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Comparison of the Mechanism of Induction of Apoptosis in Ovarian Carcinoma Cells by the Conformationally Restricted Synthetic Retinoids CD437 and 4-HPR

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Abstract All-trans-retinoic acid (ATRA) has been shown to inhibit the growth of a number of ovarian tumor cell lines while others have been found to be resistant to retinoid suppression of growth. Interestingly, two synthetic retinoids, CD437 and 4-HPR, inhibit the growth of both ATRA-sensitive (CA-OV-3) and ATRA-resistant (SK-OV-3) ovarian tumor cells. However, in contrast to ATRA, both induce apoptosis. Our goal was to elucidate the mechanism by which these two synthetic retinoids induce apoptosis in ovarian tumor cells. Since it has been documented that apoptosis induction is often mediated by the activation of a cascade of proteases known as caspases, we initially studied the role of caspases in induction of apoptosis by CD437 and 4-HPR. We found that both retinoids induced caspase-3 and caspase-9 enzyme activity. Furthermore, using caspase specific inhibitors we determined that caspase-3 and caspase-9 activity was essential for the induction of apoptosis by these synthetic retinoids since these inhibitors completely blocked CD437 and 4-HPR induced apoptosis. Interestingly, we found that treatment with bongkriekic acid (BA), a mitochondrial membrane depolarization inhibitor, blocked apoptosis, caspase-9 activation and caspase-3 activation induced by both retinoids. Finally, we were able to determine that CD437 treatment induced the translocation of TR3, a nuclear orphan receptor, whereas, 4-HPR did not. Our results suggest that CD437 and 4-HPR initially activate separate pathways to induce mitochondrial depolarization but both utilize mitochondrial depolarization, caspase-9 activation, and caspase-3 activation in the later stages of apoptosis induction. J. Cell. Biochem. 89: 262–278, 2003.

Key words: apoptosis; retinoids; ovarian cancer; CD437; 4-HPR; caspases

Retinoids are a group of natural and synthetic analogs of vitamin A that have been shown to play an important role in cell differentiation and proliferation [Kastner et al., 1995; Means and Gudas, 1995]. Retinoids have great promise in the area of cancer chemotherapy and chemoprevention since retinoid treatment has been shown to inhibit the growth of a variety of epithelial carcinomas [De Luca, 1991; Gudas et al., 1994; Moon et al., 1994]. However, many tumors are resistant to the growth inhibitory effects of natural retinoids such as all-trans-retinoic acid (ATRA). Several synthetic retinoids such as 6-[(1-Admantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437) and fenretinide *N*-[4-hydroxyphenyl] retinamide (4-HPR) have been shown to inhibit the growth and induce apoptosis in both ATRA-sensitive and ATRA-resistant ovarian carcinoma cell lines [Wu et al., 1998; Holmes et al., 2000, 2002]. Thus determination of the mechanisms employed by these synthetic retinoids to inhibit growth and to induce apoptosis of ovarian tumors could lead to the development of more effective cancer treatments.

CD437 is a conformationally restricted synthetic retinoid that has been studied in a

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number of cancer models including non-small cell lung carcinoma, cervical, breast, and ovarian carcinomas and is currently being evaluated in a number of clinical trials [Shao et al., 1995; Schadendorf et al., 1996; Chao et al., 1997; Hsu et al., 1997; Oridate et al., 1997; Piedrafita and Pfahl, 1997; Spanjaard et al., 1997; Sun et al., 1997a; Li et al., 1998; Wu et al., 1998]. Our laboratory has previously reported that CD437 inhibits growth and induces apoptosis in both ATRA-resistant (SK-OV-3) and ATRA-sensitive (CA-OV-3) ovarian carcinoma cells lines [Wu et al., 1998; Holmes et al., 2000, 2002]. Our previous studies demonstrated that CD437 induces apoptosis through mitochondrial depolarization, caspase-9 activation, and subsequent caspase-3 activation in ovarian carcinoma cell lines [Holmes et al., 2002]. Mitochondrial membrane depolarization causes the release of the proteins APAF-1, cytochrome c, and procaspase-9 [Solary, 1998; Perkins et al., 2000]. These mitochondrial proteins associate and induce activation of caspase-9 through the cleavage of procaspase-9.

We have previously found that CD437-dependent depolarization of mitochondria in CA-OV-3 cells is dependent on the translocation of an orphan nuclear hormone receptor, TR3 (nur77), from the nucleus to the cytosol (submitted for review). TR3 has been shown to associate with mitochondria in other tumor cell lines in response to apoptosis inducing agents causing the depolarization of the mitochondrial membrane [Li et al., 2000; Dawson et al., 2001; Zhang, 2002].

Fenretinide (4-HPR) is a synthetic retinoid in which the mechanism of action is not fully understood. 4-HPR has been demonstrated to induce apoptosis in ovarian tumor cells [Bast et al., 1995; Supino et al., 1996; Sabichi et al., 1998]. Previously, we have shown that 4-HPR induces growth arrest and apoptosis in ATRA-sensitive, CA-OV-3, and ATRA-resistant, SK-OV-3, ovarian tumor cell lines [Holmes et al., 2002]. We also have found that, in CA-OV-3 and SK-OV-3 ovarian tumor cells, 4-HPR acts independent of RAR function or activation (unpublished observation) but induces the activation of the caspase cascade [Holmes et al., 2002].

Previously we described a CD437-resistant ovarian carcinoma cell line, CA-CD437R, which was made resistant to the apoptotic effects of CD437 [Holmes et al., 2002]. Interestingly, this cell line, isolated from the CA-OV-3 ovarian tumor cell line, is resistant to apoptosis induced by CD437 and TNF-α treatments, but is not resistant to apoptosis induced by 4-HPR. Our results suggest that these two retinoids use separate pathways to induce apoptosis. In this investigation we describe the late stage mechanisms common for the induction of apoptosis by CD437 and 4-HPR in ovarian carcinoma cell lines. We also report that 4-HPR does not cause the translocation of TR3 but CD437 does, indicating that these synthetic retinoids induce separate pathways, which results in the depolarization of mitochondrial membranes.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

CA-OV-3 and SK-OV-3 cells were obtained from the American Type Culture Collection (Rockville, MD). Stock cultures were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin, and 100 U/ml nystatin. All cell lines were routinely split at a ratio of 1:10 weekly. The plates were then placed in a 98% humidified, 5% CO_2 incubator.

Retinoids and Reagents

6-[-(1-Admantyl)-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (CD437) was a generous gift of Galderma Research and Development (Sophia Antipolis, France). Fenretinide (4-HPR) was generously provided by R.W. Johnson Pharmaceutical Research Institute (Raritan, NJ). Retinoid stock solutions (10^{-3} M) prepared in DMSO were stored at -70°C. Recombinant human TNF-α was purchased from Promega (Madison, WI). Stock solutions (50 μg/ml) were prepared in sterilized MILLI-Q water and stored at -70°C. All procedures involving retinoids were carried out under subdued light. In each experiment, control cultures were treated with an equivalent amount of DMSO. Betulinic acid (Bet A) and bongkreikic acid (BA) were purchased from Biomol (Plymouth Meeting, PA). Stock solutions of Bet A (10 mg/ml) and BA (50 mM) were prepared in sterilized MILLI-Q water and stored at -70°C.

Cell Proliferation

Ovarian cancer cells were seeded onto 100 mm tissue culture plates at a density of

2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. The medium, with or without varying concentrations of retinoids, was changed every 2 days. On the days indicated, cells were washed with PBS, trypsinized, and counted using a hemocytometer. Cells were assessed for viability using trypan blue. All cell counts were repeated in triplicate.

Apoptosis ELISA

Detection of apoptosis in ovarian cancer cells was performed via an ELISA as described by Salgame et al. [1997]. The ELISA uses a capture monoclonal antibody (LG11-2), which is specific for residues 1–25 of the amino terminal domain of histone H2B, and a biotinylated detection monoclonal antibody (PL2-3), which is specific for the nucleosome subparticle composed of histones H2A, H2B, and DNA. The ELISA signals were quantitated by measuring absorbance at 405 nm using a V_{max} kinetic reader (Molecular Devices, Sunnyvale, CA).

Caspase Activity Assays

The activity of caspase proteases was measured using an ApoAlert CPP32 colorimetric assay kit (Clontech, Palo Alto, CA). Briefly, whole cell lysate from approximately 4×10^6 ovarian cells was incubated for 1 h at 37°C with the caspase specific substrate in the provided reaction buffer. Cleavage of the caspase specific substrate by active caspase resulted in the liberation of *p*NA (*p*-nitroanilide) into solution. The release of *p*NA was quantitated spectrophotometrically by measuring absorbance at 405 nm using a V_{max} kinetic reader (Molecular Devices). All experiments were repeated in triplicate. The following specific caspase substrates were utilized in these studies: Ac-DEVD-*p*NA (caspase-3) (Clontech), Ac-IETD-*p*NA (caspase-8), and the fluorometeric LEHD-AFC (caspase-9) (Biomol). All experiments were repeated in triplicate.

Caspase Inhibitor Studies

Ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment, 20 μg/ml of the caspase inhibitor was added. The effect of inhibiting caspase-3-like activity on retinoid induced growth arrest and apoptosis was determined by direct cell counting and/or apoptotic ELISA. The following specific caspase inhibitors were utilized in these studies: DEVD-cmk (caspase-3), IETD-fmk (caspase-8), and LEHD-fmk (caspase-9) (Biomol). All experiments were repeated in triplicate.

$DIOC_{(6)}$ Cell Staining

A method described by Barni et al. [1996] was used to determine the permeability of mitochondria after treatment with 4-HPR and CD437. After 12 h treatment with retinoids cells were stained with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) at a final concentration of 2 μg/ml of for 10 min at room temperature. The cells were then washed in phosphate buffered saline for 10 min. Culture medium was added to the cells and the fluorescent mitochondrial probe $DIOC_{(6)}$ (3,3′ dihexyloxacarbocyanine iodide) (Eastman Kodak Co., Rochester, NY) was added at a final concentration of 0.5 μg/ml for 5 min at room temperature. After two rinses for 2 min each with culture medium the cells were wet mounted with culture media and sealed with silicone. Images were visualized using a fluorescent microscope (Axioskop, Zeiss, Germany) and camera (DKC-5000 digital photo camera, Sony, Japan).

GFP-TR3 Transient Tranfection and Confocal Microscopy

The pGFP-TR3 construct was a generous gift from Xioa-kun Zhang (Burnham Institute, La Jolla, CA). This construct was generated by cloning TR3 cDNA into a pGFP-N2 vector (Clontech) [Li et al., 2000]. Briefly, 4×10^5 cells/well were cultured in 60 mm plates with cover slips. The CA-OV-3 cells were transfected with the pGFP-TR3 construct using the Effectene (Quiagen, Valencia, CA) transfection protocol. The cells were treated with CD437 24 h after transfection the cells were treated with CD437 (10^{-6} M). Treatments were performed for 0, 3, 6, and 12 h. After CD437 treatment, the medium was replaced with fresh DMEM containing the mitochondrial stain MitoTracker Red CMXRos (8-(4′-chloromethyl) phenyl-2, 3, 5, 6, 11, 12, 14, 15-octahydro-1H, 4H, 10H, 13H-diquinolizino-8H-xanthylium chloride) from Molecular Probes (Eugene, OR). CMXRos was added at a final concentration of 0.25 μg/ml the cells were stained for 15 min at room temperature. After two rinses for 2 min each with culture medium the cells were washed with phosphate-buffered

saline (PBS) and fixed for 30 min in 4% paraformaldehyde. All work was carried out in low light to prevent photo-bleaching of CMXRos and GFP. Fluorescent images were collected and analyzed by using an Olympus Fluoview confocal laser scanning microscope (Olympus America, Inc., Melville, NY).

RESULTS

Synthetic Retinoids CD437 and 4-HPR Induce Growth Arrest and Apoptosis in Ovarian Carcinoma Cell Lines

We performed experiments to determine that CA-OV-3 and SK-OV-3 cell lines were sensitive to CD437 and 4-HPR induced growth arrest and apoptosis. Retinoid treatments were performed on both cell lines at predetermined optimal concentrations: CD437 (10^{-6} M) and 4-HPR (10^{-5} M). After 8 days, viable cell numbers were determined by direct cell counts. Figure 1A,B show a comparison of the growth of the ovarian carcinoma cell lines treated with CD437 and 4-HPR, for comparison TNF-α (50 ng/ml) was used as a positive control. DMSO treatment was used as a negative control. Cell counts were plotted as percent of DMSO control. In both cell lines CD437 and 4-HPR was able to induce growth arrest over time.

It has been reported that 4-HPR and CD437 induce apoptosis in a many different cancer cell types [Oridate et al., 1995, 1997; Schadendorf et al., 1996; Wang and Phang, 1996; Hsu et al., 1997; Sun et al., 1997b, 1999; Li et al., 1998;

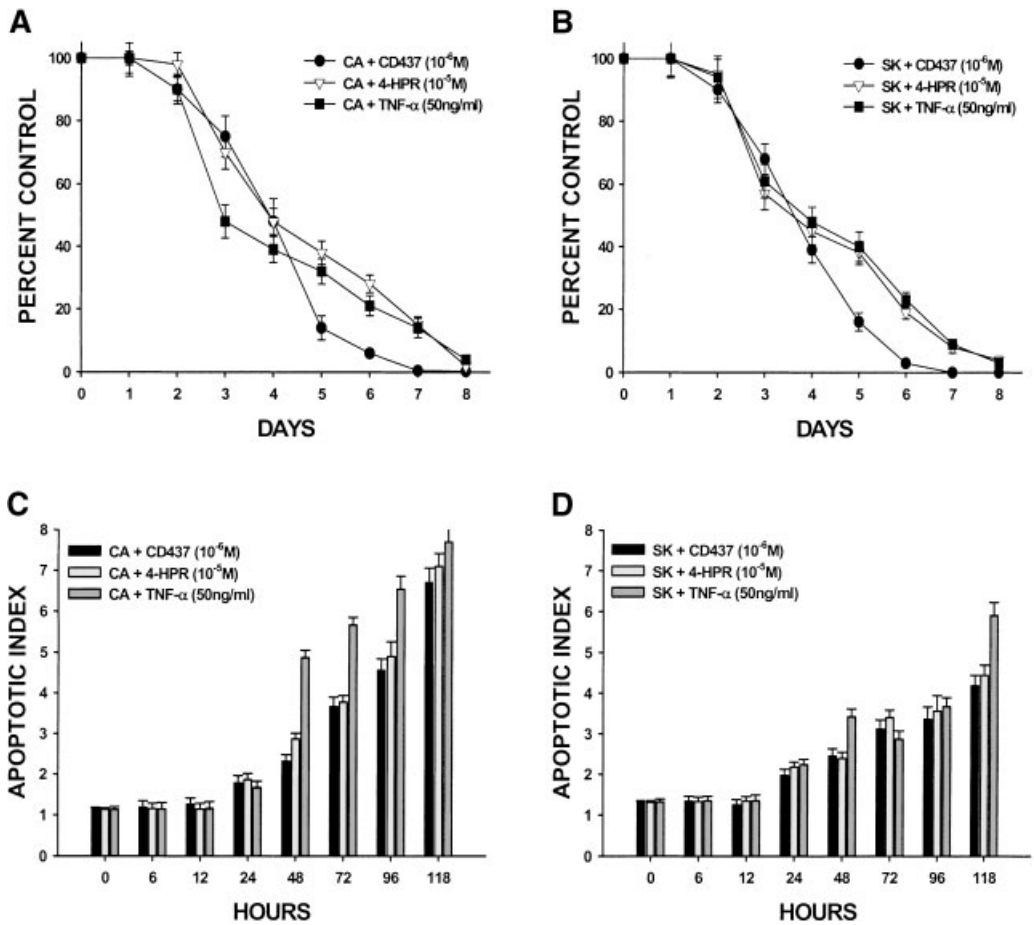

Fig. 1. The synthetic retinoids CD437 and 4-HPR induce growth arrest and apoptosis in ovarian carcinoma cell lines. Ovarian tumor cells (2×10^5) CA-OV-3 (**A**) and SK-OV-3 (**B**) were treated with CD437 (10^{-6} M) or 4-HPR (10^{-5} M) or TNF-α (50 ng/ml). Control cultures were treated with an equal volume of DMSO. Direct cell counting was performed at the times indicated after treatment using a hemocytometer. Data are expressed as percent DMSO treated control. The data represent the mean ± SD of three separate experiments performed in triplicate. **C**: CA-OV-3 and (**D**) SK-OV-3 ovarian tumor cells (5×10^5) were treated with 10^{-6} M CD437 or 4-HPR (10^{-5} M) or TNF-α (50 ng/ml). The apoptosis ELISA (Materials and Methods) was performed on day 5. DMSO treated was used as controls. The data represent the mean ± SD of three separate experiments performed in triplicate.

Marchetti et al., 1999; Maurer et al., 1999; Shen et al., 1999]. We investigated the induction of apoptosis of ovarian carcinoma cell lines in response to CD437 and 4-HPR. Retinoid treatments were performed on both cell lines at the same concentrations used for the growth studies: 10^{-6} M for CD437 and 10^{-5} M for 4-HPR. At the times indicated, cytoplasmic lysates were collected and used in an apoptotic ELISA described previously [Salgame et al., 1997]. ELISA signals were quantitated by measuring absorbance at 405 nm. Figure 1C,D show a comparison of the induction of apoptosis of the ovarian carcinoma cell lines treated with CD437 and 4-HPR, for comparison TNF-α (50 ng/ml) was used as a positive control. Data are expressed as an index of the absorbance of DMSO treatment, which was set to a value of 1 apoptotic unit. In both cell lines CD437 and 4-HPR were able to induce apoptosis in a manner similar to apoptosis induced by TNF-α.

Caspase-3 Activity is Essential for Apoptosis in Ovarian Carcinoma Cells After Synthetic Retinoid Treatment

In order to begin to understand the mechanism by which retinoids such as CD437 and 4-HPR induce apoptosis, we next examined the activation of the effector caspase associated with the final stages of apoptosis, caspase-3. Caspase-7 requires the same substrate (DEVD) and is very difficult to distinguish from caspase-3 so all measurements will reflect the activity of both caspase-3 and caspase-7 but will be referred to as just caspase-3. Figure 2A,B shows that caspase-3 activity could be detected in both the CA-OV-3 and the SK-OV-3 cells, in response to CD437 and 4-HPR. These results demonstrate that the caspase-3 protease is activated in both CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines in response to treatment with either CD437 or 4-HPR. These data are also indicative of apoptosis since activation of caspase-3 is associated with the late stages of apoptosis induction.

In order to determine if caspase-3 activation was essential for the induction of apoptosis by these synthetic retinoids, CA-OV-3 and SK-OV-3 cells were treated with a caspase inhibitor DEVD-cmk (a caspase-3-like inhibitor) one-hour prior to retinoid treatment. In Figure 2C,D, we show that use of the caspase-3 inhibitor significantly blocked CD437 and 4-HPR induced apoptosis in the CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines. These results demonstrate that caspase-3 activation is required for the induction of apoptosis by both CD437 and 4-HPR.

Caspase-8 is not Activated After CD437 and 4-HPR Treatments of Ovarian Carcinoma Cells

In order to determine the mechanism by which retinoids such as CD437 and 4-HPR induce apoptosis, we next examined the activation of the initiator caspase, caspase-8. Caspase-8 is associated with the activation of the caspase cascade during apoptosis induced by external signal stimuli, such as TNF-α. Caspase-3 cleavage is well documented as a target for activated caspase-8. Active caspase-8 targets a specific peptide (IETD) in caspase-3 and other proteins. Figure 3A shows that caspase-8 activity could not be detected in CA-OV-3 cells treated with CD437 or 4-HPR. In Figure 3B, SK-OV-3 cells were treated with either CD437 or 4-HPR, respectively and show a similar lack of caspase-8 activity in response to either retinoid treatment. TNF-α treatment was used as a positive control for both cell lines indicating that no defect exists in the activation of caspase-8 in either the CA-OV-3 or SK-OV-3 cell lines. These results demonstrate that caspase-8 protease is not activated in both CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines in response to treatment with either 4-HPR or CD437.

In order to determine if caspase-8 activation is necessary for the induction of apoptosis by these synthetic retinoids we treated with a caspase-8 specific inhibitor IETD-fmk one-hour prior to retinoid treatment. In Figure 3C,D, we show that use of the caspase-8 inhibitor did not block apoptosis induced by either CD437 or 4-HPR treatment of CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines. The slight increase in caspase-8 activity in the CA-OV-3 cells treated with CD437 for 8 h could be a result of the induction of apoptosis and not an initiator of the apoptosis signal (Fig. 3A). Taken together, these data demonstrate that caspase-8 activation is not required for the induction of apoptosis by CD437 and 4-HPR.

Caspase-9 Activity is Essential for the Induction of Apoptosis After CD437 and 4-HPR Treatments of Ovarian Carcinoma Cells

We next examined the activation of the mitochondrial membrane associated initiator

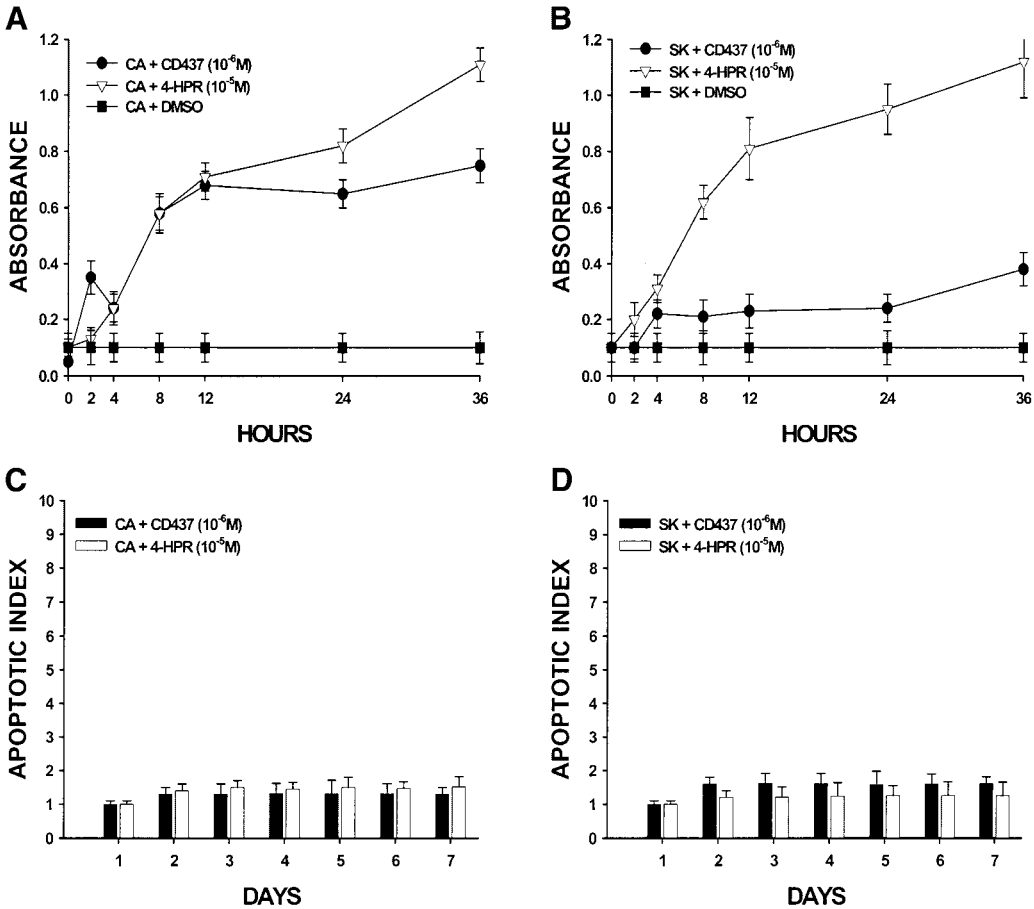

Fig. 2. Caspase-3 is essential for CD437 and 4-HPR induction of apoptosis in ovarian carcinoma cells. Ovarian tumor cells (8×10^6) were plated in 100 mm culture dishes in 10 ml of DMEM medium with 10% FBS, and treated as indicated for 48 h. Whole cell lysate from approximately 4×10^6 ovarian cells was collected at the indicated times and caspase-3 activity in cell extracts was measured by the cleavage of the colorimetric caspase substrate Ac-DEVD-*p*NA. Data are expressed as absorbance at 405 nm. The data represent the mean ± SD of three separate experiments performed in triplicate. **A**: CA-OV-3 cells treated with CD437 (10^{-6} M) (●), or 4-HPR (10^{-5} M) (▽), or DMSO (■). **B**: SK-OV-3 cells treated with CD437 (10^{-6} M) (●), or 4-HPR (10^{-5} M) (▽), or DMSO (■). Ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment, 20 μg/ml of the caspase-3 inhibitor DEVD-cmk was added. The effect of inhibiting caspase-3 activity on retinoid-induced apoptosis was determined by apoptotic ELISA described in Materials and Methods. Data are expressed as an index of the absorbance DMSO treatment, which was set to a value of 1 apoptotic unit. The data represent the mean ± SD of three separate experiments performed in triplicate. **C**: CA-OV-3 cells treated with CD437 (10^{-6} M) and 4-HPR (10^{-5} M) (**D**) SK-OV-3 cells treated with CD437 (10^{-6} M) and 4-HPR (10^{-5} M).

caspase, caspase-9. Caspase-9 is a major activator of caspase-3, and is associated with the induction of apoptosis by internal signaling mechanisms that cause the depolarization of the mitochondrial membrane. Active caspase-9 targets a specific peptide (LEHD) in caspase-3 and other proteins. Figure 4A,B show that caspase-9 activity could be detected in both cell lines, CA-OV-3 and SK-OV-3, in response to CD437 and 4-HPR.

In order to determine if caspase-9 was essential for the induction of apoptosis the synthetic retinoids CD437 and 4-HPR, we treated both cell lines with a caspase-9 specific inhibitor LEHD-fmk one-hour prior to retinoid treatment. In Figure 4C,D, we show that use of the caspase-9 inhibitor could block apoptosis induced by CD437 and 4-HPR treatment of CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines. These data demonstrate that caspase-9 activation is required for the induction of apoptosis by CD437 and 4-HPR and this induction of apoptosis most likely involves mitochondrial depolarization.

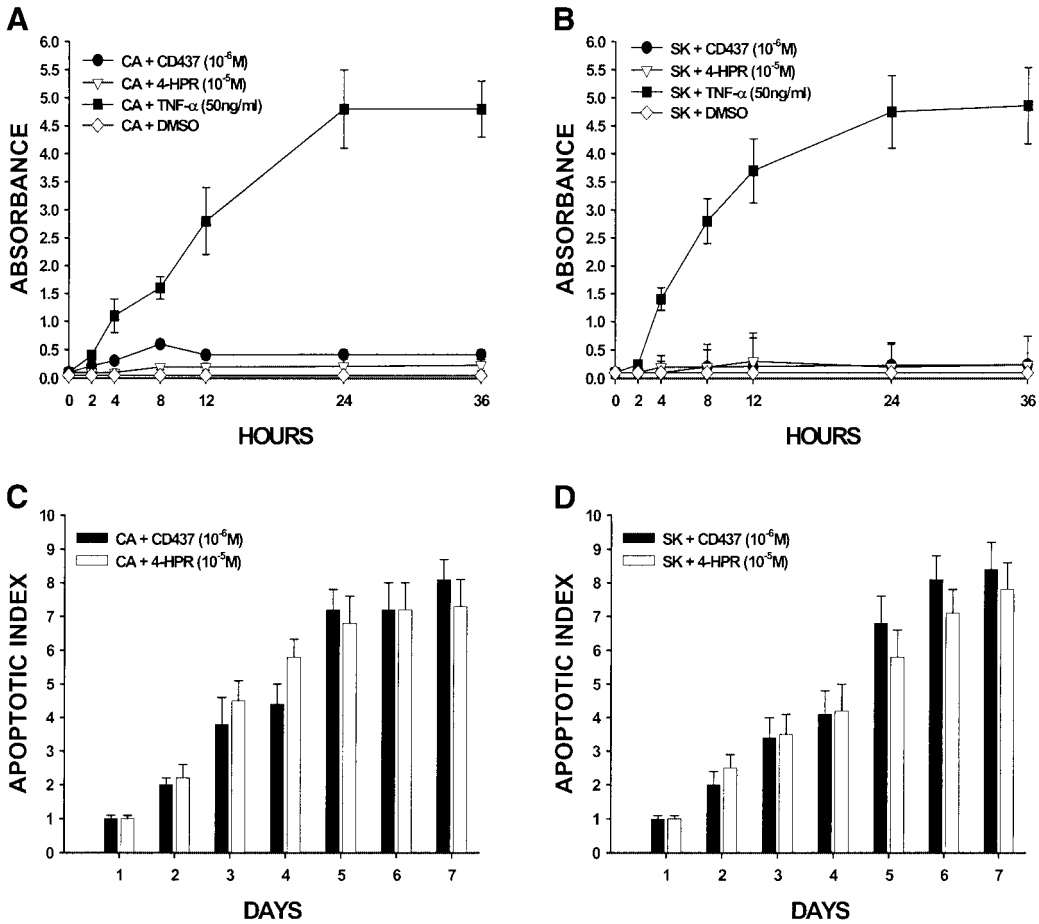

Fig. 3. Caspase-8 is not essential for CD437 and 4-HPR induction of apoptosis in ovarian carcinoma cells. Ovarian tumor cells (8×10^6) were plated in 100 mm culture dishes in 10 ml of DMEM medium with 10% FBS, and treated as indicated for 48 h. Whole cell lysate from approximately 4×10^6 ovarian cells was collected at the indicated times and caspase-8 activity in cell extracts was measured by the cleavage of the colorimetric caspase substrate IETD-*p*NA. Data are expressed as absorbance at 405 nm. The data represent the mean ± SD of three separate experiments performed in triplicate. **A**: CA-OV-3 cells treated with CD437 (10^{-6} M) (●), or 4-HPR (10^{-5} M) (▽), or DMSO (■). **B**: SK-OV-3 cells treated with CD437 (10^{-6} M) (●), or 4-HPR (10^{-5} M) (▽), or DMSO (■). Ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment, 20 μg/ml of the caspase-8 inhibitor IETD-fmk was added. The effect of inhibiting caspase-8 activity on retinoid-induced apoptosis was determined by apoptotic ELISA described in Materials and Methods. Data are expressed as an index of the absorbance DMSO treatment, which was set to a value of 1 apoptotic unit. The data represent the mean ± SD of three separate experiments performed in triplicate. **C**: CA-OV-3 cells treated with CD437 (10^{-6} M) and 4-HPR (10^{-5} M) (**D**) SK-OV-3 cells treated with CD437 (10^{-6} M) and 4-HPR (10^{-5} M).

Depolarization of the Mitochondrial Membrane Occurs After Synthetic Retinoid Treatment of Ovarian Carcinoma Cell Lines

Apoptosis characterized by induction of caspase-9 activity followed by activation of caspase-3 has been reported to be associated with permeabilization of the mitochondrial membrane [Hatai et al., 2000; He et al., 2000; Sun et al., 2000]. Mitochondrial depolarization has been reported to occur after treatment with CD437 and 4-HPR [Marchetti et al., 1999; Suzuki et al., 1999; Dawson et al., 2001; Hail et al., 2001]. Since CD437 and 4-HPR treatments induced caspase-9 and caspase-3 activity, we next examined mitochondrial permeability in both CA-OV-3 and the SK-OV-3 cell lines using the probe $DIOC_{(6)}$. $DIOC_{(6)}$ is a fluorescent stain that is visible as a green color when visualized under a UV microscope. Mitochondria accumulate the $DIOC_{(6)}$ probe unless the membranes of the mitochondria are depolarized. Hoechst 33342 was used as a counter stain of the nucleus. The micrographs in

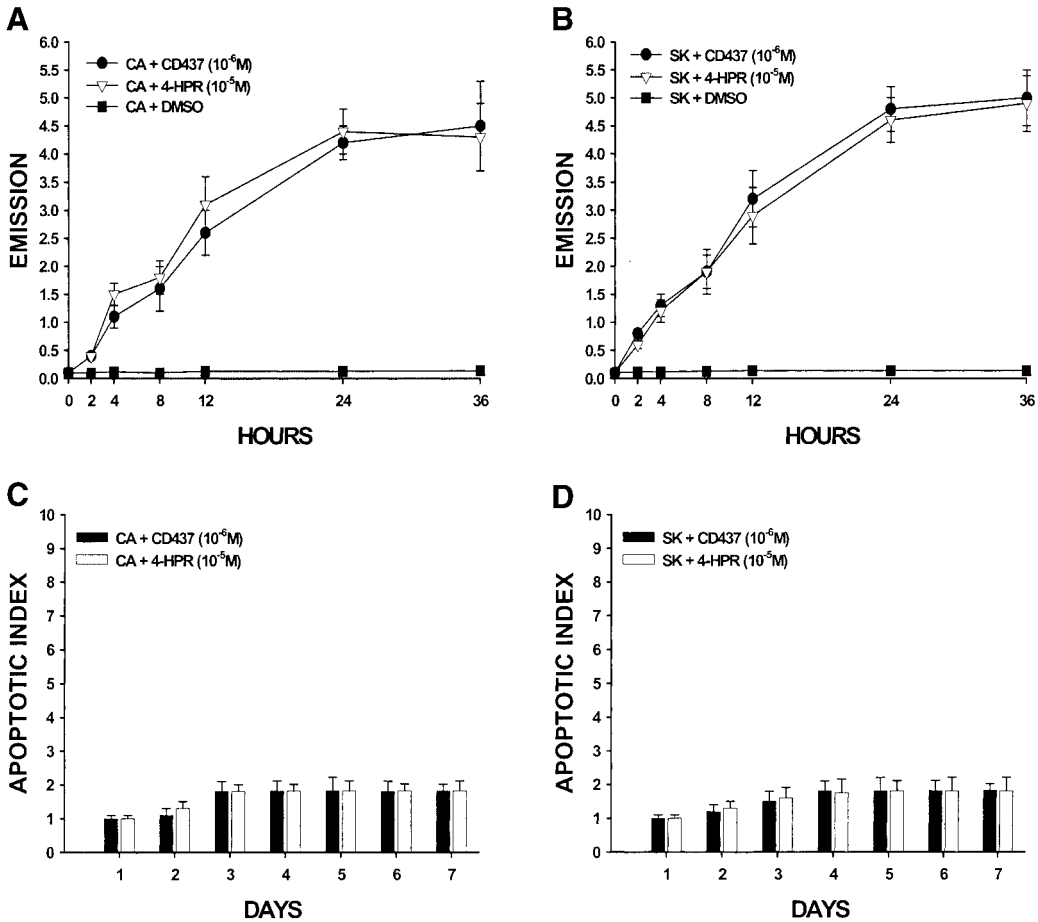

Fig. 4. Caspase-9 is essential for CD437 and 4-HPR induction of apoptosis in ovarian carcinoma cells. Ovarian tumor cells (8×10^6) were plated in 100 mm culture dishes in 10 ml of DMEM medium with 10% FBS, and treated as indicated for 48 h. Whole cell lysate from approximately 4×10^6 ovarian cells was collected at the indicated times and caspase-9 activity in cell extracts was measured by the cleavage of the Fluorometric caspase substrate LEHD-AFC. Data are expressed as emission at 505 nm. The data represent the mean ± SD of three separate experiments performed in triplicate. **A**: CA-OV-3 cells treated with CD437 (10^{-6} M) (●), or 4-HPR (10^{-5} M) (▽), or DMSO (■). **B**: SK-OV-3 cells treated with CD437 (10^{-6} M) (●), or 4-HPR (10^{-5} M) (▽), or DMSO (■). Ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment, 20 μg/ml of the caspase-9 inhibitor LEHD-fmk was added. The effect of inhibiting caspase-9 activity on retinoid-induced apoptosis was determined by apoptotic ELISA described in Materials and Methods. Data are expressed as an index of the absorbance DMSO treatment, which was set to a value of 1 apoptotic unit. The data represent the mean ± SD of three separate experiments performed in triplicate. **C**: CA-OV-3 cells treated with CD437 (10^{-6} M) and 4-HPR (10^{-5} M) (**D**) SK-OV-3 cells treated with CD437 (10^{-6} M) and 4-HPR (10^{-5} M).

Figure 5 clearly show that compared to vehicle treated control cells (Fig. 5A) the mitochondria of CA-OV-3 did not accumulate the $DIOC_{(6)}$ stain following treatment with either CD437 (Fig. 5B) or 4-HPR (Fig. 5C). This indicated that the induction of apoptosis by these retinoids involves depolarization of the mitochondrial membranes. Retinoid treatment of the SK-OV-3 cell line yielded similar results; CD437 treatment induced the depolarization of the mitochondrial membranes (Fig. 5E), and 4-HPR treatment induced depolarization (Fig. 5F) as compared to control (Fig. 5D). These data indicate that mitochondrial membranes depolarize in response to CD437 and 4-HPR treatments in both CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines.

Mitochondrial Depolarization is Essential for the Induction of Apoptosis and Caspase-9 Activation After CD437 and 4-HPR Treatments of Ovarian Carcinoma Cells

We wanted to determine if depolarization of mitochondria after treatment with CD437 and 4-HPR is necessary for the activation of caspase-9, activation of caspase-3, and apoptosis. In

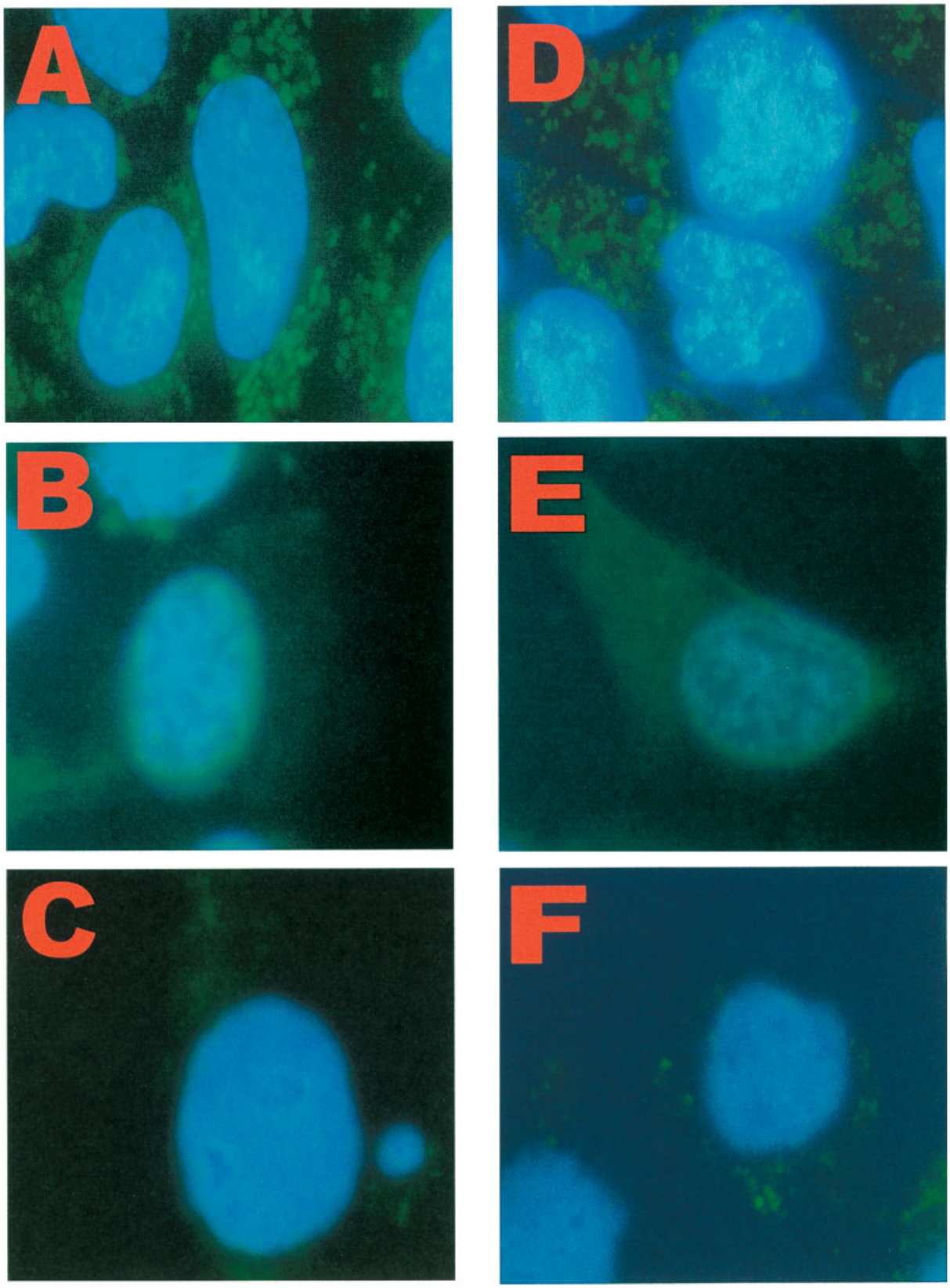

Fig. 5.

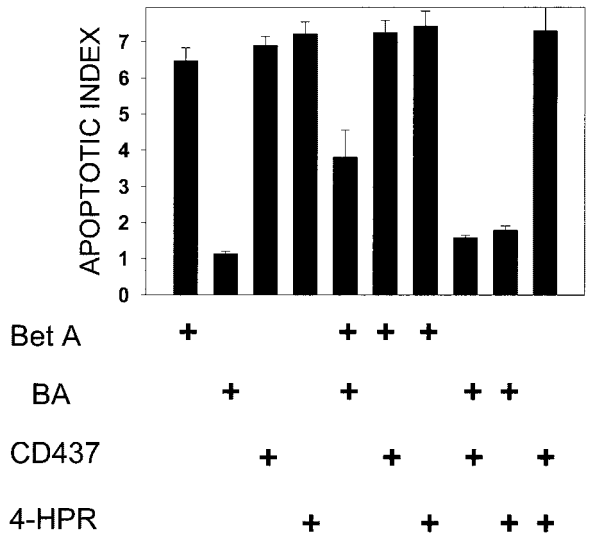

Fig. 6. Mitochondrial depolarization is essential for the induction of apoptosis by CD437 and 4-HPR treatment of ovarian tumor cells. CA-OV-3 ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment either 10 μg/ml of the Bet A an inducer of mitochondrial membrane depolarization or 50 μM BA an inhibitor of mitochondrial depolarization. Retinoid treatment with either 10^{-6} M CD437 and/or 10^{-5} M 4-HPR was carried out for four days. The effect of inhibiting mitochondrial depolarization on retinoid-induced apoptosis was determined by apoptotic ELISA described in Materials and Methods. Data are expressed as an index of the absorbance DMSO treatment, which was set to a value of 1 apoptotic unit. The data represent the mean ± SD of three separate experiments performed in triplicate.

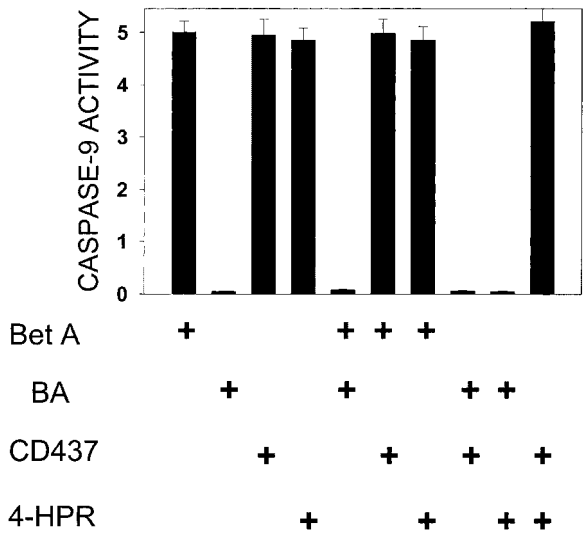

Fig. 7. Mitochondrial depolarization is essential for caspase-9 activation by CD437 and 4-HPR treatment of ovarian tumor cells. CA-OV-3 ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment either 10 μg/ml of the Bet A an inducer of mitochondrial membrane depolarization or 50 μM BA an inhibitor of mitochondrial depolarization. Retinoid treatment with either 10^{-6} M CD437 and/or 10^{-5} M 4-HPR was carried out for 12 h. Whole cell lysate from approximately 4×10^6 ovarian cells was collected and caspase-9 activity in cell extracts was measured by the cleavage of the fluorometric caspase substrate LEHD-AFC. The data represent the mean ± SD of three separate experiments performed in triplicate.

Figures 6–8 CA-OV-3 were treated with combinations of Bet A a chemical that causes the depolarization of the mitochondria membranes, BA a chemical that inhibits depolarization of mitochondria membranes, CD437 and 4-HPR.

The data in Figure 6 show that Bet A can induce apoptosis similar to CD437 and 4-HPR. BA inhibits the depolarization of mitochondria and blocks the induction of apoptosis by Bet A, CD437, and 4-HPR. This indicates that mitochondrial depolarization is essential for the induction of apoptosis by both CD437 and 4-HPR.

Using a caspase-9 activity assay described in Materials and Methods, Figure 7 shows that caspase-9 activity occurs after Bet A-induced depolarization of mitochondria similar to caspase-9 activation that occurs after CD437 and 4-HPR treatments. BA inhibits caspase-9 activity following retinoid or Bet A treatments. This indicates that mitochondrial depolarization by the synthetic retinoid treatment activates the initiator caspase, caspase-9.

Caspase-9 is activated after mitochondrial depolarization and we hypothesize that it is the activator of caspase-3 following CD437 or 4-HPR treatment. Figure 8 shows that caspase-3 activity could be detected in the CA-OV-3 ovarian carcinoma cell line in response to CD437 and 4-HPR and this activation could be blocked by an inhibitor of mitochondrial membrane depolarization, BA and the caspase-9

Fig. 5. Mitochondrial membrane depolarization after treatment with the apoptosis inducing retinoids, CD437 and 4-HPR. $DIOC_{(6)}$ staining was performed as described in the Materials and Methods. Ovarian carcinoma cells were treated for 12 h with DMSO, or CD437 (10^{-6} M), or 4-HPR (10^{-5} M) and then stained with $DIOC_{(6)}$ to determine mitochondrial membrane potential. The lack of retention of $DIOC_{(6)}$ (Green) occurs in mitochondria with a loss of membrane potential which is indicative of apoptosis induction. Hoechst 33342 (Blue) is used as a counter stain of the nucleus. CA-OV-3 cells treated with DMSO (**A**) or CD437 (10^{-6} M) (**B**), or 4-HPR (10^{-5} M) (**C**). SK-OV-3 cells treated with DMSO (**D**), or CD437 (10^{-6} M) (**E**), or 4-HPR (10^{-5} M) (**F**).

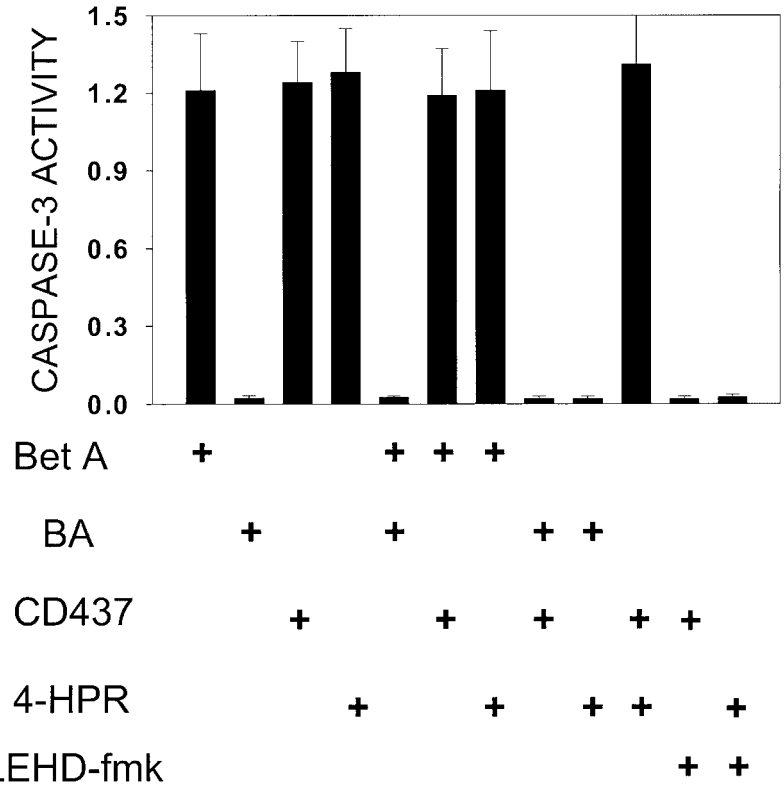

Fig. 8. CD437 and 4-HPR induced mitochondrial depolarization and caspase-9 activation is essential for caspase-3 activation in ovarian tumor cells. CA-OV-3 ovarian carcinoma cells were seeded onto 100 mm tissue culture plates at a density of 2×10^4 cells/plate. The cells were incubated for 48 h prior to treatment. One-hour prior to retinoid treatment either 10 μg/ml of the Bet A an inducer of mitochondrial membrane depolarization or 50 μM BA an inhibitor of mitochondrial depolarization or LEHD-fmk a caspase-9 inhibitor was added to the media. Retinoid treatments with either 10^{-6} M CD437 and/or 10^{-5} M 4-HPR were carried out for 12 h. Whole cell lysate from approximately 4×10^6 ovarian cells was collected and caspase-3 activity in cell extracts was measured by the cleavage of the colorimetric caspase substrate Ac-DEVD-pNA. Caspase-3 activity is expressed as absorbance at 405 nm. The data represent the mean ± SD of three separate experiments performed in triplicate.

inhibitor LEHD-fmk. This mechanism of mitochondrial depolarization causing activation of caspase-9 and subsequent activation of caspase-3 was reproduced in the SK-OV-3 cell line with almost identical results (data not shown). Furthermore, these data indicate that the synthetic retinoids CD437 and 4-HPR induce apoptosis through the depolarization of the mitochondrial membrane. This depolarization is shown to cause caspase-9 activation, which in turn activates caspase-3 leading to apoptosis.

TR3 Translocates to the Cytosol and Targets Mitochondria After CD437 Treatment but not After 4-HPR Treatment

TR3 is an orphan receptor of the steroid/thyroid receptor superfamily that has been reported to be under the transcriptional control of MEF2. We have previously reported that TR3 mRNA increases after CD437 treatment [Holmes et al., 2002]. This finding is supported by reports in other cell models that TR3 mRNA is induced during apoptosis and after CD437 treatment [Woronicz et al., 1994; Li et al., 2000; Liu et al., 2002; Zhang, 2002]. TR3 has been reported to translocate to the cytosol during apoptosis [Katagiri et al., 2000; Li et al., 2000; Liu et al., 2002]. Association of TR3 with mitochondria has been reported to cause the depolarization of the mitochondrial membrane and subsequent release of pro-apoptotic proteins such as cytochrome c [Li et al., 2000]. Recently, we have found that TR3 translocates from the nucleus to the cytosol and associates with mitochondria in CA-OV-3 cells in response to CD437 treatments (submitted for review). In Figure 9, we demonstrate that TR3 translocates from the nucleus to the cytosol and associates with mitochondria in the CA-OV-3 ovarian carcinoma cell line in response to

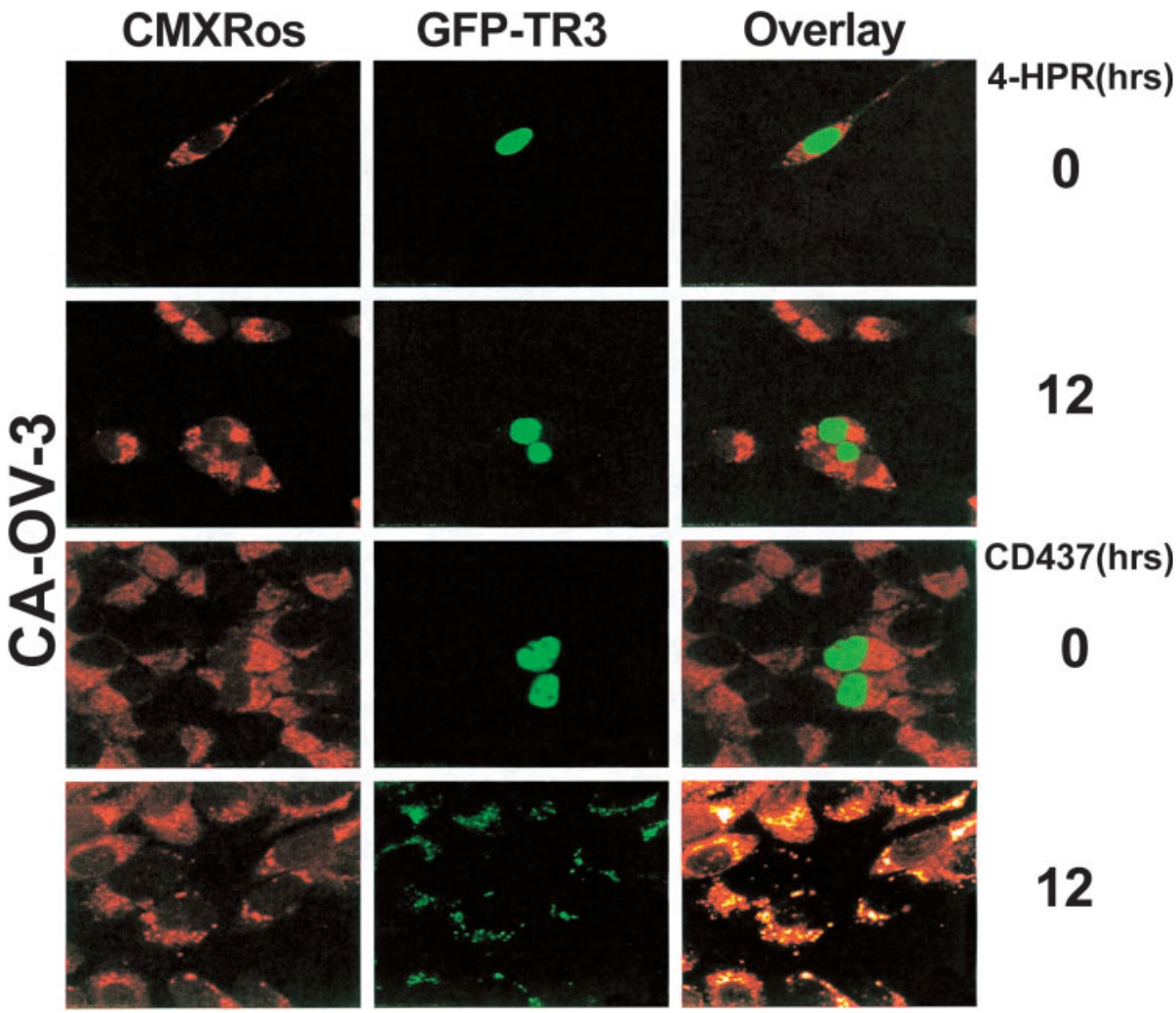

Fig. 9. CD437 treatments induce TR3 targeting of the mitochondria. CA-OV-3 ovarian carcinoma cells, cultured on cover slips in 60 mm plates, were transfected with the pGFP-TR3 construct. The cells were treated with DMSO, or CD437 (10^{-6} M), or 4-HPR (10^{-5} M) 24-h after transfection. Treatments were performed for 12 h. After retinoid treatment the cells were stained with the mitochondrial stain CMXRos. The cells were then fixed for 30 min in 4% paraformaldehyde. GFP (Green) and CMXRos (Red) were visualized using confocal microscopy, and the two images were overlayed (Overlay). TR3 translocates from the nucleus to the cytosol and associates with mitochondria (Yellow) after CD437 or 4-HPR treatments.

CD437 treatment. In cells with no CD437 treatment the GFP-TR3 protein was sequestered in the nucleus. In cells expressing GFP-TR3, all of the GFP-TR3 protein (green) was associated with mitochondria stained by CMXRos (red) resulting in a yellow color at 12 h of CD437 treatment. In contrast, 4-HPR treatment of CA-OV-3 cells expressing GFP-TR3 protein did not induce translocation of TR3 after 12 h of treatment. These data indicate that 4-HPR and CD437 activate separate pathways leading to the mitochondrial depolarization associated with apoptosis induced by these two retinoids in ovarian carcinoma cell lines.

DISCUSSION

Physiological mechanisms for the removal of abnormal cells are a fundamental requirement for maintaining healthy ovarian function [Tilly, 1996; Morita and Tilly, 1999]. Apoptosis, or programmed cell death, is a physiological mechanism for the healthy maintenance of some tissues. In theory, failure of the molecular mechanisms responsible for the physiological induction of apoptosis may result in tumorogenisis of ovarian cells [Ghahremani et al., 1999; Morita and Tilly, 1999]. It may be possible to restore or induce the disrupted apoptotic

mechanisms within the ovarian tumor cells through the use of chemotherapeutic reagents.

Natural retinoids, for the most part, do not induce apoptosis. In this report we have investigated two synthetic retinoids that are reported as potent inducers of apoptosis, CD437 and 4-HPR. Our results demonstrate that CD437 and 4-HPR induced growth inhibition and apoptosis in both CA-OV-3 and SK-OV-3 ovarian carcinoma cell lines. This indicates that, although these retinoids bind RARs, the growth inhibitory mechanism induced by these retinoids may not be the mechanism employed by natural retinoids.

The CA-OV-3 and SK-OV-3 cell lines are reported to have mutated p53. P53 protein is responsible for determination of whether a cell will undergo growth arrest and DNA repair or the induction of apoptosis in response to DNA damage. Treatment of tumor cells with CD437 or 4-HPR may be able to override or circumvent the block in the normal path leading to apoptosis commonly observed in many tumor cells. Since p53 and other proteins which control apoptosis and growth arrest are often mutated in the majority of tumor cells, it is important to understand the molecular mechanisms induced by these retinoids to further the ability to create chemotherapeutic agents for the treatment and prevention of cancer.

Studies designed to elucidate the molecular events, which constitute the apoptotic pathway induced by CD437 and 4-HPR, have suggested that the mechanism of action of these two retinoids is extremely complex and highly contradictory. For example, Adachi et al. [1998] showed that CD437 could induce growth arrest and apoptosis in Jurkat and Molt-4 cells via both caspase-dependent and caspase independent pathways, although, many do report the existence of caspase activity after CD437 treatment [Adachi et al., 1998; Marchetti et al., 1999; Mologni et al., 1999; Sun et al., 1999; Holmes et al., 2002; Zhang et al., 2002]. Likewise, many have reported on the need for caspase activation for 4-HPR induced apoptosis [Piedrafita and Pfahl, 1997; Maurer et al., 1999; Suzuki et al., 1999]. These reports indicate that activation of caspases in response to these retinoids may be cell type specific or a result of induction of other molecular mechanisms. Caspase activity is a hallmark of the induction of apoptosis. In light of these facts we needed to assay for the activation of the caspase cascade and determine if the caspases activated were essential for CD437 and 4-HPR induced apoptosis of ovarian carcinoma cells.

Caspase-3 activity is indicative of the final stages of apoptosis induction associated with apoptosis via the caspase cascade. The synthetic retinoids, CD437 and 4-HPR induced caspase-3 activity in the CA-OV-3 and the SK-OV-3 ovarian carcinoma cell lines. Using caspase-3 inhibitors, we determined that caspase-3 activation was essential for the induction of apoptosis by both CD437 and 4-HPR in both CA-OV-3 and SK-OV-3 cell lines.

Caspase activation occurs through the activation of a cascade of events transferring the death signal from initiator caspases to effector caspases [Schulze-Osthoff et al., 1998; Wolf and Green, 1999; Hingorani et al., 2000; Schneider and Tschopp, 2000]. Caspase-3 is an effector caspase, meaning that it is activated by upstream caspases known as initiator caspases [Schneider and Tschopp, 2000; Chun et al., 2001]. Caspase-8 is a very important initiator caspase that is associated with cell surface death receptors through death domain proteins [Wolf and Green, 1999; Schneider and Tschopp, 2000]. Active caspase-8 targets caspase-3. We did not detect any appreciable caspase-8 activity after treatment with either CD437 or 4-HPR. We, also, determined that caspase-8 was not necessary for the induction of apoptosis by either CD437 or 4-HPR. This finding was not too surprising since retinoids are not known to interact with cell surface signal receptors. These findings indicated that we needed to observe a different pathway for the induction of caspase-3 activity.

Caspase-9 is an initiator caspase associated with mitochondria that has been characterized as an activator of caspase-3 [He et al., 2000; Perkins et al., 2000; Cao et al., 2002]. Determinations of caspase-9 activity were positive in response to treatments with either CD437 or 4-HPR in both CA-OV-3 and SK-OV-3 cell lines. Caspase-9 activation was determined to be essential for the induction of apoptosis by both of these synthetic retinoids. This finding indicated that the pathway for the induction of apoptosis might involve the depolarization of the mitochondrial membrane.

The depolarization of mitochondria in response to apoptotic inducing agents, such as CD437 and 4-HPR, has been reported to be necessary for the activation of caspase-9 and

caspase-3 [Marchetti et al., 1999; Mologni et al., 1999; Suzuki et al., 1999; Costantini et al., 2000; Eldadah and Faden, 2000; Tartier et al., 2000]. The depolarization of the mitochondrial membrane allows for the release of the pro-apoptotic proteins cytochrome c, procaspase-9, and APAF-1. These three proteins are able to associate causing the cleavage of procaspase-9 to active caspase-9. Active caspase-9 is then able to cleave procaspase-3 to active caspase-3. We wanted to determine if this mechanism was responsible for the activation of caspase-9 and caspase-3 in ovarian cancer cells treated with CD437 or 4-HPR.

The determination of the mitochondrial membrane depolarization required the use of two chemicals, BA and Bet A. BA inhibits the depolarization of mitochondrial membranes, whereas, Bet A induces the depolarization of mitochondrial membranes inducing apoptosis through the activation of caspase-9 and caspase-3 [Fulda et al., 1997, 1998, 1999; Raisova et al., 2001]. Using these chemicals, we determined that CD437 and 4-HPR required the depolarization of the mitochondrial membrane to induce apoptosis, activate caspase-9, and activate caspase-3. This confirms that CD437 and 4-HPR require the same mechanism for the final stages of apoptotic induction.

Furthermore, we were able to determine that the activation of caspase-3 was directly dependent on caspase-9 activation, due to mitochondrial depolarization by CD437 and 4-HPR, using a caspase-9 inhibitor (LEHD-cmk). This allows us to map the order of events in the late stages of apoptosis induction by CD437 and 4-HPR: mitochondrial depolarization leading to caspase-9 activation followed by the subsequent activation of capase-3.

TR3 is an orphan receptor of the steroid/thyroid receptor superfamily that is present in the nucleus as a transcription factor but has been shown to translocate to the cytoplasm and cause the depolarization of the mitochondrial membrane [Li et al., 2000; Dawson et al., 2001; Holmes et al., 2002; Zhang, 2002]. This translocation and association has been reported to be a mechanism for the induction of mitochondrial depolarization and subsequent apoptosis in response to apoptosis inducing agents, such as CD437 [Li et al., 1998; Zhang, 2002]. We show that GFP-TR3 is sequestered in the nucleus before treatment with either CD437 or 4-HPR. CD437 treatment induces the translocation of TR3 to the cytosol and subsequent association with mitochondria in ovarian carcinoma cells. In contrast, 4-HPR does not induce TR3 translocation indicating that separate pathways are utilized by these synthetic retinoids to induce mitochondrial depolarization and subsequent apoptosis in ovarian carcinoma cell lines.

In conclusion, we have determined that the late stages of induction of apoptosis by both CD437 and 4-HPR involved the same pathway (summarized in Fig. 10). Treatment of ovarian carcinoma cell lines with either synthetic retinoid induced apoptosis through the depolarization of the mitochondrial membrane. Depolarization of the mitochondrial membrane causes the release of caspase-9, cytochrome c, and APAF-1. These three proteins associate and induce activation of caspase-9. Active casape-9 activates the effector caspase, casape-3. Activation of caspase-3, by treatment with these retinoids, results in growth arrest and apoptosis. Interestingly, CD437 induces mitochondrial depolarization via a separate pathway from that of 4-HPR. Previously, we have found that

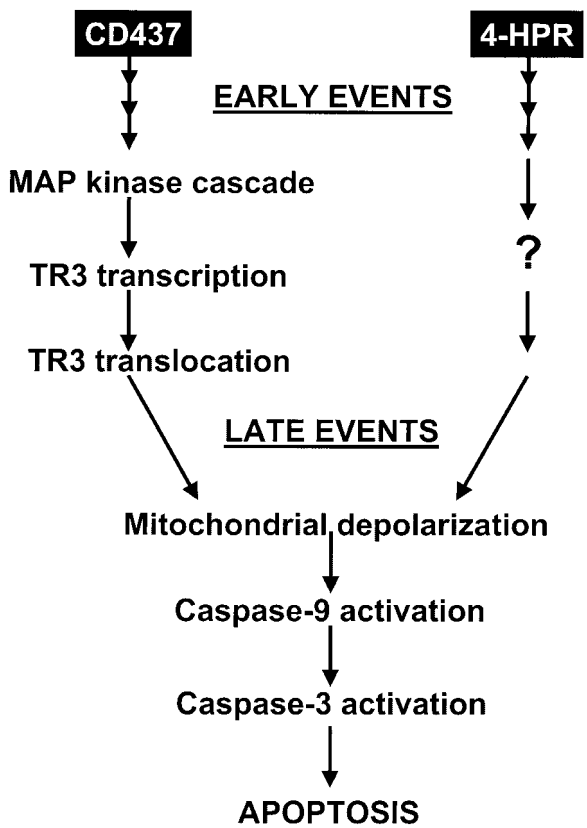

Fig. 10. Summary of the molecular events leading to the induction of apoptosis by treatment of ovarian carcinoma cells with CD437 or 4-HPR.

the early events of CD437 induced apoptosis required the MAP kinase pathway and the induction of TR3 transcription [Holmes et al., 2002]. Inhibitors of the MAP kinase pathway blocked the induction of apoptosis by CD437 but not the induction of apoptosis by 4-HPR in ovarian carcinoma cell lines. As depicted in Figure 10, we have not determined the early mechanisms by which 4-HPR induces mitochondrial depolarization. In this report, we show that TR3 translocates in response to CD437 treatment of ovarian carcinoma cells but not after treatment with 4-HPR. We have found, in our previous work and in this report, evidence that the early events induced by CD437 treatment are not induced by 4-HPR treatment of ovarian carcinoma cell lines.

These findings expand our understanding of the relation between the structures of these conformationally restricted synthetic retinoids and the molecular mechanisms activated by treatment with CD437 and 4-HPR to induce apoptosis. This may aid in the creation of other synthetic retinoids for the treatment of resistant carcinomas. Importantly, activation of separate pathways that result in the induction of apoptosis indicates that combination treatments with synthetic retinoids will prove to be more efficacious for the prevention and treatment of ovarian cancer.

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