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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Abstract

Purpose: Therapy for advanced renal cell carcinoma (RCC) is ineffective in the majority of patients. We have previously reported that retinoid-induced up-regulation of retinoic acid receptor β (RARβ) correlated with antitumor 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 inhibitors with retinoic acid (RA) would restore RARβ2 receptor expression, leading to increased growth inhibition in RCC cells.

Experimental Design: Cell proliferation, Western blot, and reverse transcription-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 done on SK-RC-39 cells treated with these combinations. Additionally, a xenograft tumor model (SK-RC-39) was used in this study to investigate the efficacy of a liposome-encapsulated, i.v. 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 on 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 histone deacetylase inhibitor therapies should be explored for the treatment of advanced RCC.

Renal cell carcinoma (RCC) accounts for ~2% to 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 (IFN-α) or interleukin–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, retinoic acid receptors (RAR) and retinoid X receptors, each with α, β, and γ subtypes (5, 6). RARs and retinoid X receptors interact with transcriptional coactivator and corepressor complexes that determine their activation state, with coactivator complexes possessing histone acetyltransferase activity and corepressor complexes possessing histone deacetylase (HDAC) activity (5, 6). Pharmacologic 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 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 whereas histone deacetylation represses transcription by chromatin compaction (15). During tumorigenesis, histone hypoacetylation, due to the disruption of histone acetyltransferase 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 catalytic site of the enzyme. There are four distinct classes of HDAC inhibitors, including short-chain fatty acids (valproic acid and butyric acid), hydroxamic acids [trichostatin A (TSA) and suberoylanilide hydroxamic acid], cyclic tetrapeptides (trapoxin and depsipeptide), and benzamides (MS-275; refs. 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 retinoic-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. (21) showed that TSA plus all-trans retinoic acid (ATRA) enhanced cell differentiation when compared with ATRA alone. 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 that 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. ATRA-IV (formerly Attagen, Antigeneics Inc., Lexington, MA) was reconstituted with 0.9% saline. Empty liposomes (Antigeneics) were diluted with 0.9% saline at a concentration equivalent to the ATRA-IV preparation.

Cell culture and drug treatments (for Western blot and reverse transcription-PCR analyses). The SK-RC-39 and SK-RC-45 RCC cell lines were derived as previously described (26). Cell lines were maintained in DMEM supplemented with 7% FCS 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 × 10⁵ to 1 × 10⁵ cells/100 mm plate and allowed to attach overnight. The cells were then harvested and fixed in 100% ice-cold ethanol, 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 units/mL in 1.12% sodium citrate; Sigma) at 37°C for 15 minutes. An equal volume of propidium iodide (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., Ashland, OR) were used to analyze the propidium iodide-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 × 10⁶ SK-RC-39 cells after sedation with Metofane (Scherer-Plough Animal Health, Madison, WI). Mice were treated in four cohorts of ten. All mice received two tail vein injections, 0.08 to 0.1 mL per injection, 3 times per week (Monday, Wednesday, and Friday) for the length of the study. Cohort 1 was treated with 1% ethanol and empty liposomes; cohort 2 was treated with ATRA-IV (0.16 μg/30 μL, 0.1 mL of a 5 μM solution) and 1% ethanol; cohort 3 was treated with empty liposomes and TSA (0.76 μg/30 μL); and cohort 4 was treated with ATRA-IV (0.16 μg/30 μL) and TSA (0.76 μg/30 μL). The length and width of the tumors were measured 3 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 blot analysis. Whole SK-RC-39 cell extracts were lysed directly in denaturing SDS sample buffer by boiling, and 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) to confirm proper transfer and equal loading. Hyper-acetylation of histone H3 was detected using a 1:20,000 dilution of anti-acetyl-histone H3 polyclonal antibody (Upstate, Lake Placid, NY). An actin polyclonal antibody (1:400:1,800 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. Primary antibody incubation was done overnight at 4°C. After 1-hour incubation with an immunoglobulin G horseradish peroxidase–conjugated secondary antibody at room temperature (anti-rabbit for acetylated histone H3, 1:20,000 dilution; anti-goat for actin, 1:10,000 dilution; Santa Cruz Biotechnology), 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% BSA (Santa Cruz Biotechnology) and 0.1% Tween 20.

RNA extraction and reverse transcription-PCR. Total cellular RNA was isolated from cultured cells using Trizol (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. SuperScript III reverse transcriptase (Invitrogen) 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 were 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 CTC GGT GGT TGA AG-3′) and RARβ2 (sense primer 5′-GAC TGT AGT GAT GTT GTC TCA G-3′ and antisense primer 5′-ATG TGG CTC GGG ACA CAG AC-3′) were designed by Sirchia et al. (17). RARs and RARβ primers are as follows: RARα sense 5′-GTC TGT CAG GAC AAG TCC TCA GG-3′; RARβ antisense 5′-GCT TGG CAC TCC TCA TCA ATG AG-3′; RARγ sense 5′-AAA TGA CAA GTT
and RAR antisense 5' - CAG ATC GAG CTG CAC GCG GTG GTC-3'. All primer pairs were designed to span an intra- 
control for genomic DNA contamination. PCR reactions were run 
accompanying the following steps: (a) initial denaturation of template at 
95°C for 5 minutes, (b) denaturation at 94°C for 30 seconds, (c) 
average for 30 minutes, and (d) extension at 72°C for 
45 seconds. After drug treatment, cells were 
trypsinization, pelleted, and fixed with 3% 
paraformaldehyde in PBS for 30 minutes on ice. Cells were stained 
with annexin V-FITC (early 
and 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining 
(marker for DNA content and condensation). After drug treatment, cells 
cells were captured by trypsinization, pelleted, and fixed with 3% 
paroformaldehyde in PBS for 30 minutes on ice. Cells were stained 
with annexin V-FITC (2.5 μg/mL) and propidium iodide 
(50 μg/mL) at room temperature in the dark. Following annexin V-FITC 
and propidium iodide staining, cells were stained with DAPI (1 μg/mL) 
for 30 minutes on ice in the dark and analyzed by fluorescence 
microscopy. 

Statistics. Growth assays were analyzed using a one-way ANOVA 
after the data were normalized to account for differences between 
experiments. The tumor xenograft model was analyzed using repeated 
measures of ANOVA 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 1 tumor size, was 
used for the repeated measures of ANOVA. A P value of <0.05 was 
considered statistically significant. Statistical analysis of the xenograft 
tumor data was done by Dr. Martin Lesser.

Results

Enhanced growth inhibition of human renal cell carcinoma in a 
exenograft model with the combination of all-trans retinoic acid and 
trichostatin A. RA-resistant SK-RC-39 and SK-RC-45 cell 
lines were assessed to determine if there was increased growth 
hindrance with the combination of ATRA and low dose of TSA 
as compared with cells treated with either drug alone. In these 
experiments, a very low dose of TSA (2 ng/mL) was used to 
study a concentration that could be achieved in vivo. This is 5 to 
100 times lower than that used in many studies of HDAC 
hindrance by TSA (27–29). Statistical analysis was done using 
a one-way ANOVA test. 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) (Fig. 1). In summary, both SK-RC-39 and SK-RC-45 
exhibited much greater growth hindrance when treated with 
a combination of ATRA and low dose of TSA versus either 
drug alone.

We next tested whether a similar additive growth hindrance 
would occur in a tumor xenograft model of RCC. The RA-
resistant line SK-RC-39 was chosen to more rigorously test 
the potential enhancement of growth hindrance 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 with 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 
RCC. Forty Swiss nu/nu mice were injected in the right flank 
s.c. with SK-RC-39 cells. Mice were treated in four cohorts of 
ten 3 times per week (Monday, Wednesday, and Friday) for a 
total of 8 weeks. Cohort 1 was treated with 1% ethanol and 
empty liposomes; cohort 2 was treated with ATRA-IV (0.16 
μg/injection) and 1% ethanol; 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 8-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% ethanol 
control was not significantly different than that observed in 
the vehicle control group (Fig. 2). Mice receiving only TSA 
had a 38% reduction in tumor growth compared with 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 done using 
repeated measures of ANOVA. Histologic 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.

Trichostatin A induces hyperacetylation of histone H3 in human 
renal cell carcinoma cell lines. To investigate further the 
biological effects of ATRA and TSA, we confirmed the HDAC 
hindr worsening 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 to 24 hours. Western blot 
analyses showed increased acetylation of histone H3 on 
treatment with TSA as compared with untreated controls 
(Fig. 3). A 10- to 12-fold increase in acetylation can be seen 
as early as 8 hours with 50 and 100 ng/mL TSA treatments over 
control. HDAC inhibition with 50 ng/mL treatment diminishes 
over the 24-hour period, with a 5- to 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- 
to 12-fold increase in acetylation, as compared with 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 all-trans retinoic acid and trichostatin A reactivates the expression of retinoic acid receptor β2.** 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. Because 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 blot analysis, and the levels of RARα, RARβ2, RARγ, and β-actin mRNAs were analyzed using semiquantitative reverse transcription-PCR (RT-PCR). ATRA alone did not result in an increase in RARβ2 mRNA (Fig. 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 (Fig. 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 (Fig. 3).

**Enhanced apoptotic response and changes in cell cycle distribution on treatment of SK-RC-39 cells with all-trans retinoic acid and trichostatin A.** 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 done using SK-RC-39 cells treated for 24 hours with combinations of ATRA and TSA (Fig. 4). These experiments were done only with SK-RC-39 cells because similar Western blot 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% to 14% 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 to 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 sub-G0 population (Fig. 4B). The sub-G0 population includes necrotic and apoptotic cells, in addition to cell debris. Based on the results from the growth assay, cell cycle analysis was also done 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% to 6% as compared with untreated control and ATRA-treated cells.

This fluorometric cell cycle distribution assay is not a measure of apoptosis; so 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, propidium iodide, and DAPI (Fig. 5). Annexin V-FITC is a marker for early apoptosis, whereas 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 propidium iodide (red) staining, reflecting dose toxicity.
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 lecithin:retinol acyltransferase (LRAT), 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). Whereas pharmacologic 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 (Fig. 1). We confirmed and extended the growth inhibition assays in a more clinically relevant model, a Swiss nu/nu mouse tumor xenograft model (Fig. 2). One mechanism by which TSA seems to exert its growth inhibitory effects is through the regulation of cell cycle progression and apoptosis (Figs. 4 and 5). Treatment of certain carcinomas with TSA was shown to up-regulate cell cycle regulators, including p21 and cyclin A, and to reduce the expression of phosphorylated retinoblastoma protein 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 (47).

Our data indicate that the positive therapeutic activity of the two drugs, ATRA and TSA (Fig. 1), does not occur via a direct effect of ATRA on the acetylation of histones because no difference in histone H3 acetylation was seen in cells treated with TSA alone as compared with the combination (Fig. 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 because 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 corepressor complexes, thereby enhancing retinoid regulated transcriptional activation. In this study, the enhanced growth inhibition observed with the drug combination (Fig. 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 (Fig. 3). However, the growth inhibition was measured at 7 days (Fig. 1) and we did not examine RARβ2 mRNA levels at this later time point. 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 combination.
Fig. 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 was evaluated using flow cytometry analysis. This experiment was repeated 3 times, with results that were very similar. A, cell cycle distribution, percentage ± SD. Percentages do not add up to 100% because this quantitative analysis includes only nonapoptotic cells with 2N to 4N genetic material. B, representative histograms of each treatment condition.
TSA- and TSA + ATRA–treated cells at 24 hours (Fig. 5). Moreover, there was an increasing percentage of cells in G_0-G_1 on treatment with increasing doses of TSA (Fig. 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

Fig. 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 (ethanol); D-F, 1 μM ATRA; G-I, 2 ng/mL TSA; J-L, 1 μM ATRA + 2 ng/mL TSA; and M-O, 100 ng/mL TSA. C, F, I, L, and O, merged images of each respective treatment condition. This experiment was repeated twice with very similar results.
and prolonged histone hyperacetylation on sodium butyrate and TSA treatment (49). Short-term treatment (<8 hours) with these HDAC inhibitors induced p21 expression and cell growth arrest, whereas prolonged dosing (>24 hours) activated additional programs of growth inhibition including differentiation, apoptosis, and growth factor unresponsiveness. Although these studies were done 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 used ATRA-IV in the xenograft model, instead of ATRA, and TSA as a potent and specific HDAC inhibitor (Fig. 2). Because 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 suberoylanilide hydroxamic acid or depsipeptide, to potentiate tumor growth inhibition is an attractive future chemotherapeutic modality for RCC.

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References


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