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Retinoids, derivatives of vitamin A (retinol), are required for the appropriate differentiation of normal human prostate epithelial cells. Human prostate cancer cells contain much lower levels of vitamin A and its metabolites that normal cells. We hypothesize that aberrant metabolism of vitamin A and dysregulation of gene expression in prostate tumor cells are related to the abnormal growth properties of the tumor cells. A rationale for using retinoids in prostate cancer chemotherapy is further supported by the effectiveness of ATRA (all-trans Retinoic Acid), a vitamin A metabolite, in the treatment of acute promyelocytic leukemia (APL). We hypothesize that the efficacy of retinoic acid can be enhanced if it is administered in combination with low doses of selective, potent histone deacetylase inhibitors such as trichostatin A (TSA(or valproic acid. The goals of this idea grant are to use mouse xenograft models to ascertain the effectiveness of various retinoids plus histone deacetylase inhibitors in inhibiting the growth and inducing the differentiation of the human prostate cancer lines LNCaP and PC-3. A second goal of the project is to understand at the molecular level the mechanisms by which the combination of retinois acid and histone deacetylase inhibitors result in human prostate tumor cell growth inhibition. In the past year, we have begun the xenograft experiments, employing 13-cis RA and valproic acid. We have also continued to perform a variety of biochemical and molecular biological assays on human prostate cancer cells treated with various combinations of the aforementioned drugs in order to gain more insight into the molecular mechanisms involved in cell growth inhibition. The studies that we have performed, and the studies proposed in the next period of this Idea Development grant should provide a much clearer rationale for new clinical treatments for prostate cancer in humans.

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INTRODUCTION

Retinoids such as retinoic acid have been used for a number of years to treat a variety of cancers. For instance, retinoic acid has been used successfully in the treatment of acute promyelocytic leukemia (APL). While ATRA (all-trans retinoic acid) administration results in remissions in most APL patients and the subsequent cures of the patients following secondary, cytotoxic cancer chemotherapy, occasional cases of patient relapse occur and the leukemia is then sometimes ATRA-resistant. In such patients, the addition of a histone deacetylase inhibitor, such as sodium phenylbutyrate, induced a complete molecular remission. The mechanism for the increased efficacy of retinoic acid in the presence of histone deacetylase inhibitors most likely involves the ability of histone deacetylase inhibitors to drive the formation of a more active transcription complex involving the retinoic acid receptors, retinoid X receptors, histone acetyltransferases, and coactivator proteins (for rev., ref. (1).

The clinical data from the acute promyelocytic leukemia patients, plus data generated in our laboratory, led us to develop the hypothesis that the combination of retinoids with histone deacetylase inhibitors should more effectively bring about cell differentiation and the control of cell growth in other types of tumors in addition to acute promyelocytic leukemia. More specifically, we hypothesize that cell differentiation is dysregulated in prostate cancer and that treatment of prostate cancer cells with pharmacological doses of all-trans-retinoic acid plus an inhibitor of histone deacetylase will result in greater tumor growth inhibition and tumor cell differentiation than treatment with either all-trans-retinoic acid or a histone deacetylase (HDAC) inhibitor alone. We propose to test aspects of this model in a more clinical setting, using a human prostate cancer xenograft model in Nu/Nu mice, and to test mechanistic aspects of this hypothesis using cultured human prostate cancer cell lines. A recent review discusses some of the clinical trials which have been performed to test various differentiation agents in prostate cancer therapy (*2*).

BODY

Task 1. To determine the mechanism by which concomitant administration of retinoids and various histone deacetylase inhibitors such as trichostatin A (TSA) inhibits human prostate cell cancer growth (months 1-36).

CELL PROLIFERATION INHIBITION

In task 1, we employed cell proliferation assays to determine if we observed increased growth inhibition using combination therapy with ATRA plus a low dose of a variety of different histone deacetylase inhibitors compared to either drug alone or to the untreated, control tumor cells. Both LNCaP and PC-3 human prostate cancer cell lines were tested. Various retinoids were tested, including 13-cis-retinoic acid, all-trans-retinoic acid, and 9-cis-retinoic acid, since all of these retinoids have been successfully used in the treatment or chemoprevention of several different human cancers. The histone deacetylase inhibitors that were tested included trichostatin A, valproic acid, and SAHA (suberoylanilide hydroxamic acid (SAHA)) (*3-6*). We utilized several histone deacetylase inhibitors because a number of these inhibitors are new, but positive results have been obtained in mouse models and for some, in human cancer therapy trials (*3-8*). We also added the drug 5-aza-deoxycytidine to our growth inhibition studies

because of much recent data that in combination with histone deacetylase inhibitors, the addition of 5-aza-CdR treatment results in demethylation and enhanced gene expression (*9-11*).

Our data, some of which are shown in this report, indicate that for LNCaP, while both valproic acid (VPA) and trichostatin A plus ATRA or retinol were growth inhibitory, VPA plus ATRA or retinol was more growth inhibitory than was TSA plus ATRA or retinol (Figure 1). The drugs 13-cisRA and 9-cisRA also resulted in growth inhibition in combination with VPA (data not shown).

In contrast, for the PC-3 cell line trichostatin A plus RA or retinol was more growth inhibitory than was VPA plus ATRA or retinol (data in 2002 report). Furthermore, the addition of aza-deoxycytidine to either VPA plus retinol or VPA plus retinoic acid led to enhanced growth inhibition of the PC-3 cells (data in 2002 report). It is of interest that a recent report (*12*) showed that stable re-expression of the androgen receptor in PC-3 cells also restored retinoid sensitivity to the PC-3 cells. This is similar to what we observed with RA plus TSA in PC-3 cells.

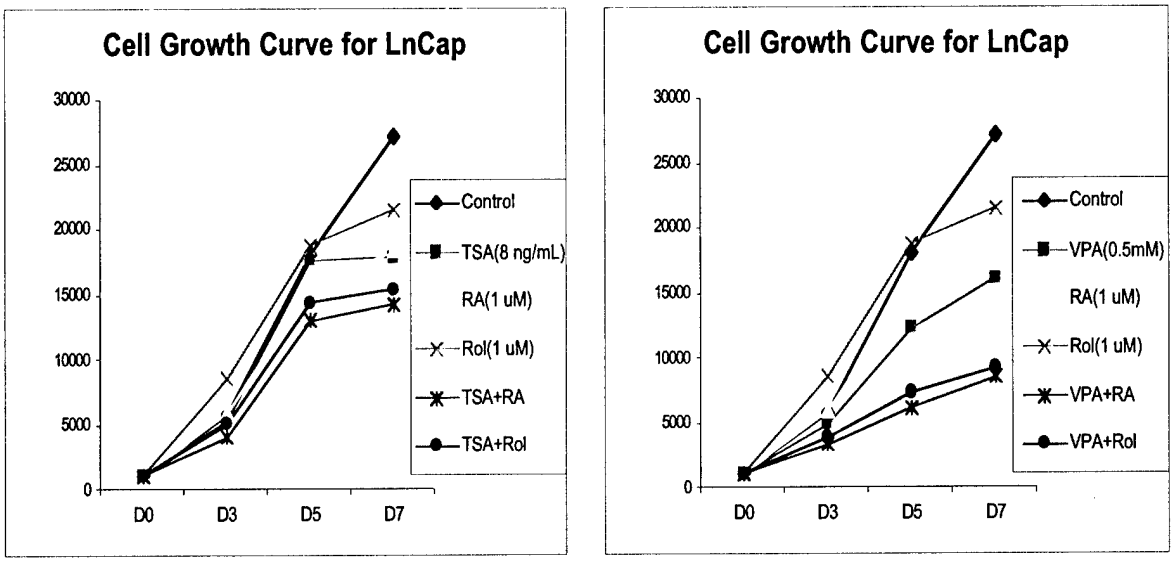

Figure 1A, B. The inhibitory effects of retinoic acid (RA, 1 μM) and retinol (Rol, 1 μM) on human LnCaP prostate cancer cells in combination with the histone deacetylase inhibitors trichostatin A (TSA, 8 ng/ml) or valproic acid (VPA, 0.5 mM). The LnCaP cells ($1x10^6$/well) were plated in 24-well cell plates for 16 hours before the indicated drugs were administered. The cells were trypsinized and counted using a Coulter counter in triplicate after incubation with the drugs for 3, 5, or 7 days.

We conclude from these growth inhibition assays that a combination of a retinoid, histone deacetylase inhibitor, and a demethylating agent such as aza-deoxycytidine is most efficacious at inhibiting the growth of prostate cancer cells in culture. Additionally, one very intriguing observation from our studies is that different HDAC inhibitors, in combination with retinoids such as retinoic acid or retinol, inhibit the growth of different prostate cancer cell lines to varying degrees, i.e. there is a cancer cell specificity to the HDAC inhibitors. It is known that there are different classes of HDAC inhibitors (reference 3 for review) but the roles of the various histone deacetylases (currently 11 different HDACs are known) in cells and the levels of expression of

the various histone deacetylases in cells are not well understood. Thus, one conclusion from our data is that different HDAC inhibitors may have roles to play in the chemotherapy of a variety of different types of cancers and molecular subtypes of prostate cancers.

EXAMINATION OF MOLECULAR MARKERS OF CELL DIFFERENTIATION IN CULTURED PROSTATE CELLS BY NORTHERN ANALYSIS, RT-PCR, AND WESTERN ANALYSES.

We have examined a number of different genes which are expressed at higher levels in differentiated, normal human prostate epithelial cells vs. prostate cancer cells. Untreated cells, as compared to cells treated with various combinations of retinoids plus HDAC inhibitors, were examined. For example, we have measured the levels of the keratin 18 and keratin 8 genes in these cells by Northern analysis. We did not detect changes in keratin 8 or 18 mRNAs in response to the combination of HDAC inhibitors plus retinol or retinoic acid treatment. However, we did detect a three to five fold increase in keratin 8 and keratin 18 mRNA levels in both cultured normal prostate epithelial cells and in prostate cancer cell lines treated with retinoic acid or retinol (1×10^{-7} M) alone. Cell cycle analysis indicated that the cells treated with retinoids plus HDAC inhibitors arrested in the G1/G0 phase of the cell cycle.

In the past year we have analyzed additional molecular markers to understand the effects of retinoids plus histone deacetylase inhibitors on human prostate cancer cell growth. Strikingly, we have found that the acetylation of histone proteins in human PC-3 prostate cancer cells is greatly increased by the addition of histone deacetylase inhibitors, and increased even further when either all-trans retinoic acid or 13-cis retinoic acid is added to the cells in addition to the histone deacetylase inhibitors. The histone deacetylase inhibitors tested were valproic acid (VPA), trichostatin A (TSA), and SAHA. Some of our results are depicted in Figure 2. As acetylation of histones is thought to be required for the activation of many genes involved in cell differentiation, these data begin to provide a molecular mechanism for the significant cell growth inhibitory effects we have observed with retinoids in combination with histone deacetylase inhibitors (e.g. Figure 1A, B).

In addition, we have analyzed gene expression in LNCap and PC-3 cells treated with retinoids with or without histone deacetylase inhibitors. We have examined the gene EZH2, a polycomb gene homologue of Drosophila's Enhancer of zeste [E(z)], which exerts its gene silencing function by forming a complex with another PcG gene, EED, and with histone deacetylases (*13*). EZH2 contains an evolutionary conserved SET domain (*14*) which is highly conserved in other chromatin-associated regulators of gene expression involved in the modulation of several cell growth pathways. In a dramatic, recent finding, Varambally and colleagues (*15*) reported that the levels of EZH2 were correlated with the malignant phenotype of human prostate tumors. Levels of EZH2 protein were found to be higher in malignant tumors than in benign prostate tumors. In contrast, no expression of EZH2 mRNA was detected in normal human prostate epithelium. Furthermore, high levels of EZH2 were found in cultured human prostate cancer cell lines, including PC-3 and LNCap. In conclusion, the authors identified an association of high levels of EZH2 with advanced prostate cancer (*15*). They also showed that EZH2 plays a role in mediating cell proliferation, most likely via "global" transcriptional repression of large numbers of genes in prostate cells.

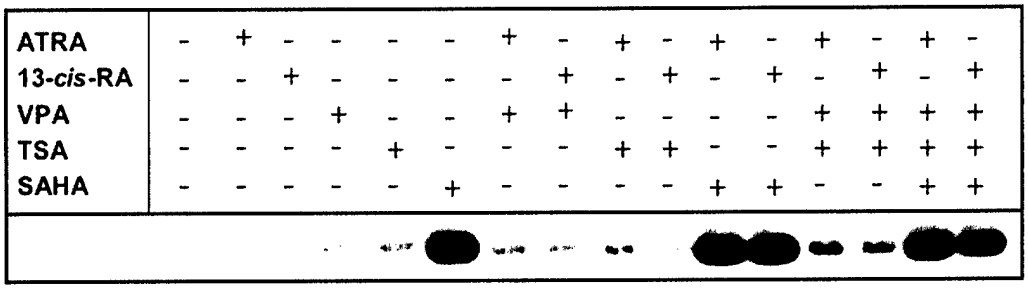

Fig. 2. 1 x 10^5 PC-3 cells were treated for 24 hours with various combinations of ATRA (1 μM), 13-*cis* RA (1 μM), VPA (200 μM), TSA (5 ng/ml), and SAHA (5 μM) or left untreated as a control. Whole cell extracts were prepared in denaturing SDS sample buffer, 10 μg protein was separated on a 15% SDS-PAGE gel, and transferred to nitrocellulose membranes. The membranes were stained with Ponceau S (Sigma, St. Louis, MO) to confirm proper transfer and equal loading (not shown). Hyperacetylation of histone H3 was detected using a 1:5,000 dilution of anti-acetyl-histone H3 antibody (Upstate, Lake Placid, NY). After incubation with a 1:10,000 dilution of anti-rabbit IgG horseradish peroxidase (HRP) conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), the membranes were developed with Supersignal Substrate (Pierce, Rockford, IL) for 5 minutes and exposed to Biomax film (Eastman Kodak, Rochester, NY). Primary and secondary antibodies were diluted in PBS containing 5% Blotto (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.1% Tween 20.

In recent studies we treated both androgen-responsive LNCap and androgen-independent PC-3 prostate cancer cell lines with retinoids and synthetic RXR agonists, alone or in combination with the histone deacetylase inhibitor valproic acid (*6*) for 48 hrs. After the specified period of time in culture, the samples were harvested and expression of the EZH2 gene was examined by Northern assay. These results are shown in Figure 3. Quantitation of normalized EZH2 mRNA levels is shown in Figure 4. As we've shown that pharmacological doses of retinoids, especially in the presence of histone deacetylase inhibitors, can reduce the expression of the EZH2 gene in prostate cancer cell lines, we hypothesize that the reduction in expression of retinoid receptors during the course of prostate carcinogenesis leads to upregulation of EZH2, subsequently resulting in the reduction of expression of many genes involved in the induction of cell differentiation by retinoids. We are currently testing this hypothesis by determining whether the decrease in EZH2 mRNA expression is concomitant or *required* for the growth inhibition we observed in the presence of retinoids and HDAC inhibitors. In order to determine this, we are attempting to delineate the mechanism by which retinoids and histone deacetylase inhibitors decrease the levels of EZH2 mRNA in cultured human prostate cancer cell lines. We will also determine if EZH2 is a direct target gene of retinoic acid and histone deacetylase inhibitors. This will be done in the next 12 months of this grant.

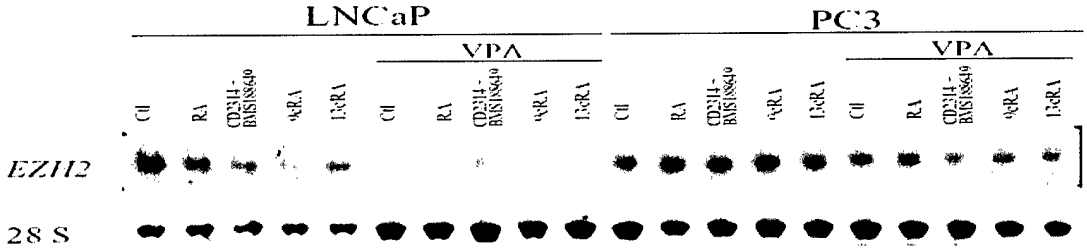

Fig. 3. Regulation of EZH2 expression by retinoids in prostate cancer cells. LNCaP and PC3 cells were treated with RA, RARβ agonist (CD2314) plus RXR agonist (BMS188649), 9cRA, and 13cRA. Control cells (Ctl) were treated with ethanol carrier only. All drugs were used at a concentration of 1μM, and they were added alone or in combination with 1mM VPA. Retinoids reduced the expression of *EZH2* mRNA in LNCaP cells and this effect was enhanced by VPA. In PC3 cells, VPA was required in addition to retinoids for the reduction of *EZH2* mRNA levels. The levels of the 28 S ribosomal subunit are shown as total RNA loading controls. This experiment has been repeated with similar results.

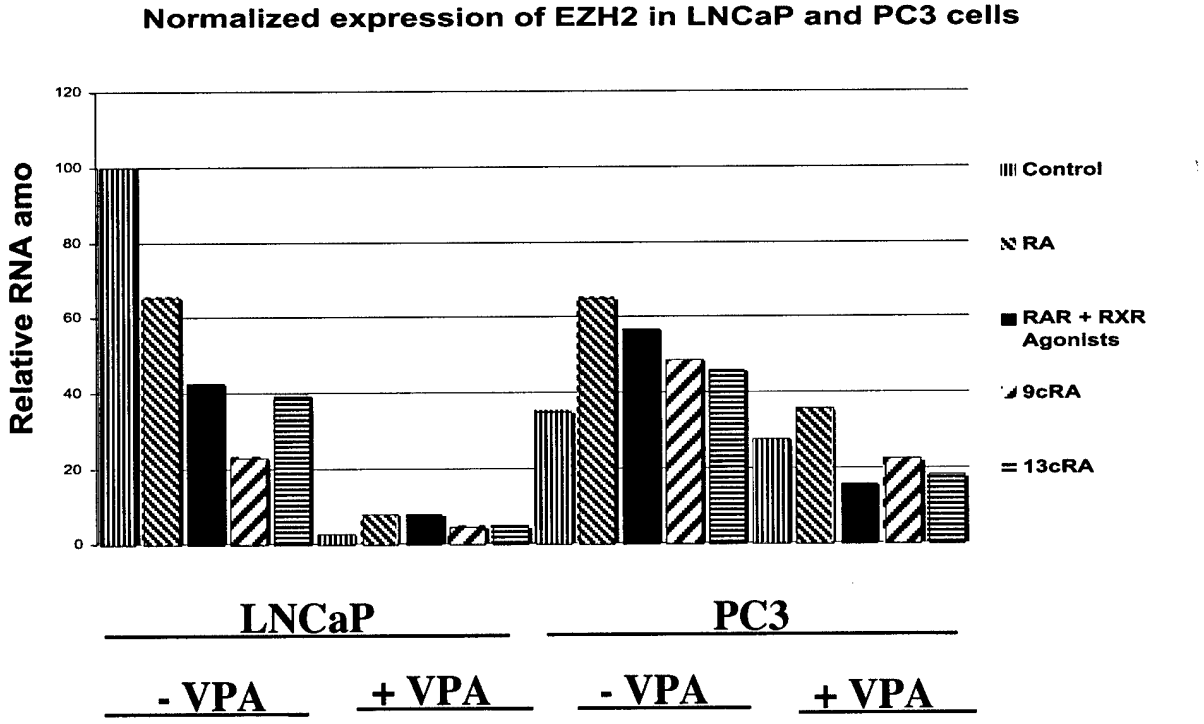

Fig. 4. Quantitative analysis of *EZH2* mRNA expression after RA treatment. Cells were treated for 48 hr with retinoids or a combination of RARβ and RXR agonists (1 μM) in the absence or presence of VPA (1mM). Controls were treated with ethanol only. The Y axis is given as percentages of normalized *EZH2* RNA levels in control LNCaP cells. Retinoids decreased the expression of *EZH2* in LNCaP cells and this effect was enhanced by VPA. In PC3 cells, VPA was required in addition of retinoids for the reduction of *EZH2* mRNA levels. For every sample, the intensity of the band after Northern blot analysis was normalized to the levels of the 28 S ribosomal subunit.

Task 2. In task 2, we propose to evaluate the efficacy of combined retinoid/histone deacetylase inhibitor administration in xenograft models (months 4 through 36). We have begun these experiments. The goal of task 2 is to establish a human prostate tumor xenograft model in Swiss nude, immunocompromised mice. Drugs are then administered in four experimental arms, including control, retinoid alone, histone deacetylase inhibitor alone, and the combination of retinoid and histone deacetylase inhibitor in combination. The tumor growth is monitored by measuring tumor area, and molecular markers of apoptosis, cell growth, and cell differentiation are to be assessed. RNA and protein from the tumors will be isolated for assessment, and both differentiation specific and pro-apoptotic genes will be measured by Northern and Western blotting. Tissue slides will be prepared and stained with hemotoxylin and eosin for histological characterization of the tumors, both untreated and drug treated.

We have been performing the xenograft experiments, and we now have some reportable data. The first xenograft experiment consisted of cohorts of ten mice in each cohort treated with either 13-cis retinoic acid alone by oral gavage or valproic acid, a histone deacetylase inhibitor, alone by intraperitoneal injection twice a day, or the combination of both drugs. The doses we used were 50 mg/kg of RA and 500 mg/kg of VPA. Valproic acid dosing was as suggested by researchers at Abbott Labs. Valproic acid has not been utilized in many animal models of cancer to date, but we wanted to use valproic acid because this drug (also known as Depakote) is FDA approved for use in epilepsy. VPA is an HDAC inhibitor. We plan to initiate Phase I clinical trials of 13-cis retinoic acid plus valproic acid within the next month or two months, and as a result, we wanted to use valproic acid in our animal studies rather than a drug such as trichostatin A. Trichostatin A is a more specific, potent inhibitor of histone deacetylases, but it is not yet approved by the FDA for human use. Thus, if our animal model is to be utilized in preparation for human trials in this translational research, it is necessary to use valproic acid in the xenograft experiments. We encountered some problems with the valproic acid injections. The animals became weak and lethargic shortly after each valproic acid injection. We also lost two of the animals in the valproic acid group and one animal in the valproic acid plus retinoic acid group. We are currently decreasing the dose of valproic acid in the xenograft model and we may go to one dose of valproic acid a day rather than two doses. We will continue these studies over the next twelve months of this grant.

KEY RESEARCH ACCOMPLISHMENTS

A. The demonstration that in *different* prostate tumor lines various histone deacetylase (HDAC) inhibitors result in different degrees of cell growth inhibition when combined with retinoids such as all-trans retinoic acid or retinol.
B. The demonstration that 5-aza-deoxycytidine, in combination with a retinoid and a histone deacetylase inhibitor, results in a greater degree of growth inhibition in human prostate cancer cell lines.
C. The analysis of a variety of molecular markers such as keratin 8, keratin 18, and PSA (prostate specific antigen) and histone acetylation in both normal human prostate epithelial cells and human prostate cancer cells cultured either as control cells, or in the presence of a variety of combinations of retinoids plus histone deacetylase inhibitors.
D. The measurements of acetylated histones, retinoid receptors, and EZH2 expression in cultured human prostate cancer cell lines following treatment with retinoids plus histone deacetylase inhibitors.

E. Initial data from xenograft experiments involving VPA, a HDAC inhibitor already approved by the FDA and in human use for epilepsy treatment.

REPORTABLE OUTCOMES

Martinez-Ceballos, E., Touma, S.E., Nanus, D., Tickoo, S., and Gudas, L.J. Measurements of Histone Acetylation and Gene Expression in Retinoid and HDAC Inhibitor Treated Human Prostate Cancer Lines.

CONCLUSIONS

First, we have shown that there are differences in the responses of various human prostate cancer cell lines to different classes of histone deacetylase inhibitors. For example, the combination of the drug valproic acid plus retinoic acid was more growth inhibitory to LNCaP cells than was SAHA or trichostatin A plus all-trans retinoic acid. In contrast, trichostatin A plus retinoic acid was much more growth inhibitory to PC-3 cells than was VPA plus all-trans retinoic acid. Such findings could have significant clinical applications. The LNCaP line in androgen responsive, whereas the PC-3 line is not. Furthermore, these data suggest that the examination of the different histone deacetylases expressed in various tumor lines could be worthwhile. Second, we have preliminary data that the combination of a retinoid and a histone deacetylase inhibitor results in growth inhibition, and there is also some apoptosis of the cells. Third, we have shown for the first time that valproic acid alone results in some growth inhibition of the LNCaP prostate cancer cell line. Fourth, we have shown that EZH2 gene expression is strikingly down-regulated by retinoids plus the HDAC inhibitor VPA. Fifth, we have some initial xenograft data from retinoid plus VPA treated animals.

In addition to gaining fundamental knowledge of the mechanisms of action of retinoids plus histone deacetylase inhibitors in combination, our studies should provide insights for future pharmacological therapies for human prostate cancer treatment.

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