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TITLE: Retinoid and Histone Deacetylase Inhibitors in the Treatment of Prostate Cancer

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Retinoid and Histone Deacetylase Inhibitors in the Treatment of Prostate Cancer

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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 than 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 retinoic acid and histone deacetylase inhibitors result in human prostate tumor cell growth inhibition. In the past year we have continued 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 have provided a much clearer rationale for new clinical treatments for prostate cancer in humans.
# Table of Contents

Cover................................................................................................................. 1  
SF 298.................................................................................................................. 2  
Table of Contents............................................................................................. 3  
Introduction........................................................................................................ 4  
Body..................................................................................................................... 4-11  
Key Research Accomplishments................................................................. 11  
Reportable Outcomes.................................................................................... 12  
Conclusions....................................................................................................... 12  
References.......................................................................................................... 13  
Appendices....................................................................................................... 14-48
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 tested aspects of this model in a more clinical setting, as well as performing molecular analyses of cultured human prostate tumor 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. 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 (1x10⁶/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. (from 2003 report).

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 x 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 year 03 we 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.
Figure 2. Western Analysis of Histone Acetylation. 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.

In year 3 we also determined how RA or retinol treatment influenced the types and metabolites of [H^3]retinol in PC-3 cells. We found that retinol treatment, with or without trichostatin A, increased both retinyl ester levels and retinoic acid levels in PC-3 human prostate cancer cells (Fig. 5, and data not shown). These data indicate that the HDAC inhibitor is not influencing retinol uptake or metabolism in the cells.
Figure 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.

Normalized expression of EZH2 in LNCaP and PC3 cells

Figure 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.
Figure 5. Analysis of the Metabolism of $[^3]$H$\text{retinol}$ in PC-3 Human Prostate Cancer Cells Cultured ± Retinol and ± Trichostatin A. PC-3 human prostate tumor cells were cultured in the presence of: (A) (top panel), 1 μM retinol for 24 hr.; (B) 1 μM RXR agonist for 24 hr.; (C) control; and (D) control. Then 100 nM $[^3]$H$\text{retinol}$ was added and the cells were cultured in this ± trichostatin A for 12 hr. Retinoids were isolated according to Guo et al. (16) and separated by HPLC. RA, 24.5 min; retinol 32-34.5 min.; retinyl esters, 50-60 min. These results have been reproduced, and only a sample of our experimental data is shown here.

Task 2. In task 2, we proposed to evaluate the efficacy of combined retinoid/histone deacetylase inhibitor administration in xenograft models (months 4 through 36). The goal of task 2 was 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 are isolated for assessment, and both differentiation specific and pro-apoptotic genes are measured by Northern and Western blotting.

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 (5). We have initiated a Phase I
clinical trial of 13-cis retinoic acid plus valproic acid for kidney, bladder, and prostate cancer patients, 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. However, we continued to encounter problems with the valproic acid injections. The animals became weak and lethargic shortly after each valproic acid injection. We continued to lose animals in the valproic acid group and animals in the valproic acid plus retinoic acid group.

We then tried a model with trichostatin A plus all-trans RA with a human kidney cancer cell line, since we also encountered problems with the prostate cancer LNCaP cells for the xenograft experiments. The results from these xenograft experiments, summarized here, are quite convincing and should be published soon. RA-resistant SK-RC-39 and SK-RC-45 cell lines were assessed to determine if there was increased growth inhibition with the combination of ATRA and low dose of TSA as compared to cells treated in culture with either drug alone. In these experiments, a very low dose of TSA (2 ng/ml) was used in order to study a concentration that could be achieved in vivo. This is 5-100 times lower than that used in many studies of HDAC inhibition by TSA (17-19). Both SK-RC-39 and SK-RC-45 exhibited much greater growth inhibition when treated with a combination of ATRA and TSA versus each drug alone (data not shown). SK-RC-39 growth was inhibited by the combination of ATRA and TSA compared to control (98%, p<0.001) and SK-RC-45 growth was similarly inhibited by the combination of ATRA and TSA compared to control (97%, p<0.001). Statistical analysis was performed using a one-way ANOVA test.

We next tested whether a similar additive growth inhibition would occur in a tumor xenograft model of RCC. The RA resistant line SK-RC-39 was chosen in order to more rigorously test the potential enhancement of growth inhibition with combination therapy. A limitation of retinoid therapy with oral free ATRA is that it induces its own metabolism and thus, it is difficult to maintain adequate drug levels over time (20). Thus, we used liposomal ATRA-IV. ATRA-IV has been shown to exhibit more favorable pharmacokinetics as compared to free ATRA (21). A low dose of TSA (0.76 µg/injection) was used in this animal study to prevent potential toxicity from long-term administration. We therefore used ATRA-IV in combination with TSA to assess the effects of this drug combination in the treatment of human renal cell carcinoma. Forty Swiss nu/nu mice were injected in the right flank subcutaneously with SK-RC-39 cells. Mice were treated in four cohorts of ten three times per week (Monday, Wednesday, and Friday) for a total of 8 weeks. Cohort 1 was treated with 1% EtOH and empty liposomes; cohort 2 was treated with ATRA-IV (0.16 µg/injection) and 1% EtOH; cohort 3 was treated with empty liposomes and TSA (0.76 µg/injection); and cohort 4 was treated with ATRA-IV (0.16 µg/injection) and TSA (0.76 µg/injection). At least seven animals remained in each cohort at the end of the eight-week study and were considered evaluable. The animals tolerated the treatment well and gained weight throughout the treatment course. Tumor growth in the cohort receiving ATRA-IV plus 1% EtOH control was not significantly different than that observed in the vehicle control group (Figure 2). Mice receiving only TSA had a 38% reduction in tumor growth compared to the control (p<0.05), whereas the combination of TSA plus ATRA-IV reduced tumor growth by 64% relative to the control (p<0.05). Statistical analysis was performed using RMANOVA. Histological analyses of the liver, lung, spleen, and kidneys by veterinary pathologists revealed no evidence of toxicity from either drug in these mice (data not shown).
Collectively, these results show that the combination of ATRA and TSA achieved enhanced tumor growth inhibition. We speculate that this therapy could be useful in treating human prostate cancer.

Figure 6: Growth of SK-RC-39 xenograft by treatment cohort. 5 x 10^6 SK-RC-39 cells were injected into the right flanks of Swiss nu/nu mice. Forty animals in four cohorts of ten were treated intravenously by tail vein injections three times per week for seven weeks as follows: vehicle control – empty liposomes plus 1% EtOH/PBS; ATRA-IV (0.16 μg/injection) plus 1% EtOH/PBS; TSA (0.76 μg/injection) plus empty liposomes; and ATRA-IV plus TSA at the same doses. Tumor area was measured with calipers and tumor volume was estimated using the formula: length x width x 1/2 the longest side.

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. Measurements of retinol metabolites in PC-3 prostate cancer cells cultured in a variety of conditions, with retinoids and with or without HDAC inhibitors.

F. Data from xenograft experiments involving another HDAC inhibitor, trichostatin A, after VPA proved to be problematic in the tumor xenograft experiments.
REPORTABLE OUTCOMES


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 the HDAC inhibitor 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 using SK-39 cells 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 have provided insights for future pharmacological therapies with retinoids and HDAC inhibitors for human prostate cancer treatment.
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Retinoic Acid and the Histone Deacetylase Inhibitor, Trichostatin A, Inhibit the Proliferation of Human Renal Cell Carcinoma in a Xenograft Tumor Model

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Abbreviations: ATRA: all-trans retinoic acid; ATRA-IV: liposome-encapsulated, intravenous form of ATRA; DAPI: 4′-6-diamidino-2-phenylindole dihydrochloride; EtOH: ethanol; FITC: fluorescein isothiocyanate; HAT: histone acetyltransferase; HDAC: histone deacetylase; LRAT: lecithin:retinol acyltransferase; PBS: phosphate buffered saline; PI: propidium iodide; repeated measures of analysis of variance: (RMANOVA); RA: retinoic acid; RAR: retinoic acid receptor; RARα: retinoic acid receptor alpha; RARβ: retinoic acid receptor beta; RARγ: retinoic acid receptor gamma; RCC: renal cell carcinoma; RXR: retinoid X receptor; TSA: trichostatin A

Running title: Retinoic acid and trichostatin A inhibit RCC proliferation

Key words: histone acetylation, kidney cancer, retinoic acid receptors, retinol, SK-RC-39, SK-RC-45, vitamin A
ABSTRACT

Purpose: Therapy for advanced renal cell carcinoma (RCC) is ineffective in the majority of patients. We have previously reported that retinoid-induced upregulation of retinoic acid receptor β (RARβ) correlated with anti-tumor effects in RCCs. Recent studies show that there is a reduction in the level of RARβ2 expression in cancer cells due in part to histone hypoacetylation. Therefore, we tested whether combining histone deacetylase (HDAC) inhibitors with retinoic acid (RA) would restore RARβ2 receptor expression, leading to increased growth inhibition in RCC cells.

Experimental Design: Cell proliferation, Western, and RT-PCR analyses of two RA-resistant RCC cell lines, SK-RC-39 and SK-RC-45, were assessed in the presence of all-trans retinoic acid (ATRA), trichostatin A (TSA), or the combination of ATRA and TSA. Analysis of apoptosis was also performed on SK-RC-39 cells treated with these combinations. Additionally, a xenograft tumor model (SK-RC-39) was utilized in this study to investigate the efficacy of a liposome-encapsulated, intravenous form of ATRA (ATRA-IV) plus TSA combination therapy.

Results: Enhanced inhibition of the proliferation of RCC cell lines and of tumor growth in a xenograft model was observed with the combination of ATRA plus TSA. Reactivation of RARβ2 mRNA expression was observed in SK-RC-39 and SK-RC-45 cells treated with TSA alone, or TSA in combination with ATRA. A partial G0/G1 arrest and increased apoptosis were observed with SK-RC-39 cells upon treatment with ATRA and TSA.
Conclusions: The combination of ATRA and the histone deacetylase inhibitor, TSA, elicits an additive inhibition of cell proliferation in RCC cell lines. These results indicate that ATRA and HDAC inhibitor therapies should be explored for the treatment of advanced RCC.
INTRODUCTION

Renal cell carcinoma (RCC) accounts for approximately 2-3% of the total new cases in the United States, with an estimated 31,900 new cases and 11,900 RCC-related deaths (1). Treatment of patients with advanced RCC is ineffective, highlighting the need for novel therapeutic approaches. More than 40% of patients diagnosed with metastatic disease are highly resistant to chemotherapy or radiotherapy (2). Immunotherapy with interferon-α or IL-2 is available; however, it is effective in only a small minority of patients (3). Therefore, it is necessary to develop more effective chemotherapeutic modalities for RCC.

Retinoids, retinol (vitamin A) and related metabolites such as retinoic acid (RA), serve as cancer chemopreventive and chemotherapeutic agents by regulating cell growth and differentiation (4). The actions of retinol and RA are primarily mediated by binding two different families of nuclear RA receptors, RARs and RXRs, each with α, β, and γ subtypes (5, 6). RARs and RXRs interact with transcriptional coactivator and corepressor complexes that determine their activation state, with coactivator complexes possessing histone acetyltransferase (HAT) activity, and corepressor complexes possessing histone deacetylase (HDAC) activity (5, 6). Pharmacological doses of RA are currently being used to treat several types of cancer and have been used in combination with IFN in the treatment of RCC, in addition to head and neck and squamous cell carcinomas of the skin (7-12).

Modifications of chromatin structure, such as histone acetylation, play a role in regulating gene expression by modulating chromatin structure (13, 14). Histone
acetylation activates transcription by creating a more open DNA conformation while histone deacetylation represses transcription by chromatin compaction (15). During tumorigenesis, histone hypoacetylation, due to the disruption of HAT and/or HDAC activity, results in the silencing of genes responsible for the regulation of cell growth, differentiation, and apoptosis (13). HDAC inhibitors have been developed to reverse gene silencing by inhibiting HDAC activity and increasing histone acetylation. These inhibitors function by binding to the enzyme's catalytic site. There are four distinct classes of HDAC inhibitors including short-chain fatty acids (valproic acid, butyrates), hydroxamic acids (trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA)), cyclic tetrapeptides (trapoxin, depsipeptide), and benzamides (MS-275) (13, 14). TSA was used for this study, as it is a potent and specific HDAC inhibitor active at nanomolar concentrations (16).

A reduction in RARβ2 expression has been observed in tumor cells relative to normal cells, resulting in part from aberrant histone acetylation and promoter methylation. A loss of cell growth inhibition and decreased expression of differentiation and retinoid-responsive genes are associated with a loss of RARβ (7). A recent, key molecular finding is that the repressive state of the RARβ2 gene in cultured, RA resistant breast cancer cells and other breast cancers can be reversed by TSA, with restoration of RA-induced RARβ2 transcription (17-19). Similar results were reported for prostate cancers (20). In cultured P19 cells, Minucci et al. showed that TSA plus ATRA enhanced cell differentiation when compared to ATRA alone (21). Combination treatments of RA plus HDAC inhibitors, tested in cell culture studies and in vivo using xenograft tumor mouse models, have been
successfully used to reduce solid tumor volume and induce differentiation and apoptosis in several types of cancer, including breast, prostate, and melanoma (7, 22-24).

Furthermore, in a clinical setting, one acute promyelocytic leukemia patient with resistance to ATRA exhibited a complete molecular remission in response to the HDAC inhibitor sodium phenylbutyrate (25). These studies suggest that therapies which include retinoids and HDAC inhibitors may have a greater therapeutic action than treatment with either drug alone.

In this report, one RA-resistant RCC cell line, SK-RC-39, and one partially RA-resistant RCC cell line, SK-RC-45, were used to investigate the efficacy of ATRA in combination with TSA. We report that there is enhanced growth inhibition of RCC cell lines in culture as well as in a tumor xenograft model with the combination of ATRA plus a low dose of TSA, indicating an additive effect between these two agents.
MATERIALS AND METHODS

Drugs: ATRA was obtained from Sigma Chemical Co. (St. Louis, MO). TSA was obtained from Wako Chemicals USA (Richmond, VA). Stocks of ATRA and TSA were prepared in 100% ethanol (EtOH). ATRA-IV (formerly Atragen®, Antigenics Inc., Lexington, MA) was reconstituted with 0.9% saline. Empty liposomes (Antigenics Inc.) were diluted with 0.9% saline at a concentration equivalent to the ATRA-IV preparation.

Cell Culture and Drug Treatments (for Western and RT-PCR analyses): The SK-RC-39 and SK-RC-45 RCC cell lines were derived as described previously (26). Cell lines were maintained in DMEM supplemented with 7% fetal calf serum and 1% penicillin/streptomycin at 37°C, 10% CO₂, and 95% humidity. SK-RC-39 and SK-RC-45 cells were seeded at a density of 0.5-1 x 10⁶ cells/100 mm plate and allowed to attach overnight. The cells were then treated for a range of 8-24 hours with combinations of ATRA (1 μM) and TSA (5-100 ng/ml).

Cell Proliferation Assays: SK-RC-39 and SK-RC-45 cells (1-5 x 10³/well) were cultured in media and treated with the following drugs (final concentrations indicated): vehicle (2 μl 100% EtOH/ml); 1 μM ATRA; 2 ng/ml TSA; 1 μM ATRA plus 2 ng/ml TSA; and 100 ng/ml TSA. Drugs were re-added and media was changed every 3 days. After seven days the cells in each well were trypsinized and counted in a Coulter Counter (Z1 Particle Counter, Coulter-Beckman Pharmaceuticals, Fullerton, CA).
Cell Cycle Analysis: SK-RC-39 cells (1.5 x 10^6 cells/dish) were plated in 100mm tissue culture dishes on day -1. Cells were either left treated for 24 hours or treated with combinations of ATRA (1 μM) and TSA (5-100 ng/ml). Up to 2 x 10^6 cells from each treatment condition were harvested and fixed in 100% ice cold EtOH, placed on ice for 15 minutes, or for longer term storage, kept at 4°C for no longer than 1 week. Cells were incubated in 250 μl of RNAse A (500 u/ml in 1.12% sodium citrate, Sigma, St. Louis, MO) at 37°C for 15 minutes. An equal volume of propidium iodide (PI, 50 μg/ml in 1.12% sodium citrate) was added to each sample, which was then incubated in the dark at room temperature for at least 1 hour. A Beckman-Coulter XL flow cytometry analyzer and FlowJo flow cytometry analysis software (Tree Star Software Inc.) were used to analyze the PI stained cells. Chris Colon, director of the Weill-Cornell flow cytometry core facility at Weill Medical College of Cornell University, provided training for both the Beckman-Coulter XL flow cytometry analyzer and FlowJo software.

Tumor Xenograft Model: Forty Swiss nu/nu mice (Taconic, Germantown, NY) were injected in the right flank with 5 x 10^6 SK-RC-39 cells after sedation with Metofane (Schering-Plough Animal Health, Madison, NJ). Mice were treated in four cohorts of ten. All mice received two tail vein injections, 0.08-0.1 ml per injection, three times per week (Monday, Wednesday, and Friday) for the length of the study. Cohort 1 was treated with 1% EtOH and empty liposomes; cohort 2 was treated with ATRA-IV (0.16 μg/injection, 0.1 ml of a 5 μM solution) and 1% EtOH; cohort 3 was treated with empty liposomes and TSA (0.76 μg/injection); and cohort 4 was treated with ATRA-IV (0.16 μg/injection) and TSA (0.76 μg/injection). The length and width of the tumors were measured three times
weekly. To estimate tumor volume, the product of the length and width was multiplied by one-half the length of the largest diameter. The animals were sacrificed at the end of the study.

**Western Analysis:** Whole SK-RC-39 cell extracts were lysed directly in denaturing SDS sample buffer by boiling, 10 μg protein/sample were 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. Hyperacetylation of histone H3 was detected using a 1:20,000 dilution of anti-acetyl-histone H3 polyclonal antibody (catalog # 06-599, Upstate, Lake Placid, NY). An actin polyclonal antibody (1:400-1:800 dilution, catalog # sc-1616; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. Primary antibody incubation was performed overnight at 4°C. After 1 hour incubation with an IgG horseradish peroxidase (HRP) conjugated secondary antibody at room temperature (anti-rabbit for acetylated histone H3, 1:20,000 dilution, catalog # sc-2054; anti-goat for actin, 1:10,000 dilution; catalog # sc-2020, 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.

**RNA Extraction and RT-PCR:** Total cellular RNA was isolated from cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) was used to carry out reverse transcription of 5 μg RNA. A 1:25 dilution of cDNA products was prepared and 5
μl of diluted cDNA was used for each individual PCR reaction. PCR primers for β-actin (sense primer 5'-ACC ATG GAT GAT GAT ATC G-3' and antisense primer 5'-ACA TGG CTG GGG TGT TGA AG-3' and RARβ2 (sense primer 5'-GAC TGT ATG GAT GTT CTG TCA G-3' and antisense primer 5'-ATT TGT CTC GGC AGA CGA AGC A-3') were designed by Sirchia et al (17). RARα and RARγ primers are as follows: RARα sense 5'-GTC TGT CAG GAC AAG TCC TCA GG-3'; RARα antisense 5'-GCT TTG CGC ACC TTC TCA ATG AG-3'; RARγ sense 5'-AAA TGA CAA GTC CTC TGG CTA CCA C; RARγ antisense 5'-CAG ATC CAG CTG CAC GCG GTG GTC-3'. All primer pairs were designed to span an intron in order to control for genomic DNA contamination. PCR reactions were run according to the following steps: 1) initial denaturation of template at 95°C for 5 minutes, 2) denaturation at 94°C for 30 sec, 3) annealing at 54°C for 30 sec, and 4) extension at 72°C for 45 seconds using the PTC-100™ thermal cycler (MJ Research, Inc.) for 23, 26, 30, and 32 cycles for β-actin, RARα, RARβ2, RARγ respectively. PCR products were resolved on 2% agarose gels and visualized by ethidium bromide staining.

Apoptosis Assays: SK-RC-39 cells were treated with EtOH, 2 μl/ml; 1 μM ATRA; 2 ng/ml TSA; 1 μM ATRA plus 2 ng/ml TSA; and 100 ng/ml TSA for 24 hours. Apoptosis was analyzed by annexin-V FITC (early apoptosis marker), propidium iodide (PI, late apoptosis/necrosis marker), and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI) staining (marker for DNA content and condensation). After drug treatment, cells were collected by trypsinization, pelleted, and fixed with 3% paraformaldehyde in PBS for 30 minutes on ice. Cells were stained for 15 min with annexin-V FITC (2.5 μg/ml) and PI (50
μg/ml) at room temperature in the dark. Following annexin-V FITC and PI staining, cells were stained with DAPI (1 μg/ml) for 30 min on ice in the dark and analyzed by fluorescence microscopy.

**Statistics:** Growth assays were analyzed using a one-way ANOVA after the data was normalized to account for differences between experiments. The tumor xenograft model was analyzed using repeated measures of analysis of variance (RMANOVA) to examine tumor size over time and across the different treatment arms. Tumor size, as calculated by the product of two diameters and the ratio of tumor size to the day one tumor size, was used for the RMANOVA. A p-value of <0.05 was considered statistically significant. Statistical analysis of the xenograft tumor data was performed by Dr. Martin Lesser, Clinical Research Methodology Core Facility, Weill Medical College of Cornell University.
RESULTS

Enhanced growth inhibition of human RCC in a xenograft model with the combination of ATRA and TSA. RA-resistant SK-RC-39 and SK-RC-45 cell lines were assessed to determine if there was increased growth inhibition with the combination of ATRA and low dose of TSA as compared to cells treated with either drug alone. In these experiments, a very low dose of TSA (2 ng/ml) was used in order to study a concentration that could be achieved in vivo. This is 5-100 times lower than that used in many studies of HDAC inhibition by TSA (27-29). Both SK-RC-39 and SK-RC-45 exhibited much greater growth inhibition when treated with a combination of ATRA and TSA versus each drug alone (Figure 1). SK-RC-39 growth was inhibited by the combination of ATRA and TSA compared to control (98%, p<0.001) and SK-RC-45 growth was similarly inhibited by the combination of ATRA and TSA compared to control (97%, p<0.001). Statistical analysis was performed using a one-way ANOVA test.

We next tested whether a similar additive growth inhibition would occur in a tumor xenograft model of RCC. The RA resistant line SK-RC-39 was chosen in order to more rigorously test the potential enhancement of growth inhibition with combination therapy. A limitation of retinoid therapy with oral free ATRA is that it induces its own metabolism and thus, it is difficult to maintain adequate drug levels over time (30). In this report we have used ATRA-IV. ATRA-IV has been shown to exhibit more favorable pharmacokinetics as compared to free ATRA (31). A low dose of TSA (0.76 µg/injection) was used in this animal study to prevent potential toxicity from long-term administration. We therefore used ATRA-IV in combination with TSA to assess the effects of this drug
combination in the treatment of human renal cell carcinoma. Forty Swiss nu/nu mice were injected in the right flank subcutaneously with SK-RC-39 cells. Mice were treated in four cohorts of ten three times per week (Monday, Wednesday, and Friday) for a total of 8 weeks. Cohort 1 was treated with 1% EtOH and empty liposomes; cohort 2 was treated with ATRA-IV (0.16 μg/injection) and 1% EtOH; cohort 3 was treated with empty liposomes and TSA (0.76 μg/injection); and cohort 4 was treated with ATRA-IV (0.16 μg/injection) and TSA (0.76 μg/injection). At least seven animals remained in each cohort at the end of the eight week study and were considered evaluable. The animals tolerated the treatment well and gained weight throughout the treatment course. Tumor growth in the cohort receiving ATRA-IV plus 1% EtOH control was not significantly different than that observed in the vehicle control group (Figure 2). Mice receiving only TSA had a 38% reduction in tumor growth compared to the control (p<0.05), whereas the combination of TSA plus ATRA-IV reduced tumor growth by 64% relative to the control (p<0.05).

Statistical analysis was performed using RMANOVA. Histological analyses of the liver, lung, spleen, and kidneys by veterinary pathologists revealed no evidence of toxicity from either drug in these mice (data not shown). Collectively, these results show that the combination of ATRA and TSA achieved enhanced tumor growth inhibition.

**TSA induces hyperacetylation of histone H3 in human RCC cell lines.** To investigate further the biological effects of ATRA and TSA, we confirmed the HDAC inhibitor function of TSA. SK-RC-39 and SK-RC-45 cells were treated with increasing doses of TSA (5-100 ng/ml) in the presence or absence of ATRA for 8-24 hours. Western analyses showed increased acetylation of histone H3 upon treatment with TSA as compared to
untreated controls (Figure 3). A 10-12 fold increase in acetylation can be seen as early as 8 hours with 50 and 100 ng/ml TSA treatment over control. HDAC inhibition with 50 ng/ml treatment diminishes over the 24 hour period, with a 5-7 fold increase in acetylation over control by 16 hours, and there are minimal differences between TSA treated and untreated samples by 24 hours. The 100 ng/ml TSA dose results in a consistent 10-12 fold increase in acetylation, as compared to untreated controls, at all three time points tested. The presence or absence of ATRA did not affect the overall degree of hyperacetylation of histone H3. These results indicate that, as expected, the presence of ATRA did not enhance the acetylation of histone H3 by the HDAC inhibitor TSA.

**Treatment with the combination of ATRA and TSA reactivates the expression of RARβ2.** Recent reports in the literature have identified the inactivation of RARβ2 expression in many types of cancer including breast, prostate, lung, gastric, and cervical cancers (22, 32-35). Decreased expression of RARβ2 has been linked to aberrant epigenetic modifications, such as histone hypoacetylation and promoter methylation. Reactivation of RARβ2 expression can be achieved by the administration of transcription modulating drugs, including HDAC inhibitors and demethylating agents.

Since RA-resistance in these RCC cell lines correlates with the lack of RARβ2 mRNA, we examined the effects of combination of ATRA and TSA on RARβ2 expression (36). Cells were treated in a manner similar to that described for Western analysis, and the levels of RARα, RARβ2, RARγ, and β-actin mRNAs were analyzed using semiquantitative RT-PCR. ATRA alone did not result in an increase in RARβ2 mRNA (Figure 3). A low dose of TSA (5 ng/ml) alone, or in combination with ATRA, was not capable of activating
RARβ2 expression. High doses of TSA alone (50-100 ng/ml), and in combination with ATRA, were capable of inducing RARβ2 mRNA expression as early as 8 hours after treatment. The pattern of RARβ2 induction at each dose of TSA is similar to the pattern of histone H3 hyperacetylation, suggesting that the degree of histone hyperacetylation as a result of the TSA treatment affects the degree of RARβ2 reactivation in SK-RC-39 and SK-RC-45 cells (Figure 3). The specificity of RARβ2 reactivation was confirmed by the fact that the levels of RARα and RARγ mRNA did not change with ATRA, TSA, or the combination of ATRA and TSA (Figure 3).

Enhanced apoptotic response and changes in cell cycle distribution upon treatment of SK-RC-39 cells with ATRA and TSA. RARβ2 controls cell proliferation via several mechanisms, including the induction of growth arrest and apoptosis (37-40). The following experiments were designed to address the effects of short-term treatment with ATRA and TSA on cell cycle distribution and apoptosis.

Cell cycle analysis was performed using SK-RC-39 cells treated for 24 hours with combinations of ATRA and TSA (Figure 4). These experiments were performed only with SK-RC-39 cells since similar Western and RT-PCR results were obtained for SK-RC-39 and SK-RC-45 and SK-RC-39 cells were used in the xenograft study. There were no differences in cell cycle distribution between the control and ATRA treated cells at 24 hours. Cells treated with lower doses of TSA (5-25 ng/ml) exhibited a dose dependent accumulation in G0/G1, with a 4-14% percent difference over untreated control and ATRA treated cells. There was also a decrease of up to 12% of cells in S phase that were treated with 5-25 ng/ml TSA. There was a high level of toxicity associated with the 50 and 100
ng/ml doses of TSA, as indicated by a distinct subG₀ population (Figure 4B). The subG₀ population includes necrotic and apoptotic cells, in addition to cell debris.

Based on the results from the growth assay, cell cycle analysis was also performed on SK-RC-39 cells treated for 6 days with 1 μM ATRA, 2 ng/ml TSA, or in combination to determine if the enhanced growth inhibition observed with the combination could be attributed to differences in cell cycle distribution (data not shown). There were no differences in cell cycle distribution among the control and ATRA treated cells. Similar to the 24 hour treatment, there was a partial G₀/G₁ arrest, with a 6% increase in TSA and ATRA plus TSA treated cells over untreated control and ATRA treated cells. The percentage of TSA and ATRA plus TSA treated cells in S phase decreased by 5-6% percent as compared to untreated control and ATRA treated cells.

This fluorometric cell cycle distribution assay is not a measure of apoptosis so in order to monitor apoptotic events, SK-RC-39 cells treated for 24 hours with combinations of ATRA and TSA were also stained with annexin V-FITC, PI, and DAPI (Figure 5). Annexin-V FITC is a marker for early apoptosis, while propidium iodide is a marker for late apoptosis and necrosis. DAPI stains for DNA content and condensation. When comparing the combination of ATRA and low dose TSA versus each drug alone, the combination treatment displayed the greatest degree of apoptosis, as indicated by annexin V (green) staining. Cells treated with high dose TSA (100 ng/ml) were more necrotic than apoptotic, as indicated by increased PI (red) staining, reflecting dose toxicity.

DISCUSSION
These data show enhanced inhibition of proliferation of human RCC cell lines in a xenograft model by combination therapy with ATRA and the HDAC inhibitor TSA. RCC is a particularly interesting target in which to study this combination as we have documented defects in the retinoid pathway in RCC (36). Levels of LRAT (lecithin:retinol acyltransferase), the primary enzyme responsible for the metabolism of retinol to retinyl esters (the storage form of retinol), are reduced in RCC, and in many other carcinomas, including oral cavity, skin, breast, bladder and prostate (41-45). While pharmacological doses of retinoids have been shown to be useful in cancer chemoprevention, retinoid responsiveness is often lost and retinoid metabolism becomes abnormal during the process of carcinogenesis. The administration of HDAC inhibitors, such as TSA, in addition to retinoids shows promise in reversing the retinoid chemoresistance that these RCC cells exhibit.

Proliferation inhibition assays revealed that increased inhibition could be achieved using combination therapy with ATRA and TSA in the SK-RC-39 and SK-RC-45 RCC cell lines (Figure 1). We confirmed and extended the growth inhibition assays in a more clinically relevant model, a Swiss nu/nu mouse tumor xenograft model (Figure 2). One mechanism by which TSA appears to exert its growth inhibitory effects is through the regulation of cell cycle progression and apoptosis (Figures 4 and 5). Treatment of certain carcinomas with TSA was shown to upregulate cell cycle regulators, including p21 and cyclin A, and to reduce the expression of phosphorylated retinoblastoma protein (Rb) and Id1, an inhibitor of cell differentiation (46-48). In addition, treatment of hepatocellular carcinoma with TSA resulted in an induction of (pro)-caspase 3 and bax expression, and an inhibition of bcl-2 expression upon TSA treatment (47).
Our data indicate that the positive therapeutic activity of the two drugs ATRA and TSA (Figure 1) does not occur via a direct effect of ATRA on the acetylation of histones, since no differences in histone H3 acetylation were seen in cells treated with TSA alone as compared to the combination (Figure 3). A more likely mechanism for the observed positive therapeutic activity of the drug combination is enhanced signaling of the retinoid pathway. It has been previously shown that increases in RARβ2 expression in RCC are correlated with growth inhibition by retinoid treatment (36). Restoring RARβ2 expression may result in increased susceptibility to growth inhibition, differentiation, and/or apoptosis, since RARβ2 itself governs the expression of differentiation and retinoid-responsive genes (37, 38). The restoration of RARβ2 expression most likely is achieved by TSA inhibition of the HDAC activity associated with nuclear co-repressor complexes, thereby enhancing retinoid regulated transcriptional activation. In this study, the enhanced growth inhibition observed with the drug combination (Figure 1) is not directly correlated with early (within 24 hours) RARβ2 expression, as the reactivation of RARβ2 mRNA expression was not observed at low doses of TSA plus ATRA (Figure 3). However, the growth inhibition was measured at 7 days (Figure 1) and we did not examine RARβ2 mRNA levels at this later timepoint. The enhanced growth inhibition observed with the combination of ATRA and TSA could also result from modifications in the regulation of other retinoid-responsive target genes, such as LRAT, that regulate retinoid signaling and metabolism.

Other biological effects were elicited by these two drugs in combination. Apoptosis was predominant in the low dose TSA and TSA + ATRA treated cells at 24 hours (Figure 5). Moreover, there was an increasing percentage of cells in G0/G1 upon
treatment with increasing doses of TSA (Figure 4). This result was independent of ATRA treatment. These responses are consistent with the ability of the HDAC inhibitor TSA to alter the expression of cell cycle regulators as well as apoptotic factors (46-48). Additionally, it has been shown in HT-29 colon carcinoma that there is a differential response to transient and prolonged histone hyperacetylation upon sodium butyrate and TSA treatment (49). Short-term treatment (<8 hours) with these HDAC inhibitors induced p21 expression and cell growth arrest, while prolonged dosing (>24 hours) activated additional programs of growth inhibition including differentiation, apoptosis, and growth factor unresponsiveness. Although these studies were performed with HDAC inhibitors alone, it would be expected that these effects would be enhanced with ATRA, based on the data presented in this report.

The promising results reported here suggest that further investigation of the mechanisms underlying the growth inhibition observed in these cell lines following TSA plus ATRA treatment is warranted. One advantage of TSA lies in its dynamic nature, in that TSA functions both independently, as well as in concert with ATRA, to yield various cellular responses. This study utilized ATRA-IV in the xenograft model, instead of ATRA, and TSA as a potent and specific HDAC inhibitor (Figure 2). Since aberrant retinoid signaling and retinoid deficiency are well documented in RCC, achieving increased levels of retinoids in the serum can occur with the use of ATRA-IV. Although TSA is not currently used in humans, this study indicates that the use of retinoids and other HDAC inhibitors, such as SAHA or depsipeptide, to potentiate tumor growth inhibition is an attractive future chemotherapeutic modality for patients with RCC.
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FIGURE LEGENDS

Figure 1: Growth assays. Cell lines were treated with vehicle (2 μl/ml EtOH); RA, 1 μM; TSA, 2 ng/ml; and RA (1 μM) plus TSA (2 ng/ml) for seven days. Each growth experiment was performed at least three times.

Figure 2: Growth of SK-RC-39 xenograft by treatment cohort. 5 x 10⁶ SK-RC-39 cells were injected into the right flanks of Swiss nu/nu mice. Forty animals in four cohorts of ten were treated intravenously by tail vein injections three times per week for seven weeks as follows: vehicle control – empty liposomes plus 1% EtOH/PBS; ATRA-IV (0.16 μg/injection) plus 1% EtOH/PBS; TSA (0.76 μg/injection) plus empty liposomes; and ATRA-IV plus TSA at the same doses. Tumor area was measured with calipers and tumor volume was estimated using the formula: length x width x 1/2 the longest side.

Figure 3: Molecular Effects of ATRA and TSA Treatment. 5 x 10⁵ SK-RC-39 or SK-RC-45 cells were treated for 8-24 hours with combinations of ATRA (1 μM) and TSA (5-100 ng/ml) or left untreated as a control. This experiment was performed in duplicate with representative results shown here. A, Histone acetylation in SK-RC-39 and SK-RC-45 cells. Whole cell extracts (10 μg/lane) were separated on a 15% SDS-PAGE gel followed by Western blot analysis. Acetylation of histone H3 was detected with an antibody specific for anti-acetyl-histone H3 (1:20,000). Immunoblot analysis of actin (1:800) served as a loading control. B, RT-PCR analysis. Total cellular RNA was isolated and reverse transcription was performed on 5 μg RNA. 5 μl of cDNA products (1:25 dilution) was
used for each PCR reaction. The expression of RARα, RARβ2, RARγ, and β-actin was analyzed along with a negative PCR control without cDNA template.

**Figure 4: Cell cycle analysis.** SK-RC-39 cells were treated for 24 hours with combinations of ATRA (1 μM) and TSA (5-100 ng/ml) or left untreated as a control. Cell cycle kinetics were evaluated using flow cytometry analysis. This experiment was repeated three times, with results that were very similar. A, Cell cycle distribution, percentage ± SD. Percentages do not add up to 100% since this quantitative analysis includes only non-apoptotic cells with 2N to 4N genetic material. B, Representative histograms of each treatment condition.

**Figure 5: Annexin-V FITC Detection of Apoptosis.** Fluorescence microscopic analysis of annexin-V FITC (green), propidium iodide (red) and DAPI (blue) staining of SK-RC-39 cells after a 24 hour treatment. A-C, vehicle control (EtOH); D-F, 1 μM RA; G-I, 2 ng/ml TSA; J-L, 1 μM RA + 2 ng/ml TSA; and M-O, 100 ng/ml TSA. C, F, I, L, and O represent the merged images of each respective treatment condition. This experiment was repeated twice with very similar results.
Figure 1
Figure 2

- Empty Liposomes + EtOH
- ATRA-IV + EtOH
- Empty Liposomes + TSA
- ATRA-IV + TSA

Tumor Volume (mm$^3$)

Weeks of Treatment
Figure 4

![Graph showing DNA content and number of cells under different conditions](image)
Figure 5