Chronic Administration of Valproic Acid Inhibits Prostate Cancer Cell Growth *In vitro* and *In vivo*

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Abstract

Valproic acid (VPA) is an established drug in the long-term therapy of seizure disorders. Recently, VPA has been associated with anticancer activity, an effect thought to be mediated through the inhibition of cellular histone deacetylase 1. We investigated the effect of various doses of VPA (0, 1.2, and 5.0 mmol/L) administered either acutely or chronically on histone acetylation, p21 gene expression, androgen receptor expression, prostate-specific antigen (PSA) expression, and cell survival and proliferation in prostate cancer cell lines. We also studied the effect of chronic VPA on tumor xenograft growth *in vivo*. Our results show that acute treatment (3 days) VPA can increase net histone H3 acetylation and up-regulate p21, AR, and cytosolic PSA expression. Interestingly, the effects on AR and PSA are reversed with chronic treatment. In addition, acute VPA reduces cell survival but has no effect on the subsequent proliferation of surviving cells following drug withdrawal. However, when VPA is chronically administered (10-14 days) to prostate cancer cells, even lower doses of VPA result in marked decreases in the net proliferation rate, correlating with increased caspase-2 and caspase-3 activation. These effects are evident in both androgen receptor-positive (LNCaP and C4-2) and androgen receptor-negative (DU145 and PC3) prostate cancer cells. Moreover, chronic VPA treatment results in statistically significant reduction of tumor xenograft growth *in vivo*. We conclude that acute treatment has nominal effects on prostate cancer cell survival and proliferation, but chronic VPA results in profound decreases in proliferation, independently of androgen regulation. (Cancer Res 2006; 66(14): 7237-44)

Introduction

Since the advent of prostate-specific antigen (PSA) testing, prostate cancer diagnosis and treatment have resulted in a "stage migration" with the most recent data suggesting improved outcomes (1) with early treatment. However, when primary treatment fails, the disease develops along an intractable course for which no long-term effective therapy exists (2). Removal of androgens may cause a significant acute reduction in cancer burden, but invariably, the cancer develops mechanisms to cope with the lack of normal concentrations of androgens (3). It is the androgen-independent prostate cancers that ultimately lead to patient death (4, 5). One approach to treating advanced prostate cancer has been the evaluation and development of various modulators of epigenetic transcriptional regulation. Acetylation and deacetylation of histones play an important role in the epigenetic regulation of gene expression (6). There are at least two classes of enzymes involved in determining the acetylation state of histones, histone acetyl transferases and histone deacetylases (HDAC), and altered states of these complexes have been associated with various malignancies (7).

HDAC inhibitors are emerging as an exciting new class of potential anticancer agents for the treatment of solid and hematologic malignancies. In recent years, an increasing number of structurally diverse HDAC inhibitors that inhibit proliferation and induce differentiation and/or apoptosis of tumor cells in culture and in animal models have been identified. Despite the large number of HDAC inhibitors that have been described, little is known about their target enzyme specificity. There is increasing evidence to support a distinct biological role for each of the HDAC enzymes, and it is likely that inhibition of specific members of the HDAC family will have specific functional consequences on gene expression, cell cycle regulation, proliferation, differentiation, and apoptosis.

Several HDAC inhibitors have shown impressive antitumor activity *in vivo* with remarkably little toxicity in preclinical studies and are currently in phase I clinical trials (8). Our results suggest that acute treatment with valproic acid (VPA) leads to growth inhibition and cell death in prostate cancer cells; however, the antiproliferation effect ceases following withdrawal of drug. More importantly, when VPA is administered chronically, a profound decrease in cell proliferation results *in vitro* with significant reduction in tumor volume at clinically relevant doses *in vivo*. The ideal application for this type of therapeutic effect would be in patients who have had biochemical recurrence of prostate cancer after radical prostatectomy. Because the tumor burden at the time of PSA recurrence is low and the number of doublings required until death is high, even minimal prolongation of the tumor doubling time, multiplied over many tumor doublings, could result in significant delay in progression. Our findings suggest that chronic VPA may be of potential therapeutic benefit in the treatment of prostate cancer.

Materials and Methods

Cell lines and chemical. Human prostate cancer cell lines LNCaP, PC3, and DU145 were obtained from American Type Culture Collection (Manassas, VA), and the C4-2 line was obtained from Dr. Leland Chung. The cells were grown in RPMI 1640 with l-glutamine (Cellgro, Herndon, VA).

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supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies, Inc., Carlsbad, CA), 5 μg/mL ciprofloxacin hydrochloride (U.S. Biological, Swampsct, MA), and 50 μg/mL gentamicin (Quality Biological, Inc., Gaithersburg, MD). Cells were allowed to grow until 80% to 90% confluent and harvested with 0.05% trypsin/0.53 mM/L EDTA (Cellgro) before each subsequent passage. VPA (1 μmol/L; VPA sodium salt; Sigma, St. Louis, MO) stock was made in PBS and filter sterilized through a 0.22-μm filter.

Western immunoblotting. Seventy percent to 80% confluent T150 flasks of LNCaP, PC3, or DU145 were treated with medium containing 0, 1.2, and 5.0 mM/L VPA for 48 and 72 hours. The cells were harvested with 0.05% trypsin/0.53 mM/L EDTA, washed in PBS, and resuspended in 100 μL Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). The BCA protein assay kit (Bio-Rad, Hercules, CA) was used to determine total protein concentration and purified bovine serum albumin (Sigma) to generate the standard curve. Concentrated proteins were separated on a 12% Tris-HCl polyacrylamide gel (Bio-Rad) and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked for an hour in blocking buffer [100 mM/L Tris-HCl (pH 7.5), 150 mM/L NaCl, 0.1% Tween 20] with 5% nonfat dry milk and then incubated with rabbit anti-acetylated histone H3 (Upstate, Charlottesville, VA) overnight followed by anti-rabbit IgG peroxidase conjugate (Sigma) for 1.5 hours at room temperature. Immuno-reactive bands were detected using the enhanced chemiluminescence plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Monoclonal anti-β-actin mouse monoclonal (Sigma) and anti-mouse IgG-peroxidase (Sigma) were used to detect β-actin in the same blots. Anti-Cip1/WAF1/p21 mouse monoclonal IgG (Upstate) and anti-mouse IgG peroxidase (Sigma) were used to test p21 expression.

Cell proliferation assay. Prostate cancer cell lines LNCaP, PC3, and DU145 were seeded at 1 × 10^4 per well in 96-well culture plates and incubated overnight with RPMI 1640 containing 10% FBS. The cells were then treated with medium containing VPA (0, 1.2, and 5.0 mM/L) for 3 days. The fraction of cells surviving after acute VPA treatment was determined using the Cell Proliferation kit 1 (Roche, Basel, Switzerland) as per manufacturer's recommendation. To determine the proliferation of the viable cells after acute VPA treatment, LNCaP, PC3, and DU145 cells were seeded and treated in the same manner as above. After the 3-day VPA treatment, cells were washed with PBS and medium changed to RPMI 1640 containing 10% FBS. Viable cells were detected using the Cell Proliferation kit 1 at 24-hour intervals for 3 days to calculate cell proliferation.

Caspase assay. Prostate cancer cell lines LNCaP, PC3, and DU145 were treated with medium containing 0, 1.2, and 5.0 mM/L VPA for 1, 3, and 14 days. The cells were harvested by 0.05% trypsin/0.53 mM/L EDTA. Caspase activity was assessed by measuring conversion of a fluorogenic substrate specific for each caspase subtype with ApoAlert Caspase Assay Plates (BD Biosciences, San Jose, CA) as per manufacturer's recommendation. The plates were read using the CytoFluorII Series 4000 (Applied Biosystems, Foster City, CA; excitation, 380 nm; emission, 460 nm).

Flow cytometry. The apoptosis assay was done using APO-BRDU kit for measuring apoptosis by dual-color flow cytometry (Chemicon International, Temecula, CA). Prostate cancer cell lines LNCaP, PC3, and DU145 were seeded in T150 flasks and incubated overnight with RPMI 1640 containing 10% FBS. The cells were then treated with medium containing 0, 1.2, and 5.0 mM/L VPA for 3 days. The cells were harvested by 0.05% trypsin/0.53 mM/L EDTA and fixed with 1% (w/v) paraformaldehyde (Sigma) for 30 to 60 minutes, washed twice with PBS, and resuspended and incubated with the fluorescein-PRB-1 antibody solution in the dark for 30 minutes at room temperature followed by propidium iodide (PI)/RNase A solution in the dark for 30 minutes at room temperature. The cells were analyzed by flow cytometry within 3 hours. Both FITC and PI were excited at 488 nm. The emission from FITC was collected on FL1 (530/30 nm bandpass filter), whereas the emission from PI was collected on FL2 (670/30 nm bandpass for linear PI) and FL5 (610/20 nm for log PI). Doublet discrimination was done using pulse width versus forward scatter method. Data are plotted as fold induction of apoptosis using untreated cells as a reference. Statistical analysis was not done on this experiment.

Establishment of tumor xenografts. Human prostate cancer cell lines LNCaP C-2, and DU145 were grown to 80% to 90% confluence and harvested. Cells were resuspended in 1× phosphate-buffered saline (pH 7.4; BioSource, Rockville, MD), mixed 1x with Matrigel (BD Biosciences, Palo Alto, California), and injected (1 × 10^6 per injection) s.c. into the lateral flanks of male athymic nu/nu mice. Once palpable tumors were established, animals were randomized into control and treatment arms with the latter receiving 0.4% VPA in drinking water. Tumor volumes were measured twice weekly until animals were sacrificed, and tumors were harvested on day 35.

Statistical analysis. Data are presented as means ± SE. All statistical analyses were done on an IBM-compatible computer, running GraphPad Prism 4.0, on Windows XP. All error bars shown represent the SE. All experiments were done with at least three replicates and as many as 20 replicates. Statistical significance was calculated by the Student's t test for paired comparisons, when appropriate, by Wilcoxon rank-sum test, or by repeated measures two-way ANOVA with post-hoc testing for comparison of dose treatment effects, where applicable. P < 0.05 was considered statistically significant. Because there are over 100 paired comparisons (including post-hoc testing), Ps are reported only as P < 0.05 when significant or P > 0.01 when highly significant. Comparisons lacking significance are specifically denoted in the legends and text.

Results

VPA increases histone H3 acetylation. Three prostate cancer cell lines were treated with VPA (0, 1.2, and 5 mM/L) for 48 and 72 hours, and then H3 acetylation was assessed by Western blot. Shown in Fig. 1A, all three cell lines show significant increases in acetylated H3 compared with the β-actin control at 48 hours. The effect was dose dependent and seemed to be maximal for LNCaP and PC3 at 72 hours and DU145 at 48-hour post-treatment. For reasons not completely understood, levels of acetylated H3 in DU145 remained consistently high at 72 hours in the absence of VPA. It is possible that this phenomenon may be a reflection of cell overgrowth given the rapid proliferation rate of DU145 cells.

Treatment of malignant cells with HDACI frequently results in increased p21 WAF1/CIP1 expression and hence was investigated as secondary confirmation of HDACI activity (9). As predicted, a dose-dependent increase p21 WAF1/CIP1 expression was shown in all three cell lines (Fig. 1B); however, in contrast to the acetylated H3 data, p21 WAF1/CIP1 was maximal at 48 hours for LNCaP and 72 hours for PC3 and DU145. This suggests that H3 acetylation is not causal for p21 elevation but rather that VPA up-regulates both independently in a dose-dependent fashion.

Effect of acute VPA treatment on cell survival. Previous studies of other HCACIs in prostate cancer have shown significant therapeutic effect (10–12). Hence, we sought to determine if VPA had similar activity. Cells were treated with VPA for 3 days and evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate drug effect on cell viability. Results are shown in Fig. 2 as percentage cell viability compared with untreated control. In all three cell lines, VPA decreased cell viability compared with untreated in a dose-dependent, statistically significant manner, although the effect was generally modest and evident in DU145 only at the highest dose level (5 mM/L VPA). The data that reach statistical significance (P < 0.05, Students t test) when compared with untreated cells are marked with an asterisk.

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Previous analyses of different HDACIs have shown apoptosis as the major mechanism of cell death in prostate cancer cells, although potency of this stimulation varies with different cell lines (13, 14). To elucidate whether apoptosis is involved in the cancer cell killing effect of VPA, LNCaP, PC3, and DU145 cells were evaluated by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assays, and the effect was measured by flow cytometry (Supplementary Fig. S1A). Data are shown as scatter plots with fold induction of apoptotic index as insets. These data show that apoptosis is indeed activated in a dose-dependent fashion for all three cell lines; however, maximal induction was <15% even at the highest dose (5 mmol/L VPA), and the effect in DU145 was only significant at the highest dose.

We next attempted to determine which pathway, intrinsic or extrinsic, was involved in the fraction of cells undergoing apoptosis. To this end, direct measurement of various cellular caspase activities (Supplementary Fig. S1B) after acute VPA treatment was done. In this experiment, the cells were treated with medium containing 0, 1.2, and 5.0 mmol/L VPA for 72 hours. Of note, subsequent studies to evaluate the effect of acute VPA on caspase activation are done at 24 hours to avoid duplication of data. The cells were then harvested, and caspase activity was assessed. LNCaP showed the most pronounced increase in caspase-2 and caspase-3 activities, and DU145 showed significant increases in caspase-2 and caspase-3 activities at the higher dose (5 mmol/L) only. PC3 had nominal increases (not significant) in caspase activity after acute treatment with VPA. In all cases, the enzymatic caspase activity was modest and seemed to favor the intrinsic pathway of apoptosis activation (mitochondrial based). These findings are consistent with other HDACIs, which also have been found to favor intrinsic apoptosis pathways (15).

**Cellular proliferation after acute VPA treatment.** Inasmuch as the majority of the cells treated after VPA survived treatment, we next sought to determine if these cells behaved any differently phenotypically. In particular, we examined whether the proliferation rate was different in surviving prostate cancer cells after acute VPA treatment. Cells were treated for 72 hours with various doses of VPA and then replated (without VPA), and the proliferation was assessed at several time points by MTT as indicated in Fig. 3A-C. The data are plotted as fold cell growth, as the various starting points of the different cells varied according to the amount of cell death, which occurred initially from the VPA treatment. In these experiments, normalization to cell number was necessary because a fraction of viable cells underwent cell death following replating, resulting in unequal cell numbers. Of those cells that survived acute treatment with VPA, the rate of cell growth was found to be identical for VPA-treated and untreated cells in all three cell lines. These data indicate that, although VPA treatment may result in death of a subpopulation of prostate cancer cells, those that survived maintained the same proliferative potential as cells that had never been treated.

**Cellular proliferation decreases after chronic VPA treatment.** We also sought to determine whether the proliferation rate was different in surviving prostate cancer cells after chronic VPA treatment. Here, we measured cellular proliferation in...
cultured cells with chronic VPA administration (typically 2-week long cultures). Cells were expanded into serially larger flasks, and total cell counts were measured to maintain an environment that would sustain log-phase cell growth. Although we initially attempted to sustain cultures beyond 2 weeks, the amount of cell death was so significant in all tested cell lines that we were unable to consistently salvage any viable cells beyond that time. As shown in Fig. 4, proliferation of the tested cell lines with chronic VPA administration was profoundly diminished. In all cases, the higher dose of VPA (5 mmol/L) not only decreased the rate of proliferation but actually caused continued cell death so that few viable cells were present at the end of the two week experiment. Of the chronically treated cells that underwent cell death via apoptosis, there were marked increases in caspase-2 and caspase-3 activities compared with acute treatment (Supplementary Fig. S2A-C). For PC3, caspase-8 and caspase-9 activities were also elevated. These findings are consistent with the observation that PC3 phenotypically behaves differently to HDACIs in the activation of apoptosis (13). The effect of chronic treatment with 5 mmol/L VPA in DU145 was so profound that we were unable to harvest sufficient cellular material to do a caspase activity assay (despite multiple attempts to scale up the starting cell number). However, given the fact that caspase-2 and caspase-3 activities were so elevated in DU145 when treated with 5 mmol/L VPA at early time points, it is likely that the intrinsic pathway was still dominant at later time points.

Androgen withdrawal and VPA treatment effects are independent. Because two of the cell lines tested in these experiments (DU145 and PC3) lack appreciable expression of androgen receptor, it is clear that VPA-induced cell death does not require the androgen receptor. However, VPA may still modulate androgen receptor activity with downstream consequences. Hence, we interrogated LNCaP for the effect of VPA treatment on androgen receptor and PSA expression by Western blot analysis, shown in Supplementary Fig. S3A and B. Acute treatment with VPA results in a slight increase in androgen receptor and, consequently, PSA expression. However, chronic treatment with VPA results in a net decrease in androgen receptor and minimal decrease in PSA expression compared with h-actin. These results are consistent with the findings of Abdul et al., who evaluated the effect of VPA on interleukin-6 (IL-6) and PSA secretion in vitro, at a time when it was thought that VPA affected gene expression by blocking calcium channels (16). Although the proposed mechanism may have been in error, their results seem to have been accurate.

Because VPA seems to alter androgen receptor expression, we hypothesized that it might also alter the sensitivity of androgen-independent prostate cancers to androgen withdrawal. To test this hypothesis in vitro, we used the androgen-independent cell line C4-2, which is capable of sustained proliferation in the absence of exogenous androgen but is nonetheless stimulated to enhanced proliferation in the presence of androgen. For these experiments, androgen stimulation was accomplished by the addition of synthetic androgen, R1881 (methyltrienolone), to medium containing charcoal-stripped FBS. When androgen is acutely withdrawn, there is an initial decrease in cellular proliferation (Fig. 5). Treatment with VPA results in decreased cell survival. However, when VPA and androgen withdrawal are combined, the observed result is identical to the predicted result. Hence, in this experiment, the two treatments are additive not synergistic, implying that the two treatment effects occur through completely independent pathways. Because the decrease in survival and proliferation is notable in androgen receptor-negative cell lines, this pathway independence is not surprising. However, because the decreases in

Figure 3. The effect of acute VPA treatment on subsequent cell proliferation. LNCaP, PC3, and DU145 cells were treated with 0, 1.2, and 5.0 mmol/L VPA for 72 hours, washed, and grown in medium without VPA. The proliferation of these cells was monitored by MTT assay. Results are for LNCaP (A), PC3 (B), and DU145 (C). Comparison of groups was done by repeated measures of ANOVA with post-hoc testing revealed no significant differences between treated and untreated groups. Cells that received acute treatment with VPA retain exactly the same proliferation rate, as cells that were never treated.
proliferation induced by VPA treatment alone is so profound, it is still possible that any conversion from the androgen-independent phenotype to the androgen-dependent phenotype was below the limits of detection by this approach.

Chronic VPA treatment inhibits progression of tumor xenografts. To assess the effects of chronic VPA treatment on prostate cancer cell growth in vivo, we established tumor xenografts in male athymic nu/nu mice using both androgen-dependent and androgen-independent prostate cancer cell lines, including LNCaP, C4-2, and DU145. Animals in the treatment arm received 0.4% VPA in drinking water with peak serum VPA measurements ranging from 3 to 70 mg/L. Tumor volumes were measured until week 5 and compared using the Wilcoxon rank-sum test and 2-way ANOVA with post-hoc testing. Animals treated with VPA showed statistically significant reduction in tumor volume compared with controls in all cell lines with 40%, 60%, and 70% reductions in LNCaP, C4-2, and DU145, respectively (Fig. 6). Toxicity studies, including complete blood count, alanine aminotransferase, and aspartate aminotransferase revealed no difference between treated and control animals.

Discussion
The primary modes of treatment for prostate cancer are surgery and radiation, either of which can result in treatment failure, typically manifested as biochemical recurrence with a rising PSA. On average, biochemical recurrence of prostate cancer after radical prostatectomy predates radiographic evidence of disease by over 8 years and death by ~13 years (17). Because the tumor burden at the time of PSA recurrence is low and the number of doublings required until death is high, even small changes in the rate of tumor growth would translate into profound clinical benefit. For instance, if 15 doublings of a tumor are required from the time of...
biochemical detection until death and the doubling time of the tumor is 10 months, then, a 15% increase in the doubling time would extend life by another 1.9 years. Given that most men with advanced prostate cancer are in the 6th or 7th decade of life, such an increase in survival may actually eliminate many prostate cancer deaths (in exchange for other unrelated causes of death). With these concepts in mind, attention has been focused on the discovery of so-called “differentiation agent” therapies so that disease to a chronic medical condition (18). In this article, we provide preliminary evidence that at least one HDAC inhibitor (VPA) may also be suitable for this purpose.

Several structurally diverse inhibitors of HDAC have been identified, many of which are natural products. HDAC inhibitors (VPA) may also be suitable for this purpose.


Figure 6. The effect of chronic VPA on prostate cancer xenografts. LNCaP, C4-2, and DU145 xenografts were established in male athymic mice. Animals in the treatment arm received 0.4% VPA in drinking water. Tumor volumes were compared using Wilcoxon rank-sum tests and 2-way ANOVA with post-hoc testing. (*, P < 0.05).

VPA (2-propylpentanoic acid) is an established drug in the long-term therapy of seizure and bipolar disorders (24). Recently, it has become evident that VPA is also capable of inhibiting HDACs (25–27), resulting in altered gene expression in certain susceptible cells. The net result is that VPA inhibits cell growth, regulates differentiation, and may have effects on tumor invasion and angiogenesis (7, 8, 28). We show here that acute treatment with VPA may have limited activity on prostate cancer cell proliferation, whereas chronic administration of VPA has a more profound effect. This phenomenon may be explained by HDACI activation of cell death and cell cycle arrest in G1-G2 (29). These effects are not mutually exclusive, and it is possible that a given cell could be arrested in the cell cycle but then stimulated to reenter the cycle and activate death at a later time. In such a scenario, chronic administration of the HDACI would be predicted to have markedly better therapeutic effect with decreased cell proliferation, as many initially quiescent cells subsequently traverse through the cell cycle checkpoints. This effect is likely not specific to VPA but rather may be present with any number of other HDACIs currently under investigation in solid epithelial malignancies.

One gene most consistently induced by HDAC inhibitors is the cyclin-dependent kinase inhibitor p21WAF/CIP1 (22), which plays an important role in the arrest of cell growth. Butyrate, trichostatin A, depsipeptide, oxamflatin, MS-27-275, and the hydroxamic acid-based HDACIs all induce p21WAF/CIP1 expression (30). Our data confirm these observations, as p21WAF/CIP1 gene expression was associated with an increased level of histone H3 acetylation after VPA treatment (Fig. 1). Although it is not possible to correlate the effects on H3 acetylation and p21 expression, it is clear that VPA causes increased expression of both in a dose-dependent manner. In addition, analysis of apoptosis markers, such as by TUNEL assay or direct caspase activity, shows that VPA initiates a modest apoptotic response through the preferential activation of the mitochondrial (intrinsic) pathway (Supplementary Figs. S1 and S2). Such results are consistent with previous findings of other HDACIs in prostate cancer cells (13). Furthermore, when VPA is given acutely (<3 days), less than a 30% to 40% reduction in cell viability results. Importantly, those cells that survive the acute VPA treatment show completely normal growth patterns when compared with untreated controls (Fig. 3).

To further investigate the effects of chronic VPA on prostate cancer cells in vivo, we established xenograft models using LNCaP, C4-2, and DU145 cells. The results show statistically significant reduction in tumor volumes of VPA-treated animals versus untreated controls for all cell lines. Tumor reduction ranged from ~40% to 70% at therapeutic doses of VPA. These effects support our in vitro work, which showed marked cell kill in both androgen-dependent and androgen-independent disease following the chronic administration of VPA. Given its ability to reduce tumor volume in vivo without significant toxicity, VPA may be particularly useful for slowing cancer progression in patients with biochemical recurrence, as the tumor burden is low, and the number of cell divisions required for measurable disease is high compared with most other malignancies.

Development of the androgen-independent phenotype can occur through a diverse variety of mechanisms ranging from deletion or inactivating mutations of the androgen receptor, mutation of androgen receptor resulting in altered activity, such as promiscuous activation with other ligands, or alteration of human cancer cell lines, frequently via intrinsic pathway caspase activation (15, 23).
one or more of the numerous androgen receptor interacting factors, including IL-6 (31), activator protein, c-Jun, HER-2/neu, protein kinase A (32), mitogen-activated protein kinase, and activated Akt (33). Recently, Chen et al. (34) showed in vitro that androgen receptor amplification alone could result in the development of the androgen-independent phenotype. Multiple recent studies have implicated androgen receptor as having a role in most androgen-independent prostate cancers (31, 35, 36).

It has been suggested that down-regulation of androgen receptor expression may have potential therapeutic benefit in the treatment of prostate cancer (35). Analysis of the effect of VPA on androgen receptor and PSA expression in LNCaP cells showed a modest initial induction followed by an eventual net decrease in total expression when compared with the β-actin control (Supplementary Fig. S3). This alteration in androgen receptor regulation suggests that, although VPA-induced cell death may not require androgen receptor, treatment with VPA still affects androgen regulation. To better assess this phenomenon, we studied the effect of chronic VPA administration on C4-2 cells, an androgen-independent subline of LNCaP. Removal of androgen stimulation causes a net decrease in proliferation as does treatment with VPA (Fig. 5). However, when the two are combined, the net effect is indistinguishable from the predicted sum of the two treatments. Hence, in this experiment, the two treatments seem additive rather than synergistic. Nonetheless, it should be noted that these data do not preclude the possibility that decreasing the expression level of androgen receptor by chronic VPA results in reversal of the androgen-independent phenotype in susceptible cells. Such determinations are better made in animal models or even in pilot clinical trials.

In summary, we show that chronic administration of VPA results in a marked decrease in proliferation of prostate cancer cells in vitro and significant reduction in tumor volume in vivo. We postulate that this enhanced activity results from capturing the resistant quiescent cells, which are temporarily cell cycle arrested, as they transition back into the cycle. The ideal application for this type of therapeutic effect would be in patients who have had biochemical recurrence of prostate cancer after radical prostatectomy, as they have the least tumor burden and largest number of tumor doublings required before progression to death. In such a scenario, even minimal prolongation of the tumor doubling time, multiplied over many tumor doublings, can result in significant delay in progression. Importantly, the androgen receptor is down-regulated with chronic VPA treatment, an effect that may have downstream consequences in the regulation of androgen sensitivity. These data provide compelling evidence that additional studies are warranted in evaluating the potential use of chronic VPA as a means of altering prostate cancer cell growth and progression.

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