *RARβ2* mRNA was detected in MDA-MB-453 cells treated with RA and Aza-dC or treated simultaneously with all three drugs (Fig. 2C). As in MDA-MB-231, *RARβ2* expression was detected in SK-BR3 cells treated with RA alone and in combination with VPA and Aza-dC (Fig. 2D). Constitutive *RARβ2* expression was detected in HS578T breast cancer cells (Fig. 2E) as reported previously (10, 27).

Real-time PCR was used to quantify changes in *RARβ2* expression in breast cancer cells induced at 96 hours following drug treatments. This approach enabled the relative quantification of low levels of *RARβ2* expression in breast cancer cells not possible with end point RT-PCR (Fig. 2). The effect of VPA alone and in combination with Aza-dC on RA-induced expression of *RARβ2* was compared with the basal levels of *RARβ2* detected in each breast cancer cell line (Fig. 3). In MCF-7, similar levels of *RARβ2* expression were found in the presence of RA, VPA, or Aza-dC (Fig. 3A), suggesting that this represents a background level of expression from a basal promoter (23). The combined use of RA, VPA, and Aza-dC resulted in ~14-fold increase in *RARβ2* mRNA expression relative to RA-treated MCF-7 cells. In MDA-MB-231 cells, RA, in combination with VPA or Aza-dC or both, resulted in a similar (6-fold) increase in *RARβ2* expression relative to RA treatment alone (Fig. 3B). Expression of *RARβ2* was detected only in MDA-MB-453 cells treated simultaneously

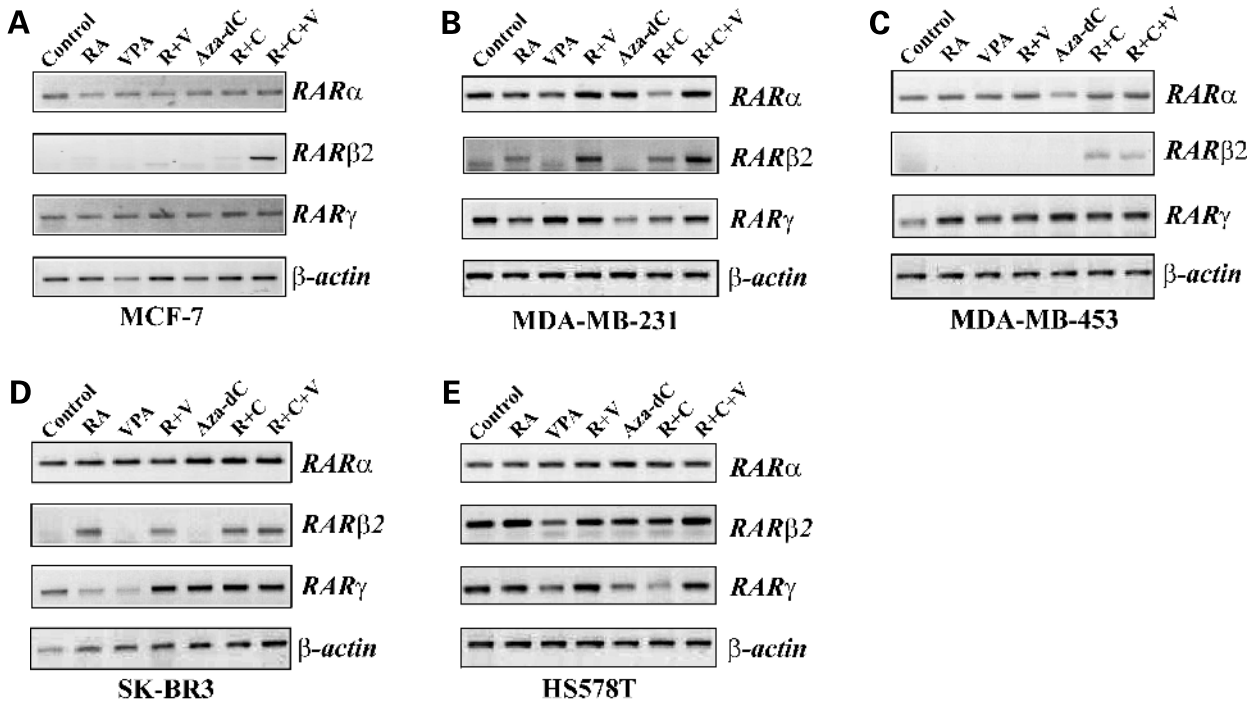

Figure 2. RT-PCR was used to detect expression of *RARα* (314 bp), *RARβ* (256 bp), and *RARγ* (394 bp) in MCF-7 (**A**), MDA-MB-231 (**B**), MDA-MB-453 (**C**), SK-BR3 (**D**), and HS578T (**E**) breast cancer cells. Amplification of *β-actin* (379 bp) was used to confirm cDNA integrity. Cells were treated twice over a 96-h period with ethanol (*lane 1*), RA (1 μmol/L; *lane 2*), VPA (250 μmol/L; *lane 3*), RA + VPA (*lane 4*), Aza-dC (2 μmol/L; *lane 5*), RA + Aza-dC (*lane 6*), and RA + Aza-dC + VPA (*lane 7*) and RT-PCR was done as described in Materials and Methods. A nonspecific amplification product was observed in control and VPA-treated MDA-MB-231 cells (**B**) and control MDA-MB-453 cells (**C**). The identity of the PCR product of each primer pair was confirmed by automated DNA sequencing. PCR products were analyzed by electrophoreses on 1.5% TAE-agarose gel and stained with ethidium bromide.

with RA and Aza-dC. However, the level of *RARβ2* mRNA was increased in MDA-MB-453 cells when VPA was used in addition to RA and Aza-dC (Fig. 3C). Combined treatment of SK-BR3 cells with RA, VPA, and Aza-dC resulted in the greatest enhancement of *RARβ2* expression relative to the RA-treated control (Fig. 3D). Although in all breast cancer cell lines tested the combined use of VPA, RA, and Aza-dC resulted in maximal reactivation of *RARβ2* expression, the different levels of *RARβ2* detected in each cell line treated with each combination of RA, VPA, and Aza-dC suggest that distinct repressive states may exist at the *RARβ2*-RARE.

VPA, Alone and in Combination with RA and Aza-dC, Inhibits Breast Cancer Cell Proliferation

We next examined the effects of VPA, alone and with RA and Aza-dC, on proliferation of MCF-7 (Fig. 4A-C) and MDA-MB-231 (Fig. 4D-F). Cells were treated twice during a 96-hour time course, with VPA (250 μmol/L), RA (1 μmol/L), and Aza-dC (2 μmol/L). Consistent with previous reports (27), MCF-7 cells were partially sensitive, whereas MDA-MB-231 cells were resistant to RA-induced growth inhibition. In MCF-7 cells, growth inhibition (Fig. 4C) coincided with restoration of *RARβ2* expression (Fig. 3A). However, growth inhibition (Fig. 4C) was also observed in MCF-7 cells treated with drug combinations that failed to increase *RARβ2* levels at 96 hours (Fig. 3A). Similarly, MDA-MB-231 cells were growth inhibited by Aza-dC treatment alone, which failed to induce expression of *RARβ2*. Growth inhibition was also observed in MDA-MB-231 cells treated with RA with Aza-dC and/or VPA (Fig. 4), which increased *RARβ2* expression. Yet, enhancement of *RARβ2* expression in MDA-MB-231 cells (Fig. 3B) by treatment with RA and VPA did not coincide with growth inhibition (Fig. 4D). Similar growth inhibition was achieved in breast cancer cells treated with RA, Aza-dC, and varying VPA (250-50 μmol/L) concentrations (Fig. 5). Hence, in MCF-7, growth inhibition could occur in the absence of detectable *RARβ2* expression, whereas in MDA-MB-231 restoration of *RARβ2* by RA and VPA failed to inhibit proliferation. These results indicate that additional tumor suppressors, in addition to *RARβ2*, play essential roles in inducing growth inhibition following treatment with RA, HDAC, and DNMT inhibitors.

Discussion

The use of chromatin-modifying drugs to restore or enhance endogenous antiproliferative mechanisms is an attractive therapeutic approach for cancer (16). Indeed, HDAC inhibitors have emerged as promising candidates for differentiation therapy of both hematologic and solid

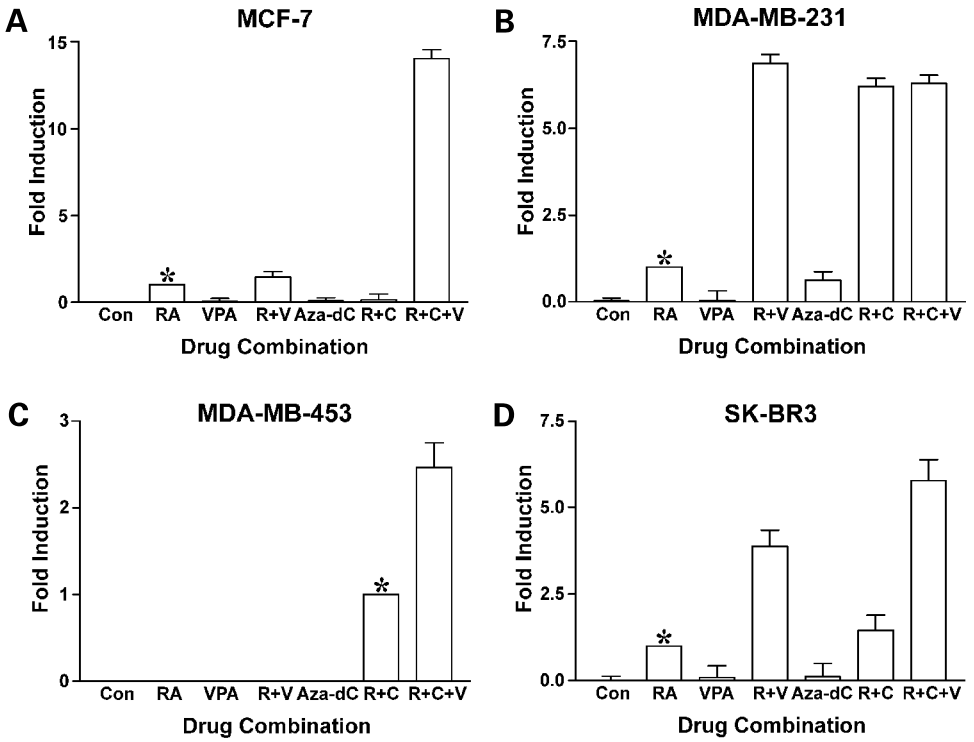

Figure 3. Real-time PCR (**A**–**D**) was done as described in Materials and Methods. Cells were treated twice over a 96-h period with RA (1 μmol/L), VPA (250 μmol/L), RA + VPA, Aza-dC (2 μmol/L), RA + Aza-dC, and RA + Aza-dC + VPA. The expression of *RARβ2* was measured relative to cellular *β-actin* levels and quantified relative to calibrator sample (*asterisks*). Real-time PCR analysis was done in triplicate for each sample. *Columns,* mean; *bars,* SD. No signal indicates that *RARβ2* was not detected under the conditions used. *R,* RA (1 μmol/L); *V,* VPA (250 μmol/L); *C,* Aza-dC (2 μmol/L); *Con,* control.

tumors (4, 17, 18). Although HDAC inhibitors are believed to influence expression of <2% to 10% of genes (37, 54), HDAC inhibitors can contribute to the reactivation of multiple genes silenced during carcinogenesis and thereby restore function of key regulatory pathways, which can inhibit of cancer cell proliferation (55). Previous studies have shown the efficacy of structurally distinct HDAC and DNMT inhibitors in inducing differentiation, growth arrest, and apoptosis of some cancer cell types (16–18). Several HDAC inhibitors are undergoing clinical trials (4); however, some may be of less therapeutic utility due to rapid metabolism and/or presence of multiple nonselective actions (ref. 37 and references therein).

In this context, VPA is an attractive candidate for cancer differentiation therapy. Whereas the VPA concentration required to induce histone acetylation *in vivo* is not currently known, VPA is an effective HDAC inhibitor *in vitro* at concentrations commonly used in the treatment of epilepsy. Furthermore, the VPA concentration used in this study (250 μmol/L) is within the range of mean serum levels (260-500 μmol/L) achieved in epilepsy patients treated with VPA (51, 52). Although VPA is largely bound to plasma proteins *in vivo* (41), this is saturatable within the therapeutic range, suggesting VPA concentrations that inhibit HDAC activity are achievable *in vivo* (ref. 51 and references therein). VPA also possesses favorable pharmacokinetic properties (37), has been in clinical use for over 35 years, and is generally well tolerated (41). VPA has been shown to induce differentiation of leukemic cells (37) and cell cycle arrest of glioma cells (39) and to inhibit angiogenesis (56). It will be interesting to determine whether VPA possesses cancer chemopreventive properties. Future studies can be done to investigate whether long-term use of VPA in the treatment of epilepsy correlates with reduced cancer incidence. Such a study may also clarify whether additional agents, such as retinoids, may be required to enhance the ability of VPA to promote cellular differentiation and reduce cancer incidence.

Retinoids have already been used in the treatment of acute promyelocytic leukemia (20, 21) and combination therapies, including retinoids, are being investigated for use in the treatment of breast cancers (57). Although the exact mechanisms by which retinoids inhibit cancer progression are not fully understood, they are believed to inhibit mitogen signaling (58) and to induce multiple downstream signaling pathways (59), which contribute to the apoptosis (60), growth arrest (22), and/or differentiation (20) of cancer cells. It is believed the proapoptotic actions of RA are modulated by *RARβ2* in breast cancer cells (25) and there is evidence that *RARβ2* influences cellular levels of important cell cycle regulators, including $p21^{CIP1}$ (61) and $p27^{KIP1}$ (62). Therefore, reduced expression of *RARβ2* results in the loss of multiple downstream growth inhibitory pathways and resistance to RA-dependent growth inhibition (27).

This study reveals for the first time that VPA, in combination with RA and Aza-dC, relieves the epigenetic repression of the *RARβ2* tumor suppressor gene in MCF-7 breast cancer cells. MCF-7 cells represent a

model of comparatively well-differentiated mammary carcinoma in which to investigate the mechanism of action of VPA at a prototypical silenced tumor suppressor promoter (*RARβ2*-RARE). Our data indicate that VPA, in combination with RA and Aza-dC, can restore *RARβ2* expression and histone H3 acetylation to the silenced *RARβ2*-RARE in MCF-7 cells. However, VPA, in the absence of Aza-dC, was unable to induce robust transcriptional activation at the *RARβ2*-RARE (Fig. 1). This is consistent with a previous study (6) and suggests that concomitant inhibition of HDACs and DNMTs is required to overcome the epigenetic barrier to RA-dependent transactivation at the *RARβ2*-RARE in MCF-7 breast cancer cells. As *RARβ2* was the only RA receptor that was not constitutively expressed in the breast cancer cells included in this study (Fig. 2), we next examined the ability of VPA to restore and enhance expression of *RARβ2* in distinct breast cancer cell types. In MCF-7, MDA-MB-453, and SK-BR3 cells, the combined use of VPA with RA and Aza-dC resulted in the greatest enhancement of *RARβ2* expression (Fig. 3).

Consistent with the hypothesis that *RARβ2* is an important mediator of the potent antiproliferative and cancer chemopreventive properties of RA (22, 24, 25, 31), the reactivation of *RARβ2* in MCF-7 cells (Fig. 3A) did coincide with growth inhibition (Fig. 4C). However, significant growth inhibition was also observed in MCF-7 (Figs. 4A and B and 5B) and MDA-MB-231 (Fig. 4D and E) cells treated with drug combinations that failed to enhance *RARβ2* expression when quantified following 96-hour drug treatment (Fig. 3A and B). We next examined whether drug combinations employing VPA concentrations (250-50 μmol/L) lower than those reported in epilepsy patients (51, 52) enhanced the restoration of expression of *RARβ2* (Fig. 5A, C, E, and G) and inhibited proliferation of a range of breast cancer cells (Fig. 5B, D, F, and H). Expression of *RARβ2* was restored in MDA-MB-231 (Fig. 5B), MDA-MB-453 (Fig. 5E), and SK-BR3 (Fig. 5G) cells treated with RA, Aza-dC, and VPA (250-0 μmol/L) and each drug combination resulted in similar growth inhibition (Fig. 5D, F, and H). Under the conditions employed, trace expression of *RARβ2* was detected in MCF-7 cells treated with RA and Aza-dC (Figs. 1A and 5A). A high level of *RARβ2* expression was detected in MCF-7 cells treated with RA, Aza-dC, and VPA (250-50 μmol/L; Fig. 5A). However, similar growth inhibition was observed for each drug combination examined (Fig. 5B). It is possible that early but transient restoration of *RARβ2* expression accounts for this growth inhibition observed in the absence of strong *RARβ2* expression at 96 hours. However, it is likely these epigenetic drug combinations promote many *RARβ2*-independent growth regulatory pathways (5, 6), which contribute to the growth inhibition observed in the absence of increased *RARβ2* expression.

Conversely, MDA-MB-231 treated with RA and VPA express *RARβ2* (Fig. 3B) but remain resistant to growth inhibition (Fig. 4D). This is analogous to the HS578T breast cancer cell line, which expresses *RARβ2* constitutively (Fig. 2E; ref. 27) yet is resistant to the growth inhibitory actions of retinoids (27). Although several previous reports have implied a crucial role for *RARβ2* in mediating the antiproliferative actions of RA (23, 24, 34, 63, 64), it is evident from this and other studies (62, 65, 66) that other genes are necessary to induce cell cycle arrest and/or

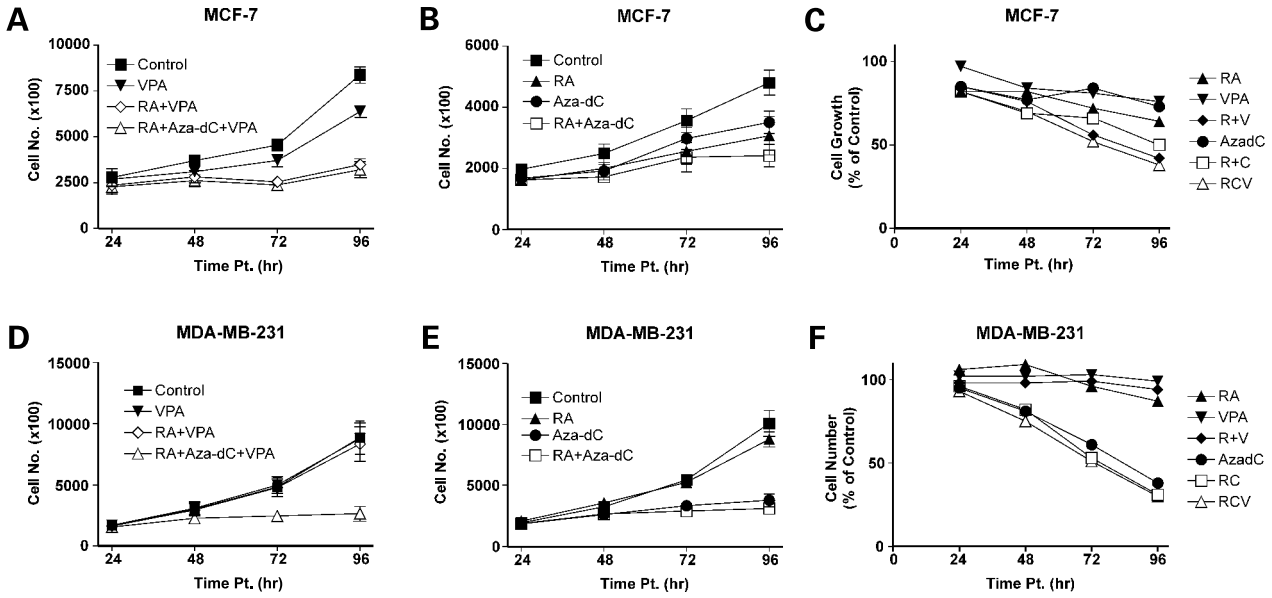

Figure 4. Proliferation of MCF-7 (**A**–**C**) and MDA-MB-231 (**D**–**F**) breast cancer cells was monitored in cells treated twice with combinations of VPA (250 μmol/L), RA (1 μmol/L), and Aza-dC (2 μmol/L) during the 96-h growth experiment. Growth inhibition expressed as percentage of untreated control MCF-7 (**C**) and MDA-MB-231 (**F**) cells is displayed to permit comparison between experiments done on different occasions. Growth assays were done on a minimum of four occasions. *Points,* mean; *bars,* SE.

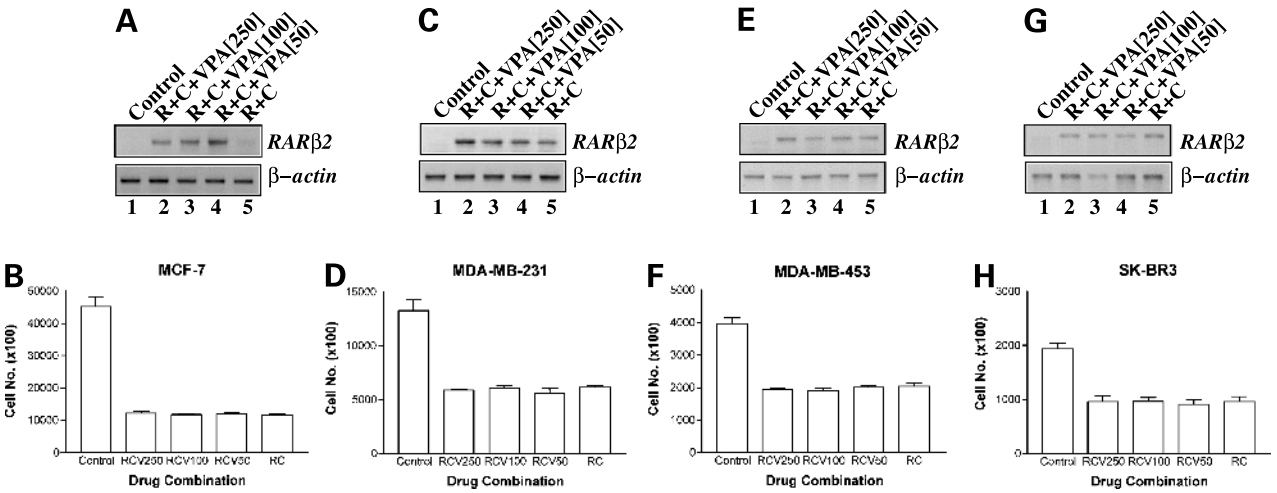

Figure 5. RT-PCR was used to detect expression of *RARβ* in MCF-7 (**A**), MDA-MB-231 (**C**), MDA-MB-453 (**E**), and SK-BR3 (**G**) breast cancer cells. Amplification of β-*actin* was used to confirm cDNA integrity. Cells were treated twice over a 96-h period with ethanol, RA (1 μmol/L), and Aza-dC (2 μmol/L) with decreasing VPA concentrations (*lane 1*); RA + Aza-dC + VPA (250 μmol/L; *lane 2*); RA + Aza-dC + VPA (100 μmol/L; *lane 3*); RA + Aza-dC + VPA (50 μmol/L; *lane 4*); and RA + Aza-dC (*lane 5*). RT-PCR was done as described in Materials and Methods. PCR products were analyzed by electrophoreses on 1.5% TAE-agarose gels and stained with ethidium bromide. Proliferation of MCF-7 (**B**), MDA-MB-231 (**D**), MDA-MB-453 (**F**), and SK-BR3 (**H**) breast cancer cells was monitored in cells treated twice with combinations of RA (1 μmol/L) with Aza-dC (2 μmol/L) and varying VPA concentrations as indicated during the 96-h growth experiment. Cell number was measured after 96-h growth. Growth assays were done in quadruplicate. *Columns,* mean; *bars,* SE.

sensitize cancer cells to the growth inhibitory pathways regulated by retinoids (59). However, restoration of retinoid signaling and *RARβ2* expression, in particular, represents an important contribution toward inhibiting breast cancer proliferation (23, 34). Indeed, recent studies are beginning to delineate the specific functions of the diverse pathways that mediate the growth inhibitory effects induced by retinoids and chromatin-targeted drugs. It was shown recently that growth inhibitory pathways induced by retinoids, HDAC inhibitors, and IFN-γ converge via the tumor necrosis factor–related apoptosis-inducing ligand proapoptotic pathway (60). Therefore, combination therapies, which target the epigenetic status of multiple tumor suppressor and growth regulatory genes and their downstream pathways (5, 6, 9, 14, 60), may be most effective in sensitizing a range of breast and other solid tumors to the growth inhibitory actions of retinoids.

In conclusion, we have shown that VPA, with RA and Aza-dC, can contribute to the reactivation and enhancement of expression of a prototypical silenced tumor suppressor gene (*RARβ2*) in breast cancer cells. It is therefore possible that combination therapies employing VPA will be useful in the treatment or chemoprevention of breast cancers. Although VPA has already shown considerable promise in inhibiting endometrial cell growth in nude mice (38) and diminishing adenoma formation in APC^{min} mice (40), it will be necessary to confirm the efficacy of VPA, in combination with therapeutic concentrations of RA and DNMT inhibitors, in a breast cancer mouse xenograft study. Such experiments will be of increasing clinical relevance when done with new classes of DNMT inhibitors (16, 67, 68), which are currently undergoing clinical trials.

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Nigel P. Mongan and Lorraine J. Gudas

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