Journal of Cellular Biochemistry 68:378–388 (1998)

Effects of Conformationally Restricted Synthetic Retinoids on Ovarian Tumor Cell Growth

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Abstract We have used conformationally restricted retinoids to investigate the role of individual RAR subtypes and RXR in mediating the growth response of ovarian tumor cells to retinoids. Our results show that treatment of all-*trans*-RA-sensitive CAOV-3 cells with retinoids that bind and activate a single RAR or RXR led to a partial inhibition of growth. Treatment of all-*trans*-RA- resistant SKOV-3 cells did not alter growth. Maximum inhibition of growth, comparable to that observed following treatment with natural retinoids such as all-*trans*-RA and 9-*cis*-RA, was obtained only following treatment with a combination of an RAR-selective compound and an RXR-selective one. These results suggest that activation of both RAR and RXR classes is required in order to obtain maximum inhibition of ovarian tumor cell growth by retinoids. In addition, one compound, AHPN, was found to inhibit both RA-sensitive CAOV-3 and RA-resistant SKOV-3 cells. Further study of the effects of this retinoid showed that AHPN acts through an apoptotic pathway. Taken together, our results suggest that retinoids may serve as effective anti-proliferative agents in the treatment of ovarian cancer. J. Cell. Biochem. 68:378–388, 1998. © 1998 Wiley-Liss, Inc.

Key words: apoptosis; growth suppression; retinoic acid receptors; ovarian cancer; AHPN

Retinoids are powerful regulators of cell proliferation and differentiation [Kastner et al., 1995; Means and Gudas, 1995]. During the past decade, with the discovery of the retinoid receptor families, our understanding of the mechanism of retinoid function has increased considerably [Mangelsdorf et al., 1994, 1995a,b]. The major biological activity of the retinoids is mediated by two families of nuclear receptors: retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [Kastner et al., 1995; Mangelsdorf et al., 1995a,b; Means and Gudas, 1995]. There are three subtypes (α, β, γ) of each of these classes of nuclear receptors. These nuclear receptors can form dimers, bind to specific DNA consensus sequences termed retinoid receptor response elements (RAREs and RXREs), and regulate the expression of target genes [Gudas et al., 1994]. The RARs bind to the natural retinoids all-*trans*-RA and 9-*cis*-RA with high affinity, whereas the natural ligand for RXRs is 9-*cis*-RA [Heyman et al., 1992; Leid et al., 1992; Levin et al., 1992]. However, the ability of these natural retinoids to bind to the subtypes, and to isomerize makes it difficult to study the role of individual RAR and RXR subtypes in mediating the biological effects of retinoids. A number of conformationally restricted synthetic retinoids can be used to address this problem [Dawson et al., 1995a,b]. These retinoids, whose bonds corresponding to the double bonds of the natural retinoids are included in aromatic rings

Contract grant sponsor: National Institutes of Health; Contract grant number: A 64945; Contract grant number: DK 49045; Contract grant number: HD 27556; Contract grant number: DK 44517; Contract grant number: P01 CA 51993; Contract grant sponsor: Research Career Development Award; Contract grant number: HD 01076.

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Received 22 September 1997; Accepted 17 October 1997

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and, therefore, constrained in a particular configuration to prevent isomerization, selectively bind and transcriptionally activate individual RAR subtypes and RXR [Lehmann et al., 1991, 1992]. Thus, these compounds are valuable reagents that permit the elucidation of the role of individual RARs and RXRs in mediating cellular responses to retinoids.

The relationship between retinoids and cancer is well established. Retinoids have been demonstrated to inhibit the development of a number of different tumor types and the growth of a variety of transformed cells [for review, see Fountzilas, 1994; Hofmann, 1992; McBurney et al., 1993]. Recently, a number of studies have shown that retinoids have a growth inhibitory effect on ovarian tumor cells [Grunt et al., 1991; Harant et al., 1993; Caliaro et al, 1994; Wu et al., 1997a,b]. We and others have recently shown that all-*trans*-RA can inhibit the growth of the ovarian tumor cell line, CAOV-3 but not the SKOV-3 cell line [Wu et al., 1997a,b; Caliaro et al, 1994]. We now have used the conformationally restricted, synthetic retinoids to examine the role of individual RAR subtypes and RXR in mediating the growth response of ovarian tumor cells to retinoids. Our results suggest that for maximum inhibition of ovarian tumor cell growth by retinoids, activation of both RARα and RXR is required. Finally, evidence is provided that at least one of these conformationally restricted retinoids, AHPN, inhibits the growth of both RA-sensitive and RA-resistant ovarian tumor cells through a unique mechanism that appears to involve apoptosis.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

CAOV-3 and SKOV-3 cells were obtained from the American Type Culture Collection (Rockville, MD). All 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. The SKOV-3 cells were routinely split at a ratio of 1:10 weekly and the CAOV-3 cells were split at 1:8 ratio weekly. The plates were then placed in a 98% humidified, 5% CO_2 incubator.

Retinoids

All-*trans*-RA and 9-*cis*-RA were kindly supplied by Hoffmann-La Roche at Nutley, New Jersey. TTAB (RAR-selective) and Am580 (RARα-selective), SR11246, and SR11237 (RXR-selective), SR11238 and SR11302 (anti-AP-1), AHPN (RARγ-selective), and SR11253 (RARγ-selective antagonist) have been described previously [Dawson et al., 1989, 1995; Bernard et al., 1992; Fanjul et al., 1994; Kagechika et al., 1988; Chao et al., 1996]. Retinoid stock solutions (10^{-3} M) in ethanol were prepared fresh every two weeks. All procedures involving retinoids were carried out under subdued light. In each experiment, control cultures were treated with an equivalent amount of ethanol.

Cell Proliferation Assay

Cell growth and viability were determined by the MTT assay [Berg et al., 1990]. Cells were seeded in 96-well flat-bottomed plates at a density of 6,000 cells/well. Two days later, the medium was changed and the cells were treated. The medium with and without retinoids was routinely changed every two days. On the last day after treatment, the medium was completely removed from all wells, and 125 µl reaction buffer (containing 100 µl complete DMEM and 25 µl 5 mg/ml MTT) was added to each well. Following a 2-h incubation in a CO_2 incubator at 370C, 100 µl extraction buffer (20% w/v SDS dissolved in a 50% solution of N,N-dimethyl formamide (DMF) in demineralized water, pH adjusted to 4.7) was added to each well. The plates were incubated at 37^{0}C overnight. Absorbance at 590 nm was measured using a Bio-Tek 96-well microplate reader, employing the extraction buffer as the blank control. All assays were performed in triplicate and repeated at least three independent times. Data are expressed as the mean ± standard deviation (SD).

Apoptosis ELISA

The method described by Salgame et al. [1997] was used. Ovarian cancer cells were seeded in 24-well plates. At different days after retinoid treatment, cells were trypsinized, pelleted, counted, and resuspended in ice-cold lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris, pH 7.5) at a concentration of 2.5×10^6 cells/ml. After 30 min incubation on ice, the lysate was spun at 1,500*g* for 5 min, and the supernatant was collected. Enzyme-linked immunosorbent assay (ELISA) was performed as follows: polyvinylchloride 96-well plates (Falcon) were coated with 100 ng/well of LG11-2 capture antibody, a

monoclonal antibody that recognizes the residues 1–25 of the N-terminal domain of histone H2B in the nucleosome. After a 2-h incubation at room temperature, plates were washed three times with phosphate-buffered saline (PBS)-0.05% Tween-20 (PT) and blocked with 50-μl/well PBS, containing 1% bovine serum abumin (BSA), 0.05% Tween-20, 0.02% sodium azide (PBTN) for 1 h at room temperature. Following three washes in PT, 50 μl of cytoplasmic lysate diluted in PBTN (6 × 103 cells/50 μl) was added to each well, and incubation was continued at room temperature for 2 h. Plates were washed four times with PT and incubated for 1.5 h at room temperature with 125 μl/well of N-hydroxysuccinimidobiotin-biotinylated (Pierce, Rockford, IL) PL2-3 detection monoclonal antibody, which is specific for the nucleosome subparticle composed of histones H2A, H2B, and DNA . Following three washes with PT, 50 μl/well alkaline phosphatase-conjugated streptavidin (Southern Biotechnology Associates, Birmingham, AL) diluted 1:2,000 in PBTN was added. After 30 min, the plates were washed three times in PT and then twice in substrate buffer (10 mM diethanolamine, 0.5 mM $MgCl_2$), treated with p-nitrophenylphosphate (150 μl/well of 1 mg/ml substrate buffer) and permitted to develop in the dark at room temperature for 1 h. Absorbance was then measured at 405 nm, using a Bio-Tek 96-well microplate reader.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Ovarian cancer cells were treated with either AHPN or all-*trans*-RA at a concentration of 10^{-6} M for 0, 1, 2, and 3 days. Total cellular RNA was then extracted using RNAzol B (Tel-Test, Friendswood, TX) according to the manufacturer's instructions as described previously [Pena et al., 1993]. RNA was quantitated by absorbance at 260 nm and purity assessed by absorbance at 280 nm.

RT was performed to synthesize cDNA by employing SUPERSCRIPT RT RNase H- Reverse Transcriptase (Gibco BRL, Gaithersburg, MD) with 5 μg of total RNA and 500 ng oligo-dT (Promega). For PCR, 1 out of 20 μl from each RT reaction was used. 5′ and 3′ primers for Bcl-2 cDNA were 5′-GACTTTGCCGAGATGTCCAGCCAG-3′ and 5′-CAAACTGAGCAGAGTCTTGAGAGA-3′, respectively. 5′ and 3′ primers for Bax cDNA were: 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. The β-actin cDNA, used as a normalization control, was amplified using the 5′ primer: 5′-CATTGTGATGGACTCCGGAGACGG-3′ and the 3′ primer: 5′-CATCTCCTGCTCGAAGTCTAGAGC-3′. The β-actin cDNA was used in order to normalize each sample for variation in quality and quantity of RNA. After PCR, 1/10 of each reaction mixture was run on a 10% polyacrylamide gel, which was stained with ethidium bromide, visualized on a UV transilluminator, and photographed.

RESULTS

Effect of Natural and Synthetic Retinoids on the Growth of CAOV-3 and SKOV-3 Cells

We have previously shown that the ovarian cell line CAOV-3 is sensitive to growth inhibition by all-*trans*-RA, while the cell line SKOV-3 is resistant. The role of individual RARs and RXR in mediating the inhibition of ovarian tumor cell growth by retinoids was examined by the use of a series of conformationally restricted synthetic retinoids. These synthetic retinoids have previously been shown to bind and activate specific RAR subtypes and RXR. The specificity of these retinoids is shown in Table I. Figure 1 shows the growth response of CAOV-3 cells following treatment for 7 days with each of these individual retinoids at concentrations ranging from 10^{-10} to 10^{-6} M with growth determined by a MTT assay. We found that treatment of CAOV-3 cells with 9-*cis*-RA, which at this concentration is known to activate both RARs and RXRs, resulted in a level of growth inhibition which was comparable or even slightly greater than that of all-*trans*-RA (Fig. 1A). In contrast, the RARγ antagonist SR11253 showed virtually no inhibition of growth. Likewise, the RXR-selective retinoids, SR11246 and

TABLE I. Selectivity of Conformationally Restricted Synthetic Retinoids

Retinoids	Selectivity	Reference
TTAB	RAR	Dawson et al. [1989]
Am580	RAR-α	Graupner et al. [1991]
SR11246	RXR	Dawson et al. [1995]
SR11237	RXR	Lehmann et al. [1992]
SR11238	Anti-AP1	Fanjul et al. [1994]
SR11302	Anti-AP1	Fanjul et al. [1994]
AHPN	RAR-γ (agonist)	Bernard et al. [1992]
SR11253	RAR-γ (antagonist)	Chao et al. [1996]

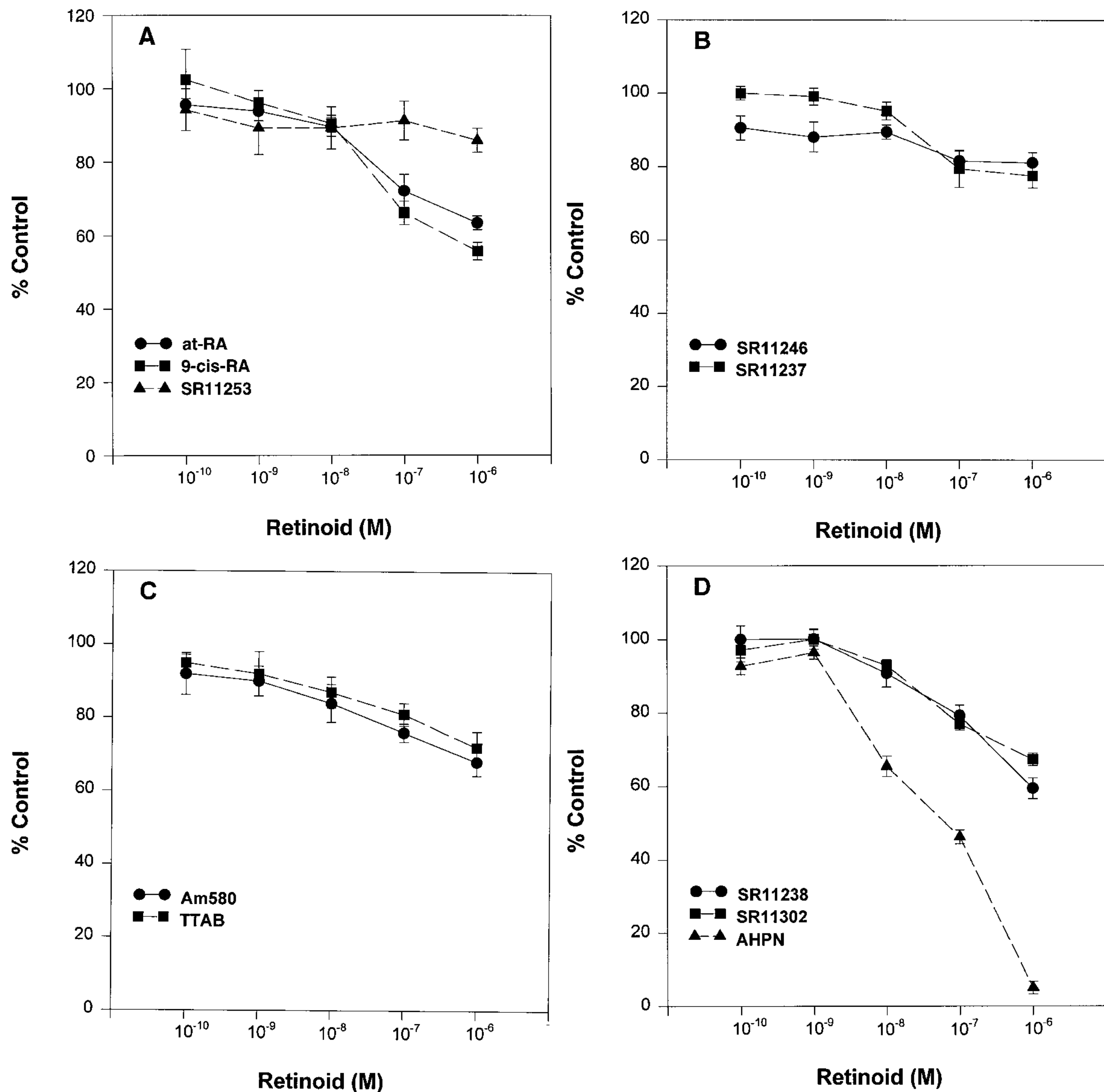


Fig. 1. Effect of natural and synthetic retinoids on the growth of CAOV-3 and SKOV-3 ovarian cancer cells. Ovarian cancer cells were seeded in 96-well plates at a density of 6,000 cells/well. Two days after plating, the cells were exposed to different retinoids at a concentrations ranging from 10^{-6} to 10^{-10}M. The MTT assay was performed at day 7 after treatment. Controls were treated with an equal volume of ethanol. Data are expressed as % control ± standard deviation. **A:** all-trans-RA (-●-), 9-cis-RA (-■-); SR11253 (-▲-). **B:** SR11246 (-●-), SR11237 (-■-). **C:** TTAB (-■-); Am580 (-●-). **D:** SR11238 (-●-), SR11302 (-■-), AHPN (-▲-).

SR11237, only exhibited minimal growth inhibitory activity (~20%) at the highest concentrations (Fig. 1B). Treatment with either RAR-selective TTAB or RARα-selective Am580 resulted in a significant reduction in CAOV-3 cell growth (~30%); however, the extent of inhibition was considerably less than that of all-*trans*-RA (Fig. 1C). The compounds SR11238 and SR11302, which are reported to exhibit anti-AP-1 activity [Fanjul et al., 1994], also inhibited CAOV-3 cell growth to an extent comparable to the RAR-selective retinoids (Fig. 1D). Interestingly, 6-[3-(1-adamantyl-4-hydroxyphenyl]-2-naphthalenecarboxylic acid (AHPN), a retinoid previously shown to induce Waf-1/p21/CIP1 [Shao et al., 1995], showed the highest amount of growth inhibition (~90%), which was significantly greater than that routinely observed with all-*trans*-RA and 9-*cis*-RA (~50%) (Fig. 1D).

It should be noted that when we treated all-*trans*-RA-resistant SKOV-3 cells with this

battery of retinoids, we found that the SKOV-3 cells were resistant to all of these compounds except AHPN. AHPN inhibited SKOV-3 cell growth to virtually the same extent as it inhibited CAOV-3 growth (90%).

Inhibition of Ovarian Tumor Cell Growth by Treatment With a Combination of RAR-Selective and RXR-Selective Ligands

Since maximal RARE-dependent transcriptional activation requires formation of heterodimers between RARs and RXRs and isomerization commonly occurs with the natural retinoids all-*trans*-RA and 9-*cis*-RA, we next examined the effect of treating CAOV-3 cells with a combination of synthetic retinoids that would activate both RAR and RXR. Figure 2 shows that while the RXR-selective retinoid SR11246 alone failed to inhibit CAOV-3 and the RAR-selective TTAB and Am580 inhibited growth by only approximately 20–30%, treatment with the combination of SR11246 with TTAB or Am580 inhibited the growth of CAOV-3 cells to the same extent as all-*trans*-RA. This result confirms that for maximum inhibition of ovarian tumor cell growth by retinoids, interaction of these retinoids with both RAR and RXR is required.

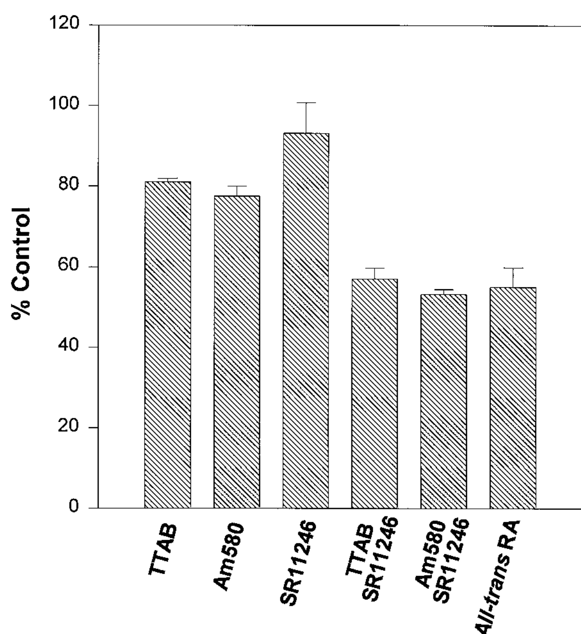


Fig. 2. Effect of RAR- and RXR-selective ligands on the inhibition of ovarian cancer cell growth. Ovarian cancer CAOV-3 cells were seeded in 96-well plates as described in the legend to Fig. 1. Two days after plating, the cells were treated with either RAR-selective (TTAB or Am580), RXR-selective (SR11246) or a combination of TTAB or Am580 with SR11246 at a final concentration of 10^{-6} M. The MTT assay was performed 6 days after treatment. Controls were treated with an equal volume of ethanol. The data are expressed as percentage control ± standard deviation.

Dose-Dependent Inhibition of CAOV-3 and SKOV-3 Cells Growth by the Synthetic Retinoid AHPN

It was notable that SR11248 was the only retinoid that inhibited the growth of both RA-sensitive and RA-resistant cell lines and did so to an extent which was significantly greater than that of all-*trans*-RA or 9-*cis*-RA. To investigate this further, we performed dose-response experiments on CAOV-3 and SKOV-3 cells treated with various concentrations of AHPN ranging from 33.7 to 1,000 nM. After 6 days of treatment, the growth of the cells was determined by the MTT assay. Figure 3A,B shows that treatment of both cell lines with concentrations of AHPN as low as 125 nM resulted in an inhibition of growth. Determination of an IC_{50} for each cell line showed that the CAOV-3 cells were slightly more sensitive (IC_{50} = 300 nM) than the SKOV-3 cells (IC_{50} = 550 nM).

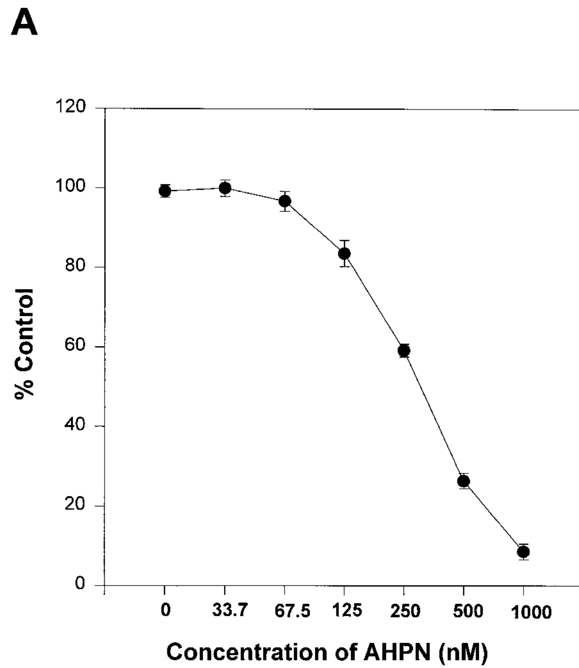
Time Course of Growth Inhibition of CAOV-3 and SKOV-3 Cells by the Synthetic Retinoid AHPN

We next performed a time-course experiment. Figure 4A,B shows that as early as 3 days after treatment with AHPN, both cell lines exhibit a reduction in growth. It is important to note that by 6 days of treatment, cell numbers were significantly reduced not only in comparison to the ethanol-treated controls, but also in comparison to the starting cell number on day 0. This observation suggested that treatment of both CAOV-3 and SKOV-3 cells with AHPN resulted in cell death.

Induction of Apoptosis by Treatment of CAOV-3 and SKOV-3 Cells With AHPN

In order to determine whether AHPN was causing apoptosis in these ovarian tumor cell lines, we next performed an apoptotic ELISA assay. This assay specifically detects and quantitates the presence in the cytoplasm of nucleosome subpopulations composed of histones H2A, H2B, and DNA. Figure 5A,B shows that AHPN treatment of CAOV-3 cells and SKOV-3 cells resulted in significant increases in the apoptotic index beginning 3 days after treatment.

A



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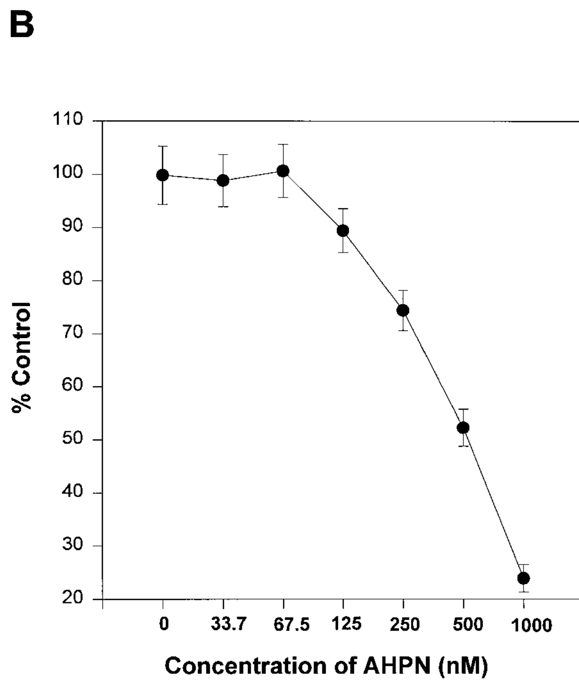
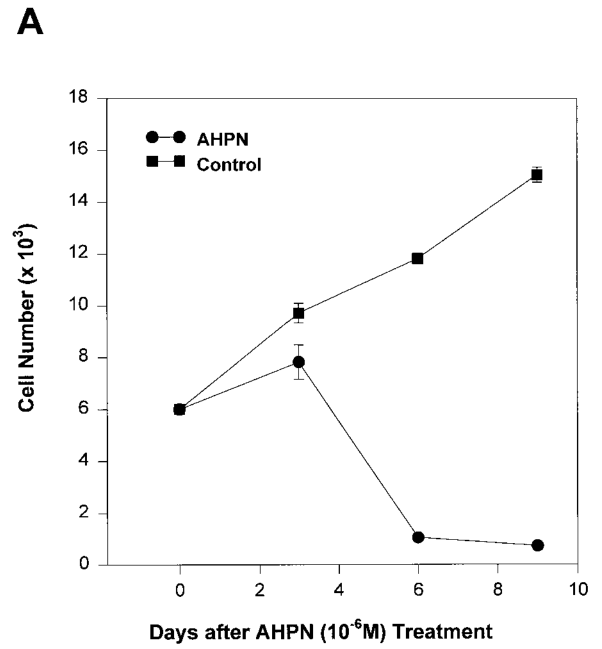


Fig. 3. Dose-dependent inhibition of CAOV-3 and SKOV-3 cell growth by the synthetic retinoid AHPN. Ovarian cancer cells were seeded in 96-well plates and treated with various concentrations of AHPN as described in Materials and Methods. The MTT assay was performed 6 days after treatment. Controls were treated with an equal volume of ethanol. The data are expressed as % control ± standard deviation. **A:** CAOV-3. **B:** SKOV-3.

A



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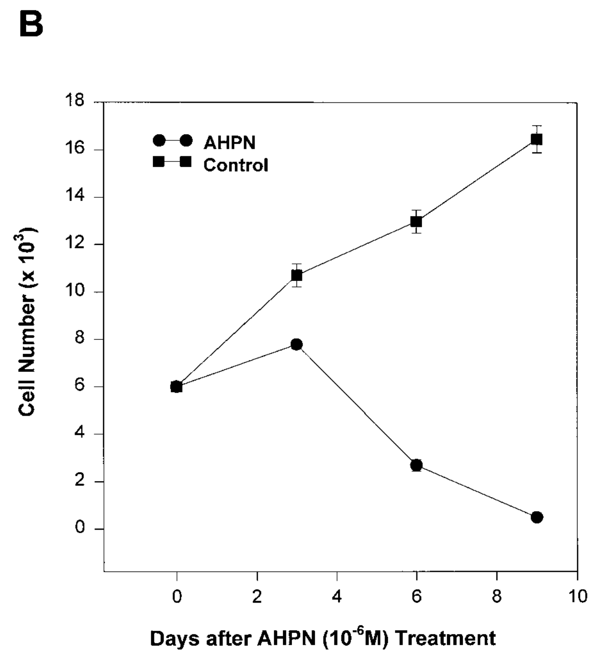

Fig. 4. Time course of growth inhibition of CAOV-3 and SKOV-3 cells by AHPN. Ovarian cancer cells were plated and treated with AHPN as described in Fig. 1. At days 0, 3, 6, and 9 after treatment, the cells were processed and the MTT assay was performed. Controls were treated with an equal volume of ethanol. The average O.D. values ± standard deviation are reported. **A:** CAOV-3; **B:** SKOV-3.

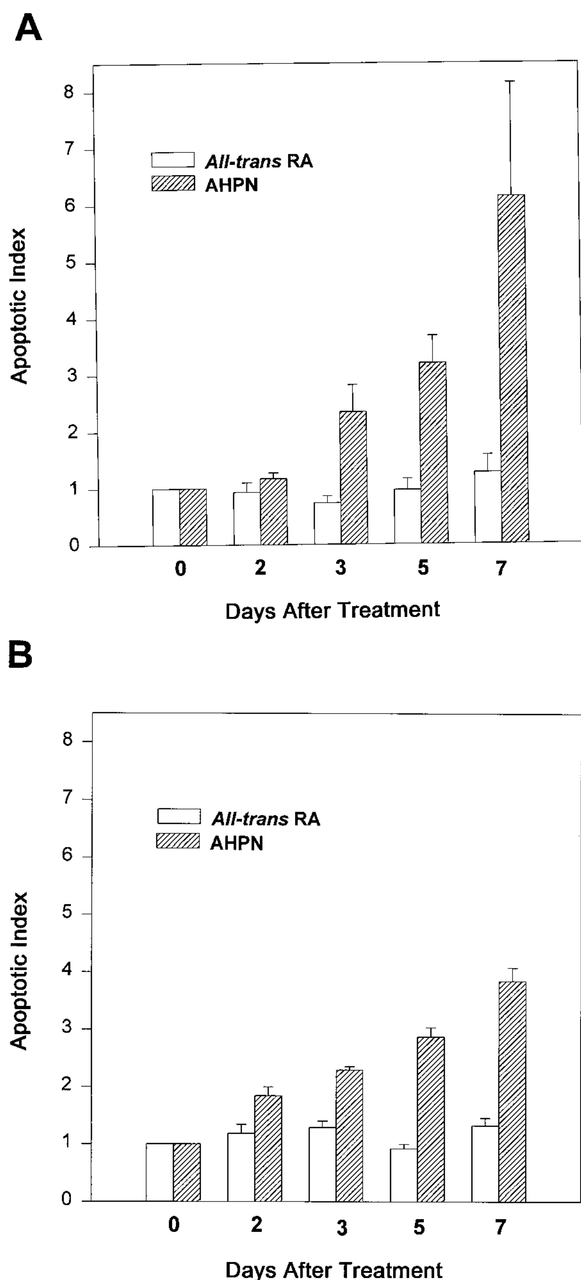

Fig. 5. Induction of apoptosis by treatment of CAOV-3 and SKOV-3 cells with AHPN. Ovarian cancer cells were seeded in 24-well plates and were treated with either all-*trans*-RA or AHPN (10^{-6} M): Cells treated with an equal volume of ethanol were used as a negative control. The apoptosis ELISA was performed on cells harvested 2, 3, 5, and 7 days after treatment as described in Materials and Methods. The apoptotic index of the control was arbitrarily set to a value of 1. The apoptotic indexes of retinoids on ovarian cancer cells were calculated by comparison with the control and expressed as index ± standard deviation. Each time point was performed in triplicate. **A:** CAOV-3. **B:** SKOV-3.

All-*trans*-RA was used as a negative control for these experiments, since we have previously established that all-*trans*-RA does not act through an apoptotic mechanism (Wu et al., 1997a]. It would appear that at least initially, both cell lines were equally sensitive to induction of apoptosis by AHPN. However, by 7 days of treatment the CAOV-3 cells exhibited an apoptotic index that is greater than that of the SKOV-3 cells.

To further prove that AHPN treatment induces apoptosis in ovarian tumor cell lines, we chose to examine the alteration in expression of two well-established molecular markers for apoptosis, Bcl-2 and Bax. Bcl-2 expression decreases during apoptosis while Bax expression increases [White, 1996; Nagata, 1997; Reynolds et al., 1994]. CAOV-3 and SKOV-3 cells were treated with 10^{-6}M of either all-*trans*-RA or AHPN. On various days after treatment, cells were harvested, total RNA was isolated and RT-PCR analysis performed. It can be seen in Figure 6A that all-*trans*-RA treatment of either cell line did not alter the steady-state levels of Bcl-2 or Bax. On the other hand, within 2 days of AHPN treatment of either cell line, Bcl-2 mRNA levels were clearly reduced. Conversely, Bax mRNA levels increased 2–3 days after AHPN treatment. These results confirm at a molecular level that the synthetic retinoid AHPN modulates the levels of apoptotic molecular markers consistent with the possibility that AHPN induces apoptosis in ovarian tumor cells.

DISCUSSION

Our studies have revealed the following new information about retinoid inhibition of ovarian tumor cell growth. First, maximum inhibition of growth requires interaction of a combination of both retinoid receptor classes (one RAR subtype and a RXR); interaction with of an RAR alone leads to only partial inhibition of growth, whereas interaction with RXR alone has no significant effect on growth. Second, since SR11238 and SR11302, which exhibit anti-AP-1 activity, but not transciptional activation from an RARE, were as effective as the RAR-selective transcriptional agonists at inhibition of growth, growth inhibition may at least in part proceed via a block of the AP-1 pathway rather than direct activation of RA-responsive genes. Third, AHPN was unique in that it inhibited the growth of both RA-sensitive and RA-

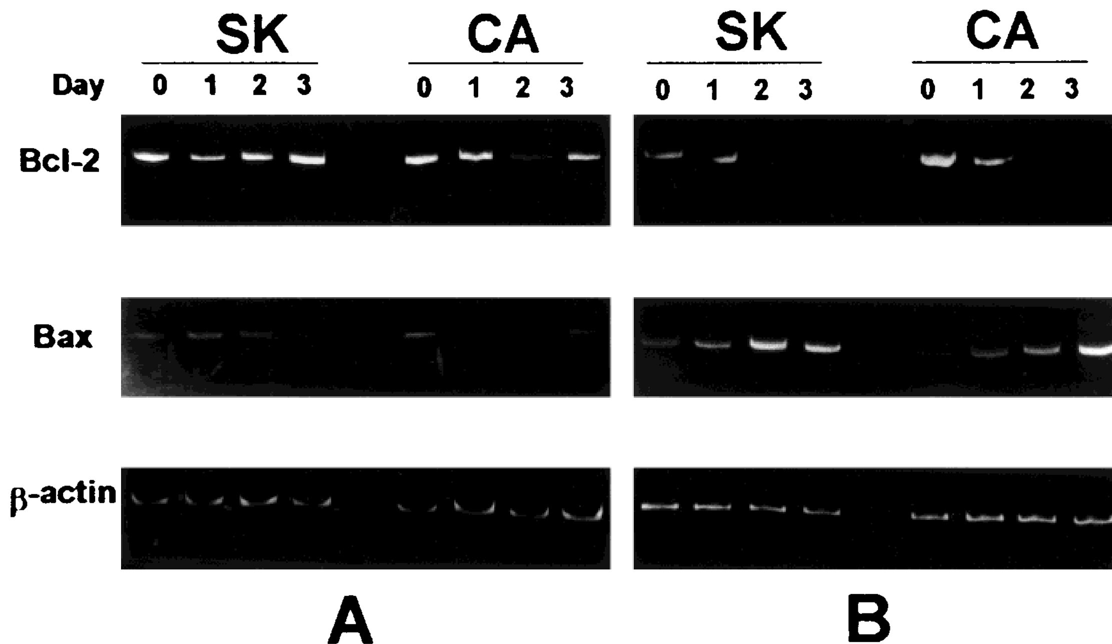

Fig. 6. RT-PCR analysis of the expression of apoptosis-related genes Bcl-2 and Bax in CAOV-3 and SKOV-3 cells treated with AHPN or all-*trans*-RA. Steady-state levels of Bcl-2 and Bax mRNA were determined by RT-PCR analysis using total RNA isolated from ovarian cancer cells on days 0, 1, 2, and 3 after treatment with either all-*trans*-RA (**A**) or AHPN (**B**). β-actin was used for normalization.

resistant ovarian tumor cells and did so to a significantly greater extent than all-*trans*-RA or 9-*cis*-RA. Fourth, follow-up studies assaying for expression of molecular markers for apoptosis and an apoptosis ELISA suggest that all-*trans*-RA inhibits ovarian tumor cell growth by a nonapoptotic mechanism, whereas AHPN uses an apoptotic pathway.

The finding that both RAR and RXR needed to be liganded in order to obtain maximum ovarian tumor cell growth inhibition was somewhat surprising in light of recent studies by Hembree et al. [1996], who showed that RXR-selective ligands antagonized the growth inhibitory effects of RAR-selective ligands on HPV-16-immortalized human cervical epithelial cells. In addition, a number of previous reports studying induction of gene expression by RA suggested that RXR was not liganded when RAR/RXR heterodimers bind to the DR5 RARE [Mangelsdorf and Evans, 1995]. However, several recent studies have shown that at limiting ligand concentrations, maximum expression of a variety of RA-responsive genes occurs when both RAR and RXR are ligand-activated [Roy et al., 1995; Lotan et al., 1995]. Likewise, Taneja et al. [1996] found that EC cell differentiation was synergistically induced by treatment of cells with a combination of a RAR-specific synthetic retinoid and a pan-RXR-specific retinoid. Also, in agreement with our studies, Lotan et al. [1995] reported that maximum inhibition of cervical carcinoma cell growth was obtained by treatment with a combination of RAR- and RXR-selective ligands. More recently, we have used antisense and dominant negative technology to show that both RAR and RXR are important mediators of ovarian carcinoma growth inhibition by retinoids [Wu et al., 1997b].

These studies in the ovarian cancer model are consistent with a number of other published reports that suggest that RARα plays an important role in mediating inhibition of MCF-7 breast tumor cell growth by retinoids. For example, Dawson et al. [1995a] showed that maximum inhibition of breast carcinoma cell growth is mediated by retinoids that bind selectively to RARα. Interestingly, in that study, evidence was presented which suggested that retinoid-dependent adherent growth inhibition was not necessarily mediated by transcriptional transactivation of RA-responsive genes. In agreement with our results, they found that breast carcinoma cell growth was inhibited by reti-

noids capable of antagonizing AP-1 without activating gene transcription. We also have recently reported that in the ovarian model system, there is a relationship between ability of all-*trans*-RA to antagonize AP-1 transcriptional activity and the ability to inhibit growth [Soprano et al., 1996]. Thus, it would appear that growth inhibition of two different gynecological cancers (ovarian and breast) by retinoids proceeds at least in part through an anti-AP-1 pathway.

In contrast to other natural and synthetic retinoids assayed, AHPN inhibits the growth of both RA-sensitive and RA-resistant ovarian cancer cell lines and does so via a mechanism which involves apoptosis. AHPN was originally characterized as a RARγ-selective agonist [Bernard et al., 1992]. It has also been previously studied in the breast cancer cell model [Shao et al., 1995]. In this study, treatment of both estrogen receptor-positive, RA-sensitive, and estrogen receptor-negative, RA-resistant breast cancer cells lines with AHPN induced G1 arrest and eventually, apoptosis. No correlation was found between AHPN-mediated transcriptional activation via RARE elements and its ability to inhibit growth, suggesting that this compound acts via an RAR/RXR-independent mechanism. Moreover, AHPN was shown to inhibit the growth of RA-resistant HL-60R cells. HL-60R cells contain a defective RARα gene and are RARβ and RARγ negative [Shao et al., 1995]. In the breast cancer model, evidence suggests that AHPN treatment brings about growth arrest and eventually apoptosis in an RAR/RXR independent fashion, perhaps by inducing Waf-1/p21/CIP-1. Interestingly, it apparently does this via a unique p53-independent mechanism. We do not know at this time the role of RARs and RXRs in mediating apoptosis in our ovarian model.

Retinoids have been previously shown to induce apoptosis in several types of transformed and/or tumor cell lines [Delia et al., 1995; Zhang et al., 1995]. RA treatment of HL-60 cells resulted in apoptosis after a period of 6–8 days [Martin et al., 1990; Nagy et al., 1995]. The synthetic retinoid N-(4-hydroxyphenyl) retinamide (4-HPR), a potent inhibitor of tumor cell growth, has been shown to induce apoptosis in many types of tumor cell cultures including the ovarian cancer cell lines A2780 and IGROV-1 [Delia et al., 1995; Kalemkerian et al., 1995; Supino et al., 1996]. The precise mechanism by which retinoids induce apoptosis is still unclear. Deregulation of apoptosis- related genes such as Bcl-2, Bax, p53 and c-myc may at least in part be responsible [Delia et al., 1995]. We found that expression of Bax increased, while the expression of Bcl-2 decreased within one day after addition of AHPN in both RA-sensitive and RA-resistant ovarian tumor cells. This expression pattern is consistent with the molecular events known to occur prior to induction of apoptosis by other agents in other cell lines [White, 1996; Nagata, 1997; Reynolds et al., 1994]. However we do not know if AHPN directly affects the transcription of Bcl-2 or Bax or acts indirectly via induction of WAF-1/p21/Cip-1. Additional follow-up studies in ovarian cancer cells will be required to elucidate the precise nature of this mechanism.

ACKNOWLEDGMENTS

This work was supported by grants CA 64945 and DK 49045 (to K.J.S.), HD 27556 and DK 44517 (to D.R.S.), and P01 CA 51993 (to M.I.D.), from the National Institutes of Health. D.R.S. is the recipient of a Research Career Development Award (HD 01076). We thank F. Hoffmann-LaRoche and Co. for the all-*trans* and 9-*cis* retinoic acid used in these studies. We also thank Drs. Marc Monestier and Padmini Salgame of the Department of Microbiology and Immunology for their invaluable help with the apoptosis ELISA.

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