Comparing the effect of ATRA, 4-HPR, and CD437 in bladder cancer cells

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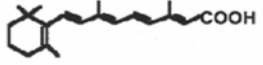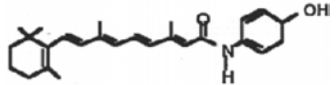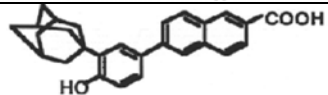
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1. ABSTRACT

Clinical trials have explored the use of natural and synthetic retinoids for the prevention of bladder cancer recurrence. Natural retinoids have been shown to inhibit bladder cancer growth. Here, we compared the effects of natural and synthetic retinoids in bladder cancer cells. Bladder cancer cell lines were treated with all-trans-retinoid acid (ATRA), N-4-hydroxyphenyl-retinamide (4-HPR) and 6-[3-(1-adamantyl)-4 hydroxyphenyl]-2-naphthalene carboxylic acid (CD437). Their effects on cell growth, apoptosis, cell cycle, gene expression, and retinoid acid receptors (RARs) and the JWA-retinoid response gene were assessed. Most of the bladder cancer cells were resistant to ATRA (1 and 10 μM). 4-HPR inhibited cell growth by 90% at 10 μM; however, CD437 showed the same effect at 1 μM. 4-HPR and CD437 increased G1 and decreased S phase. The three retinoids differentially affected p53, RARs, and JWA. Only CD437 increased Caspase 3 expression. The results demonstrated that 4-HPR and CD437 were more potent growth inhibitors and apoptosis inducers than ATRA. However, 4-HPR was effective at a concentration at least 10 μM. The *in vitro* results suggested the higher dose of 4-HPR in chemoprevention trial be considered.

2. INTRODUCTION

Retinoids are a group of compounds that regulate cell growth and differentiation. In a clinical trial (1), a synthetic retinoid has been shown to reduce bladder cancer recurrence with low toxicity generating increasing attention towards the use of synthetic retinoids in bladder cancer chemoprevention and treatment (2-3). N-(4-hydroxyphenyl) retinamide (4-HPR), a synthetic amide of retinoic acid, is being used in a clinical trial conducted by the Southwestern Oncology Group and the University of Texas MD Anderson Cancer Center, to prevent and treat recurrent superficial bladder cancer. However, the result is inclusive. 4-HPR has been shown to inhibit carcinoma formation in rodents in the mammary gland, lung, prostate, bladder, and liver, and is less toxic than other retinoids (3-4), making it an excellent candidate for cancer chemoprevention. Several clinical trials have used 4-HPR to prevent the recurrence of head and neck cancer, breast cancer, prostate cancer, and bladder cancer in man (5-8). CD437 is a selective synthetic retinoid and strong apoptosis inducer (9), and has been investigated in many different cancer types. CD437 has an anti-proliferative effect in breast cancer, lung cancer, ovarian cancer, leukemia, and melanoma (10-14). Although it has been reported that

Comparing the effect of ATRA with 4-HPR and CD437 in bladder cancer cells

Table 1. Retinoid structure

Retinoids	Structure
All-trans retinoic acid (ATRA)	
N-4-hydroxyphenyl-retinamide (4-HPR)	
6-[3-(1-adamantyl)-4 hydroxyphenyl]-2-naphthalene carboxylic acid (CD437)	

CD437 transactivates both RARβ- and RARγ- selective ligand when used at 1 μM (10-16), apoptosis induction by CD437 through RARs is controversial (14-17). Reports demonstrate that CD437 not only induced RAR-γ-dependent differentiation in contrast to ATRA but also induced RAR-γ-independent apoptosis (14-17), suggesting that this new retinoid is worthy of attention.

Nuclear retinoid receptors are the proximate mediators of many of the effects of retinoids on gene expression. Two types of receptors have been identified: retinoic acid receptors (RARs) and retinoid X receptors (RXRs). The RARs bind to ATRA and 9-cis-retinoic acid (9cRA), a natural retinoic acid isomer, which binds to both RARs and RXRs (18-22). RARs can form heterodimers with RXRs and bind to retinoic acid response elements, specific DNA sequences that are characterized by direct repeats of (A/G)GGTCA separated by two or five nucleotides that act as ligand-dependent transcriptional regulators for retinoic acid-responsive genes (21, 22).

JWA is a novel putative cytoskeleton associate and retinoic acid response gene first isolated from human tracheal bronchial epithelial cells by Zhou *et al* (23). Although several JWA homologue genes have been identified, little is known about the critical biological function of the gene. In previous studies (23-25), a JWA homologue gene was identified from rat tracheal epithelial cells. It was found that four nucleotides in its coding regional sequence are different from that in man. A promoter fragment of the gene (621-base pairs) was cloned from human samples and a complete TPA (phorbol-12-myristate-13-acetate) responsive element (TRE), (TGACTCA) was identified. Several *in vitro* cell culture models have been initiated to study the structure and function of this gene (24, 25).

We compared the natural retinoid ATRA with two synthetic retinoids, 4-HPR and CD437, in growth and apoptosis induction, cell cycle analysis, and cell cycle checkpoint gene expression by real time Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR), and Western blot. We also compared the effect of these retinoids on JWA expression.

3. METHODS

3.1. Bladder cancer cell lines and retinoids
The characterization of the human bladder cancer cell lines has been reported (26). Cells were grown in a 1:1

(v/v) mixture of Dulbecco's modified Eagle's minimal essential medium and Ham's F12 with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

ATRA were purchased from Sigma (St. Louis, MO). 4-HPR, a synthetic retinoid, was obtained from the Cancer Prevention Branch at the National Cancer Institute. CD437 was obtained from Dr. Reuben Lotan, MD Anderson Cancer Center. The structure of the three retinoids is shown in Table 1. The retinoids were dissolved in 0.1 mM dimethylsulfoxide as stock solutions and stored in an atmosphere of N_2 at −80°C.

3.2. Effects of retinoids on cell proliferation in monolayer cultures
Cells were plated in 96-well plates at a concentration of 10^4 cells/well and grown for 24 h. The cells were then incubated with 1 μM, or 10 μM of ATRA, 4-HPR or CD437 for 5 days. Control cultures received the same amount of DMSO as did the treated cultures. Growth inhibition was determined using the crystal violet method as described previously (26). Briefly, after 5 days of treatment, cells were fixed by 5% glutaraldehyde in phosphate-buffered saline (PBS), rinsed with distilled water, and dried completely. Cells were incubated in a 1:1 (v/v) mixture of 200 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 9.5) and 0.2% crystal violet at 25°C for 30 min, and then were washed and dried. The fixed and stained cells were solubilized with 10% glacial acetic acid, and the absorbance at A590 nm was determined using a plate reader. Growth inhibition was calculated according to the equation: inhibition = (1-Nt/Nc) x 100, where Nt and Nc are the numbers of cells in treated and control cultures, respectively. All experiments were performed in triplicate and the mean ± standard deviations were calculated.

3.3. Cell cycle analysis by propidium iodide (PI) staining
Cells were treated with 1, 5 and 10 μM ATRA and 4-HPR or 1 μM CD437 for 2 days. Cells were harvested by trypsinization, fixed in 4% paraformaldehyde pH 7.4, and washed and then incubated in 70% ethanol containing 1% HCl at -20°C for 10 min. Cells were stained with 500 μl of propidium iodide/RNase A solution in the dark for 30 min at room temperature. Cells were then analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA) with a 15 mW

Argon laser used for excitation at 488 nm. Fluorescence was measured at 585/42 nm. Computer analysis of the data using BD Biosciences Cellquest Pro, ModFit LT by Verity Software provided information on the proportion of cells in G_1, S, and G_2 phases of the cell cycle.

3.4. Analysis of apoptosis induced by ATRA, 4HPR or CD437

In-situ apoptosis staining. Cells were treated with 10 µM ATRA, 4HPR or 1 µM CD437 for 2 days. Following this treatment, the attached and floating cells were collected. Cytospins were prepared, fixed in 10% formalin for 10 min at room temperature, and kept at -20°C until use. Slides were washed in distilled water with 0.5% triton X-100 for 10 min 2-3 times, and then incubated with 20 µg/ml Proteinase K for 15 min at 37°C. The slides were washed again and incubated with 1 X TDT (Terminal Deoxynucleotidyl Transferase) buffer (Gibco/BRL) for 15 min at room temperature, and incubated with 0.3 µg/µl TDT plus biotinylated dUTP 20mM in TDT buffer (Gibco/BRL) for 1 hr at 37°C. The slides were then washed and stained with Avidin-Biotin-peroxidase Complex Vectastain- (ABC kit).

DNA ladder formation (26). Cells were incubated for 3 days in medium supplemented with 10 µM of ATRA, 4HPR, or 1 µM CD437. The cells were resuspended in Tris-EDTA buffer, pH 8.0 and lysed in 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.5% Triton X-100 on ice for 15 min. The lysates were centrifuged at 12,000 g for 15 min to separate soluble (fragmented) from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 µg/mL) at 37°C for 1 hr, followed by treatment with proteinase K (100 µg/mL) in 0.5% SDS, at 50°C for 2 hrs. The residual material was extracted with phenol/chloroform, precipitated in ethanol, electrophoresed in a 1.8% agarose gel, stained with ethidium bromide, and examined for DNA ladder formation by observation under UV light.

Terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-deoxyuridine-triphosphate (dUTP) nick-end labeling (TUNEL) assay (26). Following incubation with 10 µM ATRA or 4-HPR or 1 µM CD437, cells were collected at 6, 12, 24 and 48 hours and fixed in 1% formaldehyde in PBS (pH 7.4). For the assay, cells were first suspended in 1 mL wash buffer containing cacodylic acid, Tris-HCl buffered solution, and sodium azide in a Phoenix flow cytometry kit (Phoenix Flow Systems, San Diego, CA). Approximately 10^5 cells were resuspended in 50 µl staining buffer containing Tris-HCl buffer, TdT, and fluorescein-12-dUTP (Phoenix Flow Systems). Cells were incubated at 37°C for 60 min and stained with 500 µl of propidium iodide/RNase A solution in the dark for 30 min at room temperature. They were then analyzed by flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA) with a 15 mW argon laser with excitation at 488 nm. Fluorescence was measured at 585/42 nm. The Phoenix flow cytometry kit included suspensions of cells that served as negative and positive controls for apoptosis. Computer analysis used Cellquest Pro (BD Biosciences), and data processing

ModFit LT by Verity Software to provide information on the percentage of apoptotic cells.

3.5. Analysis of nuclear retinoic acid receptors by real time Q RT-PCR

Real time Q RT-PCR was performed utilizing the 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Specific quantitative assays for RARα, β, and γ were developed using Primer Express software (Applied Biosystems) following the recommended guidelines based on sequences from Genbank. Total RNA was extracted from bladder cells by Tri-reagent, cDNA was synthesized and RT-PCR was performed as previously reported (26). Due to the inherent inaccuracies in quantitating total RNA by absorbance, the amount of RNA in each sample was determined by measuring GAPDH transcript levels. The final data were normalized to GAPDH and are presented as the molecules of transcript/molecules of GADPH x 100 (% GAPDH).

Data analysis for relative quantitation using a housekeeping gene involves running identical cDNA against both a gene-of-interest (i.e. RAR) and a housekeeping gene (GAPDH), then calculating the cycle change between treatment and control groups. The purpose of the housekeeping gene is to make a final adjustment for unequal amounts of starting material. For this type of analysis, it is critical that the housekeeping gene does not change across experimental conditions. The $\Delta\Delta Ct$ for each gene is caluclated according to the equation: $\Delta\Delta Ct = Ct_{treatment\ condition} - Ct_{control\ condition}$

$2^{\wedge}(-\Delta\Delta Ct)$ = Fold Change, "Ct" means: the cycle at which the reaction crossed the threshold.

If Fold Change > 1.0, the gene is up-regulated relative to the control

If Fold Change is <1.0, the gene is down-regulated relative to the control.

3.6. Western blot analysis

Nuclear and cellular proteins were extracted from control (0 µM) and treated (1 µM CD437, 10 µM ATRA or 4-HPR) cells after incubation for 2 days as described in our earlier study (26). Nuclear proteins (30 µg/lane) were electrophoresed in 8% polyacrylamide gels in the presence of 0.1% SDS, then transferred to nitrocellulose membranes. The membranes were incubated with mouse IgG monoclonal antibody against p53, p21, GADD45, (Santa Cruz Biotech, Santa Cruz, CA) and JWA monoclonal antibody was blotted. Blots were washed and then incubated with a peroxidase-conjugated anti-mouse antibody (Amersham). Immunoreactive bands were detected using an enhance chemiluminescence reagent (Amersham). The blots were stripped and then incubated with mouse anti-β-actin antibody (Sigma, St. Louis, MO), followed by a second antibody to assess protein loading.

3.7. Immunofluorescence microscopy

Immunofluorescence staining was performed after T24 cells were treated with 1 and 5 µM ATRA or 4-HPR for 3 days. Briefly, cells were fixed with 100%
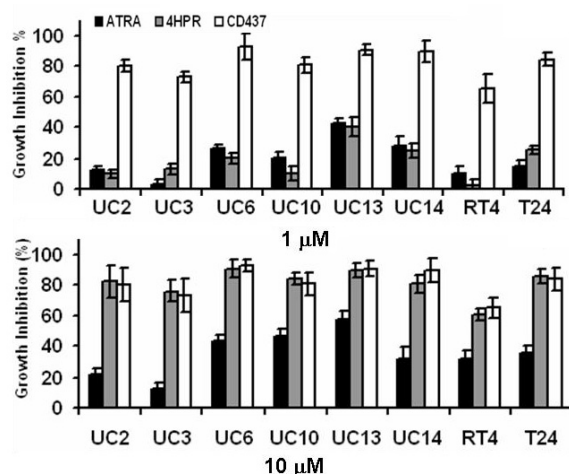
Figure 1. Effect of three retinoids on growth of human bladder cancer cells in monolayer cultures. Cells were grown for 5 days in the absence (control) or presence of 1 µM (upper panel) or 10 µM (lower panel) concentration of ATRA, 4-HPR, or CD437. The culture medium was changed on day 3. Values are means ± SD of triplicate cultures. The percentage of growth inhibition was calculated using the equation: % GI = (1-Nt/Nc) x 100; where Nt and Nc represent the numbers of cells in treated and control cultures, respectively.
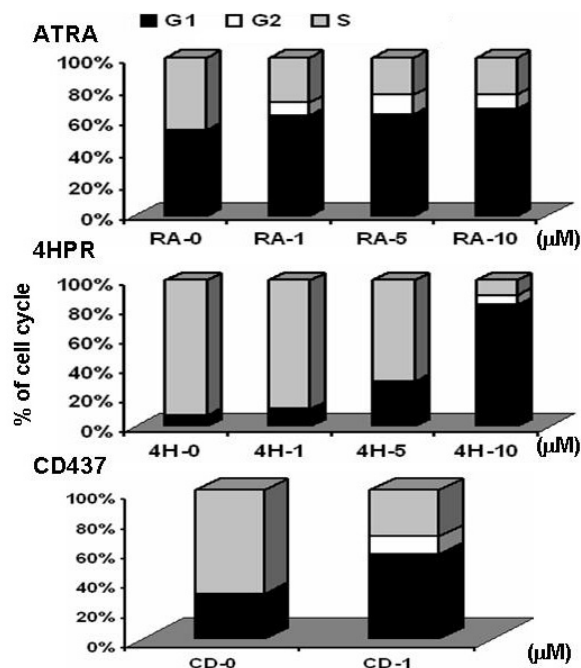
Figure 2. T24 cells were harvested and then stained with propidium iodide for cell cycle analysis. The data on DNA content distribution are presented as bar graph. The percentage of cell populations in the G1, G2, and S phases were analyzed at different concentrations of retinoids. ATRA: upper panel, 4HPR: mid-panel, and CD437: lower panel.

methanol for 5 min and permeabilized by 1% (v/v) Triton X-100 in PBS for 5 min. The cells were washed with PBS four times and incubated with the appropriately diluted antiserum containing 1:100 normal goat sera as a blocking agent. Cells were incubated with a 1:300 dilution of polyanti-JWA and species-specific anti-IgG antibodies conjugated to FITC (Sigma, St. Louis, MO). Fluorescence was observed using an IX-70 invert system fluorescence microscope (Olympus, Japan) with a 100×oil immersion objective. Images were recorded with a PM30 automatic photomicrographic system.

4. RESULTS

4.1. Growth inhibitory effect of different retinoids on human bladder cancer cell lines

Three retinoids were compared at 1 µM or 10 µM for their effect on the growth of 8 human bladder cancer cell lines grown in monolayer cultures. As we previously reported, most of the bladder cancer cell lines were resistant to ATRA at 1 µM. Increasing the concentration of ATRA increased the growth inhibitory effect by over 50% in 3 out of 8 cell lines (Figure 1). 4-HPR had little effect on bladder cancer growth at 1 µM. However at 10 µM, 4-HPR resulted in increased cell growth inhibition (Figure 1). 4-HPR inhibited the growth of all 8 cell lines by 70-80% after 5 days of treatment at 10 µM (Figure 1). CD437 had greater efficacy with 70-90% growth inhibition in all the cell lines at 1 µM (Figure 1). Increasing the concentration to 10 µM did not alter CD437 induced cell growth inhibition.
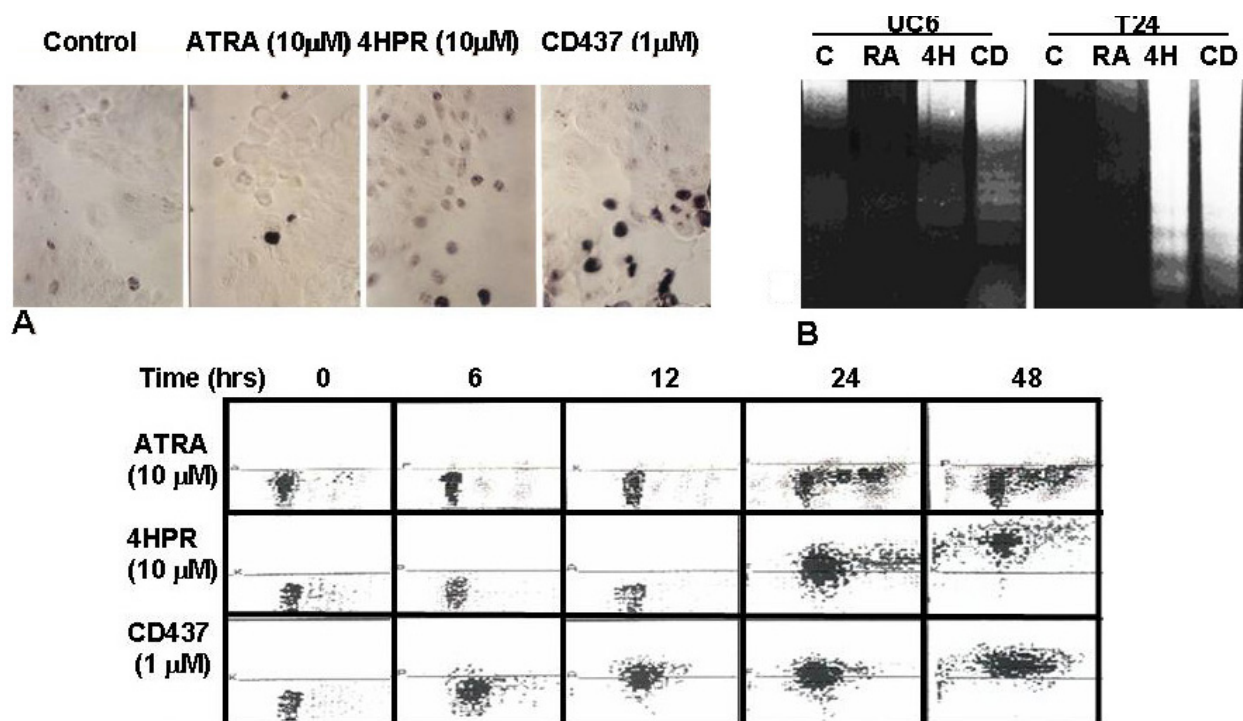
4.2. Cell cycle analysis in bladder cancer cells

Both the synthetic retinoids 4-HPR and CD437 effectively induced apoptosis in bladder cancer cells. DNA content and cell cycle analysis were performed on T24 cells after treatment with the three retinoids. ATRA increased the G1 phase and decreased the S phase only slightly compared with 4-HPR treated cells (Figure 2, upper and mid panel). Increasing concentrations of ATRA only slightly changed the G1 or S phase (Figure 2, upper panel). 4-HPR increased G1 and decreased S phase in a dose-dependent manner (G1 phase in control was 6.53%, 1 µM – 11.05%, 5 µM -29.80%, and 10 µM G1 was 93.5%; Figure 3, mid panel). CD437 at 1 µM had a similar effect as 4-HPR with an increase in G1 from 30.21% to 56.96%. CD437 also decreased the S phase (Figure 2, bottom panel).

4.3. Retinoid-induced apoptosis

The effect of the three retinoids on the induction of apoptosis was tested in bladder cancer T24 cells. After treatment (from 6 to 48 hours), apoptosis was analyzed by *in-situ* apoptosis labeling, DNA fragmentation, and TUNEL assay. ATRA had little effect on apoptosis induction (Figure 3a); CD437 induced stronger and earlier apoptosis than 4-HPR.

No ladder could be detected in control cells (treated with DMSO) nor in ATRA treated cells. The intensity and pattern of DNA laddering were similar in the 4-HPR and CD437-treated groups (Figure 3b).

Figure 3. Apoptosis induction in bladder cancer cells determined by *in situ* apoptosis labeling, DNA fragmentation, and TUNEL analysis. UM-UC 6 and T24 cells were treated with DMSO-control (c), 10 μM of ATRA (RA), 4-HPR (4H), or 1 μM CD437 (CD). Floating and attached cells were collected after 2 days. 3a. T24 cells: Cytospining cells into pathologic slides, and slides were washed and incubated with TDT (Terminal Deoxynucleotidyl Transferase) labeled buffer, 0.3 μg/μl TDT plus biotinylated dUTP 20mM (see Methods). 3b. Soluble DNA from UM-UC 6 (UC6) and T24 cells was extracted and electrophoresed on a 2% agarose gel. The gel was stained with ethidium bromide and photographed under UV illumination. 3c. Time course analysis of apoptosis of T24 cells were determined by TUNEL assay. Cells were harvested and incubated with TdT in the presence of biotin-labeled dUTP, stained with propidium iodide (PI) in RNase A solution. Apoptosis is represented by dark dots (fluorescence of individual cells) above the line in the upper panel. The percentage of apoptotic cells is given in the lower panel.

Time course of DNA content and apoptosis induction in bladder cancer cell lines were analyzed by TdT labeling and flow cytometry. ATRA results showed that even at 10μM, there was little apoptosis induction (Figure 3c). At 10μm, 4-HPR was more potent than ATRA in inducing apoptosis (Figure 3c). 4-HPR induced apoptosis was observed at 24 hr and reached a higher peak after 48 hours (Figure 3a). CD437 induced apoptosis at 1 μM at 6 hours and increased over time. These data was consistent with the *in-situ* apoptosis labeling.

4.4. Expression and induction of nuclear retinoid receptors

Expression of nuclear retinoid receptors was analyzed in bladder cancer cell lines detected by real-time Q RT-PCR. All three retinoids increased RARβ expression in T24 cells (Figure 4). However, in UM-UC-6 cells, ATRA increased RARβ expression, CD437 increased RARγ expression, and 4-HPR decreased all three receptors (Figure 4).

### 4.5. Modulation of gene expression by ATRA, 4-HPR, and CD437

We examined the effects of retinoids on the expression of the apoptosis-associated genes p53, p21, GADD45, and bcl-2. Cells were treated with 1 μM of the retinoids and

p53 expression was detected in UM-UC-3, UM-UC-10, UM-UC-14, and RT4 cells. 4-HPR and CD437 increased p53 expression in UM-UC-10 and UM-UC-14 cells. CD437 only induced expression of p53 in UM-UC-2 cells (Figure 5). The expression of p21 was barely detectable in these cells and was not altered by retinoids (data not shown). Bcl-2 was detected in all eight bladder cancer cell lines tested, and its expression was decreased by 4-HPR and CD437 (Figure 5). ATRA did not induce expression of these genes (Figure 5).

The dose response of the three retinoids on p53, Caspase 3, and JWA gene expression was assessed in UM-UC-10 and UM-UC-14 cells. The three retinoids increased p53 expression in a dose-dependent manner (Figure 6a and 6b). The three retinoids induced JWA gene expression in UM-UC-14 cells (Figure 6a and 6b). ATRA and 4-HPR failed to alter Caspase 3 expression, and only a high concentration of CD437 increased Caspase 3 expression (Figure 6a and 6b).

4.6. JWA gene expression detected by immunofluorescence staining

JWA gene expression was induced by both ATRA and 4-HPR in a dose-dependent manner (Figure 7). Immunofluorescence staining showed stronger fluorescence
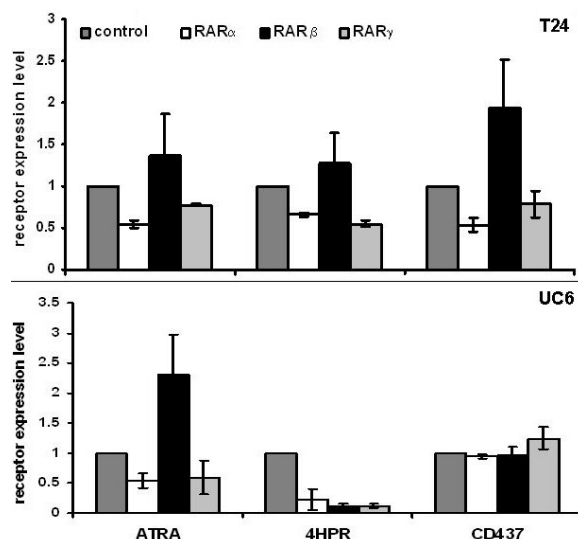
Figure 4. Expression of RARα, RARβ, RARγ, and RxRα in bladder cancer cell lines determined by Q RT-PCR analysis. Bladder cancer cells were treated with three retinoids for 3 days. The cells were then harvested, and the total RNA was extracted and analyzed by Q RT-PCR. . If fold change > 1.0, the gene is up-regulated relative to the control (equal to 1). If fold change is <1.0, the gene is down-regulated relative to the control.
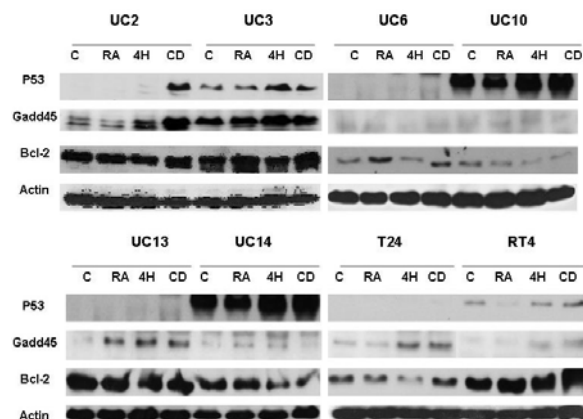
Figure 5. Effect of retinoids on p53, GADD45, and bcl-2 protein expression in bladder cancer cells. Nuclear proteins were extracted from cells treated with 1 μM ATRA (RA), 4-HPR (4H), or CD437 (CD) for 3 days. Fourty micrograms of nuclear proteins per lane was subjected to SDS-PAGE (polyacrylamide gel electrophoresis). The p53, GADD45, and bcl-2 proteins were identified by blotting with monoclonal antibodies. Immunoreactive bands were visualized using an enhanced chemiluminescence method (see Methods). The blots were stripped and then reblotted to mouse anti-β-actin antibody for assessment of loading in each lane.

at higher concentrations of both ATRA and 4-HPR. The effect of ATRA on JWA gene expression was stronger than 4-HPR, which coordinated with protein expression (Figure 6).

5. DISCUSSION

Synthetic retinoids have been used in clinical trials to prevent bladder cancer recurrence (1, 27-28). This study is the first to compare natural and synthetic retinoids, which have different receptor-binding preferences. We demonstrated that the synthetic retinoids (4-HPR and CD437) had stronger effects on growth inhibition and apoptosis induction than the natural one (ATRA). Both natural and synthetic retinoids induced RARs expression. However, 4-HPR and CD437 induced apoptosis in bladder cancer cells but ATRA did not.

ATRA, which binds to RARs, had little effect on the growth and apoptosis of the bladder cancer cell lines we tested previously (26). In this study, only 3 out of 8 cell lines were growth inhibited >20% with ATRA used at 1 μM, which might be related, in part, to RARβ constitutive expression. ATRA increased the percentage of G1 phase slightly, suggesting that it may block cells in the G1 phase causing growth inhibition but not inducing apoptosis in these cells. However, RARβ mediates a growth inhibitory effect and induces apoptosis induction in breast cancer cells (29).

4-HPR induced apoptosis more strongly and more quickly than ATRA in bladder cancer cells. 4-HPR induced apoptosis after 12hr treatment but only at 10 μM. 4-HPR may induce apoptosis through the retinoid receptor pathway or other pathways such as those involving oxygen species and mitochondria (30-32).

CD437 induced the strongest effect on apoptosis and could be detected 6 hours earlier than 4-HPR. Since we compared ATRA, 4-HPR and CD437 at the same concentration and time course, the maximal efficacy of each individual agent may not have been achieved. CD437 induced apoptosis has been reported in various cell types and different tumors, and through different mechanisms (10-17). In our study, CD437 was the only agent which induced Caspase 3 expression, and this increase was also dose-dependent (Figure 6) and suggests that retinoids may act through different pathways in bladder cancer.

Nuclear retinoid receptors are thought to be the ultimate mediators of retinoid actions. Alterations in their expression could affect cancer cell growth, differentiation and apoptosis. The receptors were modulated by the three retinoids. RARβ was induced in T24 cells in this study and in our previous study (26). ATRA induced RARβ and CD437 induced RARγ expression in UM-UC-6 cells. However, other receptors were either not altered or decreased, suggesting that receptor responses to a given retinoid are different. The natural retinoids ATRA and 9cis RA, and RARβ may induce apoptosis (29). However, most bladder cancer cell lines were resistant to natural retinoids, such as ATRA and 9cis RA treatment (26), and loss of expression of RARβ was found in most bladder cancer cell lines (26). Although ATRA strongly induced RARβ expression in UM-UC-6 cells, this agent failed to induce significant growth inhibition and had little effect on inducing apoptosis. There are reports that RARβ affects cell growth and apoptosis (15, 16). In T24 cells, the three
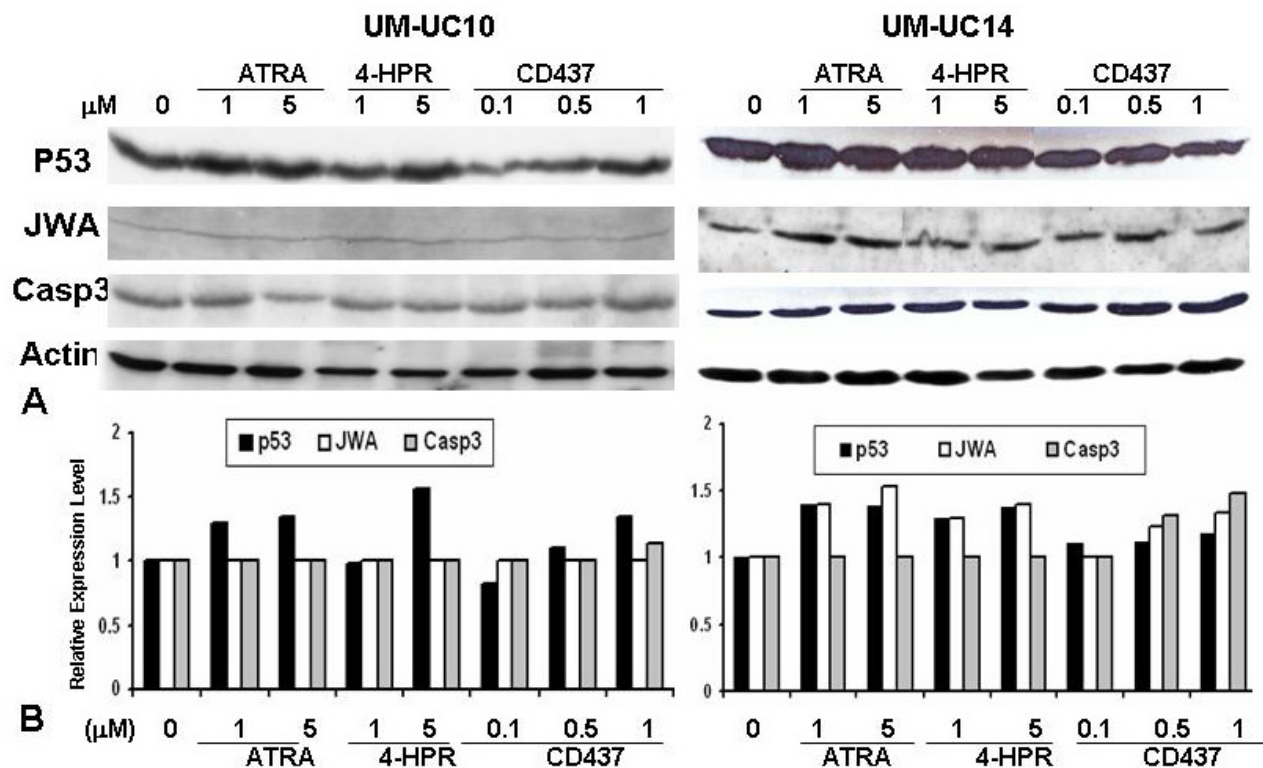
Figure 6. Dose-response of p53, JWA, and Caspase 3 expression. Nuclear proteins were extracted from cells treated with different concentration of retinoids for 3 days. Fourty micrograms of nuclear proteins per lane was subjected to SDS-PAGE (polyacrylamide gel electrophoresis). The p53, JWA, and Caspase 3 proteins were identified by blotting with monoclonal antibodies. The blots were stripped and then reblotted to mouse anti-β-actin antibody for assessment of loading in each lane. a. Immunoreactive bands were visualized using an enhanced chemiluminescence method. b. Data quantified for gene expression.
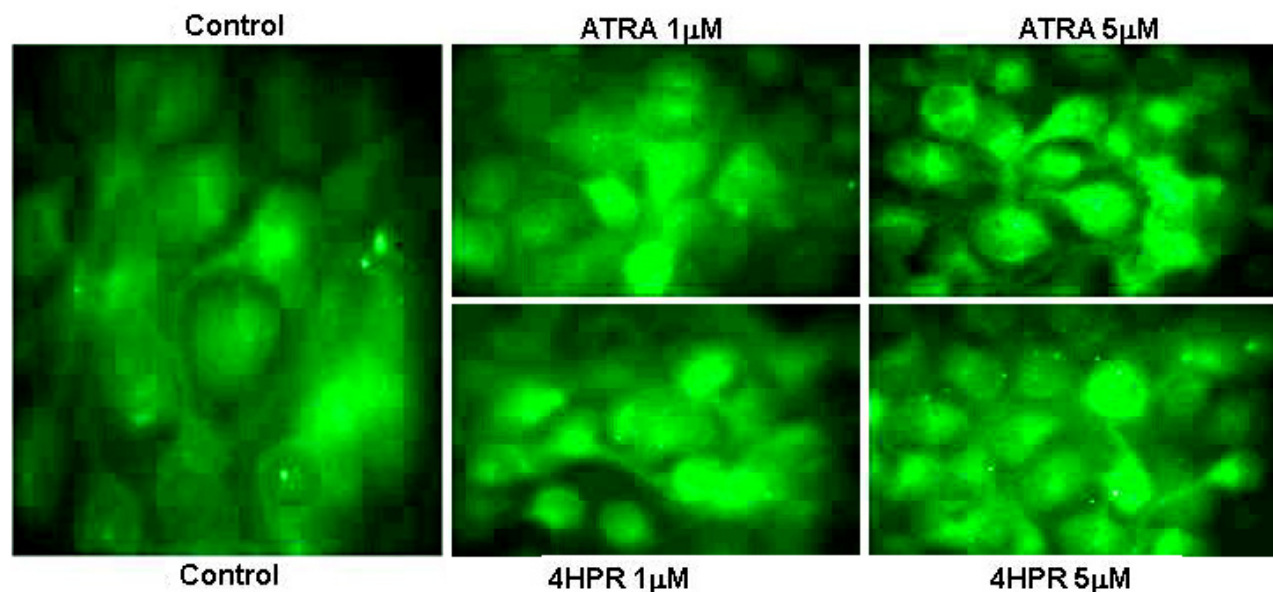
Figure 7. JWA retinoid response gene expression detected by immunofluorescence staining. T24 cells were fixed with 100% methanol. The cells were washed and incubated with polyclonal anti-JWA and species-specific anti-IgG antibodies conjugated to FITC. The fluorescent images were viewed under an IX-70 invert system fluorescence microscope with a 100×oil immersion objective.

retinoids regulated RARβ but not RARγ expression . 4-HPR and CD437 induced strong apoptosis in these cells. The mechanism of apoptosis induction in bladder cancer is most likely not through one receptor. It is unlikely that lack of RARβ or RARγ expression had any effect on growth inhibition or apoptosis. An important fact is that the three retinoids can induce expression of the retinoid response gene JWA, which suggested that at least some of the receptors mediated retinoid function in bladder cancer cells. The JWA gene regulates cell differentiation and apoptosis in human primary acute promylocytic leukemia (APL) cells (24, 25).

Mutations of the p53 gene have been associated with the development and progression of a number of human malignancies including bladder cancer (43-46). In our study, p53 expression was increased after treatment with ATRA and 4-HPR and the growth inhibitory effect of these retinoids may be dependent on p53 expression. ATRA was reported to decrease the amount of p53 mRNA and protein in F9 embryonal carcinoma cells (47-48) and in neuroblastoma cells (49, 50). ATRA is able to increase the stability of wild type p53 in certain non-small cell lung cancer (NSCLC) cell lines (51), increase p53 protein level in normal human bronchial epithelial (NHBE) cells, and decrease p53 levels in NSCLC cell lines (51).

4-HPR modulated p53 function in human breast cancer cell lines (52), ovarian cancer cells (53), and prostate cancer cells (54, 55). 4-HPR enhanced the expression of GADD45 in bladder cancer cells with wild-type p53 (UM-UC-6 and UM-UC-9), but failed to do so in cells with p53 mutations (UM-UC-3). Our results suggest that p53 may not be directly regulated by 4-HPR (56). The mechanism of p53-induced apoptosis is not well understood, but it may be that the equilibrium of bax and bcl-2, two principal and opposing protein components of apoptosis regulation that form neutralizing heterodimer complexes, is shifted by p53 in favor of cell death. ATRA decreased bcl-2 level in HL-60 myeloid leukemia cell lines (57) and in P19 embryonal carcinoma cells (49, 50) and induced apoptosis in these cell lines. Overexpression of bcl-2 can inhibit ATRA-induced apoptosis in embryonal carcinoma cells (49, 50) and delay the induction of apoptosis by 4-HPR (49). We reported previously that ATRA and 4-HPR decreased the level of bcl-2 protein in NSCLC cell lines (58). In the present study, we found that 4-HPR could down-regulate the expression of bcl-2 in bladder cancer cells.

6. CONCLUSIONS

The precise mechanism by which retinoids exert their chemopreventive effects is still unknown. ATRA effects on carcinogenesis might occur through transactivation of receptors. Loss of RARβ may contribute to the resistance of bladder cancer cells to ATRA, which may not be true for other retinoids. 4-HPR could increase retinoid receptor and JWA gene expression. However, its growth inhibitory effect is not dependent on RAR expression and induction. These results suggest that retinoid-induced growth inhibitory effect does not require retinoid receptor transactivation. Our results demonstrate that 4-HPR is a stronger growth inhibitor and apoptosis inducer than ATRA. It increased G1 and decreased S, and regulated p53 expression. 4-HPR shows promise as a chemopreventive agent for the prevention and treatment of bladder cancer when used at the propriety concentration. However, dose-response was important. CD437 was a stronger growth inhibitor and apoptosis inducer than ATRA and 4-HPR. It also increased G1 and decreased S, and regulated p53 expression. Importantly, it regulated Caspase 3 gene expression. The JWA retinoid response gene was regulated by the three retinoids in bladder cancer cells, suggesting that this receptor function was important but not the only regulation factor.

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