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Unique Property of Some Synthetic Retinoids: Activation of the Aryl Hydrocarbon Receptor Pathway

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ABSTRACT

Potential pharmacological applications in the areas of oncology, dermatology, diabetes, and atherosclerosis of synthetic analogs of retinoic acid that target a specific nuclear receptor and/or biological response have generated great interest in the development of new retinoid and rexinoid drugs. The pan-retinoic acid receptor antagonist AGN 193109 has been previously reported to elevate CYP1A1 levels, implicating the aryl hydrocarbon receptor (AhR) as an additional target for this retinoid. AhR is a cytosolic ligand-dependent transcription factor that, in conjunction with the AhR nuclear translocator (Arnt), binds to dioxin response elements (DREs) located in the promoter region of target genes, such as *CYP1A1*, and induces their transcription. The purpose of these studies was to determine whether additional synthetic retinoids were capable of elevating CYP1A1 levels and to examine the mechanism of this increase in CYP1A. Two additional retinoids, AGN 190730 and AGN 192837, were found to be potent inducers of DRE-driven transcriptional activity; AGN 190730 was the most potent. Moreover, electrophoretic mobility-shift assays demonstrate that AGN 190730 can transform AhR into its active DNA recognition form. In addition, trypsin digestion of AGN 190730-treated AhR reveals a conformational change in the protein similar to the conformational change of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-bound AhR. Finally, competitive binding studies demonstrate that AGN 190730 can inhibit the binding of TCDD to AhR. The sum of the data demonstrates that some synthetic retinoids in addition to activating the retinoic acid receptor/retinoid X receptor pathway are capable of binding to AhR and activating the AhR/Arnt pathway.

Retinoic acid (RA), the most active natural vitamin A metabolite, and its synthetic analogs are potent regulators of a diverse group of biological processes, including growth, differentiation, cell proliferation, and morphogenesis (Gudas et al., 1994). On the other hand, pharmacological doses of RA and several synthetic analogs have been shown to be effective in the prevention and treatment of a number of types of cancers (Hong and Itri, 1994) and in the treatment of a variety of dermatological conditions (Peck and DiGiovanna, 1994). Currently, great efforts are being directed toward the development of additional highly selective synthetic retinoids agonists and antagonists including rexinoids (retinoids that selectively bind to and activate retinoid X receptors) to be used as pharmacological agents for cancer chemotherapy, cancer chemoprevention, type II diabetes, atherosclerosis, obesity, and dermatological conditions (Nagpal and Chandraratna, 2000; Sporn and Suh, 2000; Thacher et al., 2000).

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The biological effects of RA and its synthetic analogs are mediated by a group of nuclear proteins called retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [for review, see Chambon (1996)]. Three subtypes, called α, β, and γ, of both RAR and RXR have been studied extensively. RARs and RXRs are RA-inducible transcriptional regulatory proteins that in dimeric form transduce the RA signal at the level of regulation of gene expression via specific *cis*-acting DNA sequences located in the promoter of target genes. In vitro binding studies have demonstrated that the natural metabolites all-*trans*-RA and 9-*cis*-RA are high-affinity ligands for RARs, whereas only 9-*cis*-RA has been shown to bind RXRs (Heyman et al., 1992; Levin et al., 1992). Although each of the RAR and RXR subtypes displays similar affinity for RA, many retinoid agonists and antagonists have been synthesized that display RAR subtype or RXR selectivity.

Exposure to xenobiotic agents, including benzo[*a*]pyrene, 3-methylcholanthrene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), elicits a variety of biochemical, immunological,

ABBREVIATIONS: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TCDD 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; Arnt, aryl hydrocarbon receptor nuclear translocator; DMSO, dimethyl sulfoxide; TCDBF, 2,3,7,8-tetrachlorodibenzofuran; DRE, dioxin response element; RLU, relative luciferase unit; TBST, Tris-buffered saline-Tween 20; EMSA, electrophoretic mobility shift assay; HAP, hydroxylapatite.

reproductive, dermatological, and neoplastic effects in animals. The actions of these agents are mediated by the aryl hydrocarbon receptor (AhR) and the AhR nuclear translocator (Arnt), both members of the basic helix-loop-helix periodicity/Arnt/simple-minded family of transcription factors. These xenobiotic agents bind in the cytoplasm to AhR and the ligand-bound AhR translocates to the nucleus where it dimerizes with Arnt. This ligand-bound AhR/Arnt heterodimeric complex binds to specific *cis*-acting regulatory DNA sequences termed dioxin response elements (DREs) located in the promoter of target genes, including *CYP1A1* and *CYP1A2*, to enhance their rate of transcription [for review, see Schmidt and Bradfield (1996) and Hankinson (1995)]. Interestingly, AhR-null mice seem to be resistant to the teratogenic effects of TCDD and the carcinogenic effect of benzo[*a*]pyrene, suggesting that most if not all of the adverse effects of these agents are mediated by AhR/Arnt (Fernandez-Salguero et al., 1996; Mimura et al., 1997; Shimizu et al., 2000).

A high-affinity natural ligand for AhR has yet to be demonstrated. However, recent studies using AhR-deficient mice suggest that AhR has important physiological functions beyond mediating the response to environmental contaminates, such as liver development and immune function (Fernandez-Salguero et al., 1995; Schmidt et al., 1996). Interestingly, a large number of naturally occurring compounds have been reported to be relatively weak AhR ligands (compared with TCDD) and to activate AhR including of indoles (Gillner et al., 1985; Fernandez et al., 1988; Bjeldanes et al., 1991), tryptophan and tryptophan metabolites (Heath-Pagliuso et al., 1998), bilirubin and biliverdin (Sinal and Bend, 1997; Phelan et al., 1998); benzocoumarins (Liu et al., 1993), and substituted flavonoids (Lu et al., 1996; Ciolino et al., 1998). Recently, we have reported that the synthetic retinoid AGN 193109 can elevate CYP1A1 mRNA levels in mouse embryos and in Hepa-1c1c7 cells and have suggested that the AhR/Arnt pathway may mediate this response (Soprano et al., 2001).

The purpose of this study was to determine whether additional synthetic retinoids were capable of elevating CYP1A1 transcriptional activity and to examine the mechanism of this induction of CYP1A1 mRNA levels. Two additional retinoids, AGN 190730 and AGN 192837, were found to be potent inducers of CYP1A1 transcriptional activity; AGN 190730 was the most potent. Moreover, AGN 190730 was found to increase AhR-dependent activation of gene expression, to induce AhR transformation and DNA binding, to cause a conformational change in AhR similar to that induced by TCDD, and to competitively inhibit TCDD binding to AhR. Taken together, these data demonstrate that some synthetic retinoids in addition to activating the RAR/RXR pathway are capable of binding to AhR and activating the AhR/Arnt pathway.

Materials and Methods

Chemicals. All AGN-series retinoids were synthesized at Allergan, Inc. (Irvine, CA). SR11254 and SR11253 were a generous gift from Dr. Marcia Dawson (The Burnham Institute, La Jolla, CA). AM-80, AM-580, AZ-80, LE135, LE540, and LE555s were a generous gift from Professor Koichi Shudo and Dr. Hiroyuki Kagechika, University of Tokyo, Japan. All synthetic retinoids were prepared as a 10^{-3} M stock solution in dimethyl sulfoxide (DMSO). All-*trans*-retinoic acid (RA) was a generous gift from Hoffman-LaRoche (Nutley, NJ) and was prepared as a 10^{-3} M stock solution in ethanol. Both TCDD and 2,3,7,8-tetrachlorodibenzofuran (TCDBF) were obtained as 50 μg/ml stock solutions in nonane from ChemSyn Laboratories (Lenexa, KS) and were diluted in DMSO. α-Naphthoflavone was purchased from Sigma and was prepared as a 10^{-3} M stock in DMSO.

Cell Culture. Hepa-1c1c7, taoBpRc1, and BpRc1 (termed BP^{r}c1 in Miller et al., 1983) cell lines were purchased from the American Type Culture Collection (Manassas, VA). Both taoBpRc1 and BpRc1 cells are derived from the parental Hepa-1c1c7 cells; however, taoBpRc1 cells have a defect rendering them AhR-negative and BpRc1 cells have a defect rendering them Arnt-negative (Miller et al., 1983). Hepa H1L1.1c2 cells were a generous gift from Dr. Michael Denison (University of California, Davis, CA). Hepa H1L1.1c2 cells are a clone of the Hepa-1c1c7 cell line that has been stably transfected with the plasmid pGudLuc1.1 (Garrison et al., 1996). The pGudLuc1.1 vector contains the firefly luciferase gene under the control of a portion of the mouse mammary tumor virus long terminal-repeat promoter, which lacks functional glucocorticoid response elements and a 484-bp fragment from the upstream promoter region of the *CYP1A1* gene containing four dioxin response elements (DRE). This stably transfected vector confers TCDD-inducible expression of the luciferase gene in these cells (Garrison et al., 1996).

All stock cells were maintained in α-Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO), 2 mM glutamine (Invitrogen), 100 units/ml penicillin (Cellgro/Mediatech, Herndon, VA), and 100 μg/ml streptomycin (Cellgro) at 37°C in a 98% humidified, 5% CO_2 atmosphere.

Bioassay of Retinoids. Hepa H1L1.1c2 cells were plated at a density of 250,000 cells/60-mm^2 tissue culture dish. On the next day, the cells were treated with the indicated concentrations of each compound and the cells were incubated for various periods of time ranging from 0 to 24 h. Control plates were treated with an equal volume of either DMSO or ethanol carrier. At the time of harvest, cells were washed one time with phosphate-buffered saline followed by lysis in 1× passive lysis buffer (Promega, Madison, WI). Cell suspensions were centrifuged in a microcentrifuge for 20 s at 12,000*g* to pellet cellular debris. The clear supernatant was used for the assay of luciferase activity.

Luciferase activity was quantitated using the luciferase assay reagent obtained from Promega according to the manufacturer's protocol. Relative luciferase units (RLU) were measured using a Zylux luminometer (Zylux Corporation, Maryville, TN). RLUs were normalized to the micrograms of protein in the cell extract. Protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard protein. Fold inductions were calculated as the fold increase in RLU per microgram of protein in the compound-treated cells relative to the RLU per microgram of protein in the DMSO- or ethanol-treated control cells.

Western Blot. Total cellular protein extracts were prepared from Hepa-1c1c7, taoBpRc1, and BpRc1 cells after treatment with the indicated retinoids or DMSO carrier by homogenization in 1 volume per packed cell volume of resuspension buffer (50 mM $NaPO_4$, pH 7.4, 0.1 mM EDTA, and 10% glycerol), followed by centrifugation in a microcentrifuge for 15 min at 4°C. The supernatant was removed and the protein concentration was determined using the Bio-Rad protein assay reagent. Samples were stored at −70°C.

CYP1A1 protein levels were measured by Western blot analysis essentially as described previously (Tairis et al., 1994; Soprano et al., 2001). Typically, 50 μg of total cellular protein was fractionated by discontinuous SDS-polyacrylamide gel electrophoresis using a 5% polyacrylamide stacking gel and a 9% polyacrylamide separating gel. Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) according to the method of Burnette (1981). The membranes were blocked for at least 1 h at room temperature in 5% (w/v) nonfat dry milk in TBST [20 mM

Tris-HCl, pH 7.4, 150 mM NaCl; 0.1% (v/v) Tween 20]. After blocking, the membrane was incubated with goat anti-CYP1A1 polyclonal primary antibody (DAIICHI), which was diluted 1:500 in 5% (w/v) nonfat dry milk in TBST for 30 min at room temperature. After removal of the primary antibody, the membrane was washed 3 times with TBST and then incubated for 30 min with rabbit anti-goat IgG conjugated horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), which was diluted 1:5000 in 5% (w/v) nonfat dry milk in TBST. After incubation with the secondary antibody, the membrane was again washed three times with TBST. The protein was visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Electrophoretic Mobility Shift Assay. EMSA was performed using in vitro transcribed and translated AhR and Arnt. AhR and Arnt were in vitro transcribed and translated using the TNT coupled reticulocyte lysate system and SP6 RNA polymerase following the manufacturer's protocol (Promega). Murine AhR and Arnt expression plasmids (Dolwick et al., 1993) were a generous gift from Dr. Christopher Bradfield (McArdle Laboratory for Cancer Research, Madison, WI). In some experiments, Amino Acid Mixture Minus Leucine was substituted with 10 μCi of [^{35}S]methionine (1175 Ci/mmol; ICN Pharmaceuticals, Costa Mesa, CA) and the samples were analyzed on a SDS-10% polyacrylamide gel. Greater than 90% of the AhR and Arnt protein synthesized was found to be full length.

The two probes used in the EMSA consisted of complementary oligonucleotide sequences that were end-labeled using [^{32}P]γ-ATP (7000 Ci/mmol; ICN) and T4 polynucleotide kinase (Promega) as described previously (Soprano et al., 1996). The DRE probe (5′-GATCTGGCTCTTCTCACGCAACTCCG-3′ and 5′-GATCCGGAGTTGCGTGAGAAGAGCCA-3′) contains the DRE from the *CYP1A1* gene promoter. The DREmm probe (5′-GATCTGGCTCTTCTCaaCAACTCCG-3′ and 5′-GATCCGGAGTTGttTGAGAAGAGCCA-3′) contains two mutations (indicated in lower case letters) in the DRE from the *CYP1A1* gene promoter. Oligonucleotides were purchased from Ransom Hill Biosciences (Ransom Hill, CA).

The activation and binding reactions were carried out as described previously by Ikuta et al. (2000). Briefly, 30 μl of rabbit reticulocyte AhR lysate and 30 μl of rabbit reticulocyte Arnt lysate were mixed, 1 μl of each compound (or solvent carrier) at the indicated concentration was added, and the samples were incubated at 30°C for 2 h. After this incubation, 10 μl of the activated lysate was mixed with 10 μl of 2× binding buffer [20 mM HEPES, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 6 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 mg/ml herring sperm DNA, 12% (v/v) glycerol, and 0.001% bromphenol blue] and incubated at room temperature for 20 min. Then 100,000 cpm of ^{32}P-labeled DRE probe or ^{32}P-labeled DREmm probe were added to each reaction and incubated at room temperature for an additional 20 min. In cold competition experiments, a 100-fold molar excess of unlabeled double stranded DRE or DREmm DNA was added along with the ^{32}P-labeled DNA probe. After incubation, the samples were loaded onto a prerun 4% polyacrylamide gel containing 2.5% glycerol and electrophoresed at 200 V for approximately 1.5 h at 4°C until the samples had entered the gel. Then the voltage was increased to 300 V until the dye had migrated approximately 3/4 of the way through the gel. The gel was dried and exposed to a phosphor screen (Packard, Meriden, CT) and analyzed using a Cyclone filmless autoradiographic system (Packard) and OptiQuant software (Packard).

Ligand Induced Conformational Analysis. Ligand-induced conformational analysis was performed essentially as described by Kronenberg et al., 2000 using in vitro transcribed and translated AhR and Arnt. AhR and Arnt were prepared as described above except that the AhR protein was radiolabeled with [^{35}S]methionine. The in vitro transcribed and translated ^{35}S-AhR and Arnt were activated as described above except that 60 μl of the activated lysate was mixed with 60 μl of 2× binding buffer and incubated at room temperature for 20 min. Next, 1 ng of unlabeled double stranded DRE was added for an additional 20-min incubation. Trypsin (type I from bovine pancreas, 11,200 units/mg of protein; Sigma) at a concentration of 10 μg/ml was added to each sample and the samples were incubated at room temperature for up to 10 min. At each indicated time point, 20 μl of the trypsin-treated sample was removed to a new microcentrifuge tube containing 20 μl of 2× sample buffer [100 mM Tris-HCl, pH 6.8, 40% glycerol (v/v), 2% SDS, 0.02% bromphenol blue, and 5% β-mercaptoethanol, freshly added] and immediately boiled for 5 min. The protein samples were fractionated by discontinuous SDS-polyacrylamide gel electrophoresis using a 5% polyacrylamide stacking gel and a 15% polyacrylamide separating gel. After drying, the gel was exposed to a phosphor screen and analyzed using a Cyclone filmless autoradiographic system (Packard) and OptiQuant software (Packard)

Competitive Binding Studies. The binding of AGN 190730 to AhR in Hepa-1c1c7 and mouse liver cytosolic extracts was measured by determining the ability of AGN 190730 to compete with [^{3}H]TCDD for specific binding using the hydroxylapatite (HAP) method essentially as described by Gasiewicz and Neal (1982). Cytosolic extracts were prepared from both the liver of C57BL/6J mice and Hepa-1c1c7 cells. Livers were removed and homogenized in 3 volumes of ice-cold HEDG buffer per gram of tissue (25 mM HEPES, pH 7.5, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) and Hepa-1c1c7 cells were homogenized in 2 volumes of ice-cold HEDG buffer per packed cell volume. After homogenization, the homogenate was centrifuged at 10,000*g* at 4°C for 20 min. The supernatant was collected and centrifuged at 105,000*g* at 4°C for 60 min. After this centrifugation, the supernatant below the lipid layer was collected, aliquoted, and immediately stored in liquid nitrogen. Protein concentration was determined using the Bio-Rad protein assay reagent using bovine serum albumin as a standard protein. Typically, the protein concentrations of the mouse liver extract and Hepa-1c1c7 cell extract were 20 mg/ml and 3 mg/ml, respectively.

In a standard competition assay, 250 μl of cytosolic extract (3 mg of protein/ml) was mixed with 0.2 nM [^{3}H]TCDD (22.2 Ci/mmol; ChemSyn Laboratories), 100 μM citral (Sigma), and the indicated concentration of each retinoid (AGN 190730, AGN191440, and all-*trans*-RA) or solvent (DMSO or ethanol) alone. To determine nonspecific binding, the retinoid/solvent was substituted with 50 nM TCDBF. Samples were incubated with gentle rotation at 20°C for 2 h. After incubation, 25 μl of the sample was placed in a scintillation vial for determination of the total amount of [^{3}H]TCDD in each sample and 200 μl was placed in a fresh tube containing 250 μl of HAP suspension [DNA-grade HAP (Bio-Rad) was washed with HEDG buffer until the pH of the washes remained at 7.4 followed by resuspension of the HAP in 2 volumes of HEDG at 4°C] for the determination of the amount of bound [^{3}H]TCDD. The samples were incubated on ice for 30 min with gentle shaking every 10 min. At the end of this time, 1 ml of ice-cold HEDG containing 0.5% (v/v) Tween 80 was added to each sample. The tubes were centrifuged at 3500 rpm and 4°C for 5 min in a microcentrifuge. The HAP pellet was washed an additional three times with 1 ml of HEDG containing 0.5% Tween 80. After the last wash, 1 ml of absolute ethanol was added to the HAP pellet and the HAP/ethanol suspension was transferred to a scintillation vial. The tube and pipette tip were washed with an additional 0.5 ml of ethanol, which was also added to the scintillation vial. Scintisafe liquid scintillation cocktail (5 ml; Fisher, Pittsburgh, PA) was added to each vial and the radioactivity was quantified by liquid scintillation counting using an LS6500 liquid scintillation counter (Beckman Coulter, Fullerton, CA). Specific binding was calculated by subtracting nonspecific binding (disintegrations per minute in the [^{3}H]TCDD plus 50 nM TCDBF sample) from total [^{3}H]TCDDbinding (disintegrations per minute in the [^{3}H]TCDD plus solvent carrier sample). Specific [^{3}H]TCDD bound was set to 100%. The percentage of [^{3}H]TCDD bound for each retinoid in the competition assay was calculated by dividing the disintegrations per minute of specific [^{3}H]TCDD bound in the retinoid containing sample by the disintegrations per minute of specific [^{3}H]TCDD bound in the solvent containing sample.

Results

Transcriptional Activation of the CYP1A1 Promoter by Retinoids. H1L1.1c2 cells were used to rapidly screen a number of retinoids for their ability to activate the AhR pathway and induce transcription of the CYP1A1 promoter. Preliminary time course and dose response experiments were performed with TCDD and AGN 193109. As reported previously by Garrison et al. (1996), treatment of H1L1.1c2 cells with TCDD caused a dramatic dose-responsive increase in transcriptional activation of the CYP1A1 promoter which reached a plateau at a concentration between 10^{-10} M and 10^{-9} M with a ~60-fold increase in luciferase activity (data not shown). Time course (0, 4, 8, 16, and 24 h) and dose response (10^{-10} to 10^{-5} M) studies with the retinoid AGN 193109 demonstrated a maximal increase in luciferase activity when H1L1.1c2 cells were treated with 10^{-5} M AGN 193109 for 4 h (data not shown). This is consistent with our previous report, which demonstrated a maximal increase in the CYP1A1 mRNA and protein levels in Hepa-1c1c7 cells 4 h after treatment with 10^{-5} M AGN 193109 (Soprano et al., 2001). Therefore, we screened all retinoids for their ability to activate transcription of the luciferase reporter gene in H1L1.1c2 cells by treating the cells with a concentration of 10^{-5} M retinoid for 4 h.

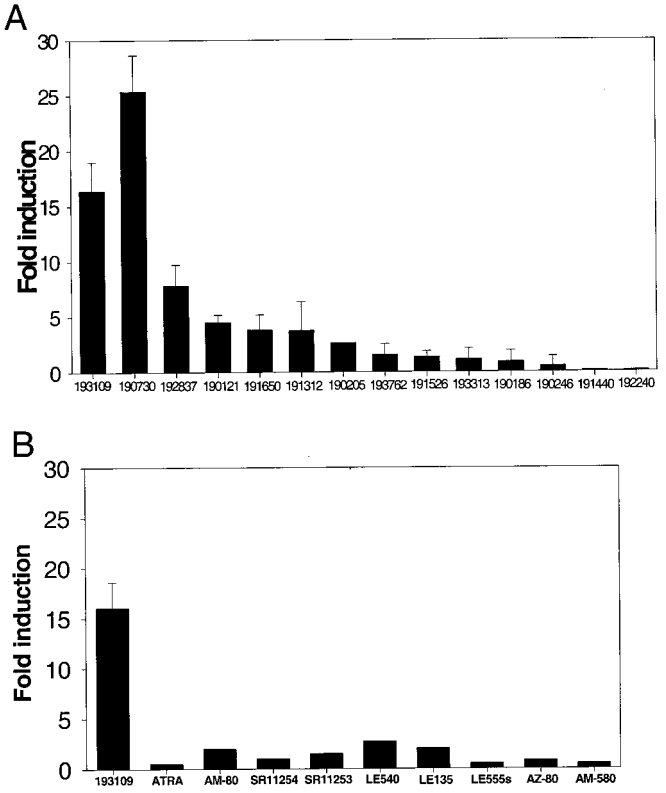


Fig. 1. Transcriptional activation of the CYP1A1 promoter by synthetic retinoids. HepaH1L1.1c2 cells were treated with 10^{-5} M of either AGN series retinoids (A) or other retinoids (B) for 4 h. After treatment cells were harvested and assayed for luciferase activity. Luciferase units were normalized with protein concentration and fold inductions were calculated by comparing retinoid treated and untreated [DMSO carrier for all retinoids except ethanol carrier for all-*trans*-RA (ATRA)] samples. Graphs show the fold inductions of all compounds tested at a concentration of 10^{-5} M, maximal fold induction for each retinoid. Each bar represents the mean ± S.E.

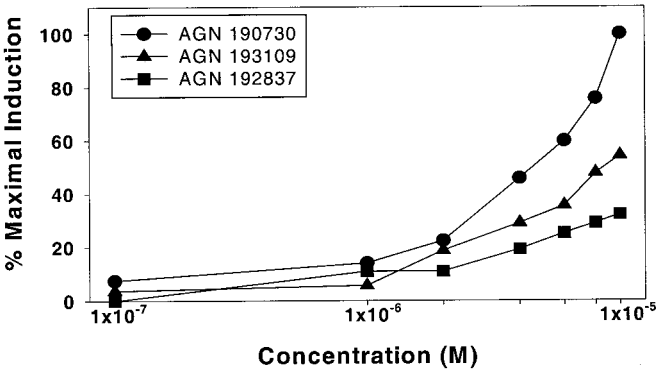


Fig. 2. Transcriptional activation of the CYP1A1 promoter by varying concentrations of AGN 193109, AGN 190730, and AGN 192837. HepaH1L1.1c2 cells were treated with AGN 193109, AGN 190730, or AGN 192837 for 4 h with the indicated concentrations of each retinoid. After treatment, cells were harvested and assayed for luciferase activity. Luciferase units were normalized with protein concentration and fold inductions were calculated by comparing retinoid-treated and untreated (DMSO carrier) samples. The percentage maximal induction represents the fold induction for each treatment relative to the fold induction measured when cells were treated with 10^{-5} M AGN 190730. Each data point represents the mean of duplicate experiments.

As seen in Fig. 1, 14 AGN-series retinoids (including AGN 193109) and 9 other retinoids (including the natural retinoid RA) were examined as possible CYP1A1 transcriptional activators. Three retinoids, AGN 190730, AGN 193109, and AGN 192837, caused a substantial increase in luciferase activity (25-, 15-, and 7-fold, respectively). Dose response experiments with these three retinoids are shown in Fig. 2. The increase in CYP1A1 transcriptional activity observed for each retinoid treatment was dose-dependent over a concentration of 10^{-6} to 10^{-5} M. It should be noted that no plateau in luciferase activity was observed at the 10^{-5} M concentration for any of these three retinoids, suggesting that at this concentration, they are not yet saturating. Unfortunately, because of the limited solubility of retinoids in aqueous solutions, concentrations higher than 10^{-5} M are not readily achievable; therefore, it is not possible to determine the maximum induction in CYP1A1 transcriptional activation by these retinoids. Four additional retinoids (AGN 190121, AGN 191650, AGN 191312, and AGN 190205) were found to elevate luciferase activity to a lesser extent, approximately 3- to 5-fold. Finally, seven AGN-series retinoids (AGN 193762, AGN 191526, AGN 193313, AGN 190186, AGN 190246, AGN 191440, and AGN 192240) and the remaining nine retinoids (Fig. 1B) examined caused an increase of less than 3-fold in luciferase activity and were not characterized further.

Increase in CYP1A1 Protein Levels by AGN 190730 and AGN 192837. Previously, we have demonstrated that AGN 193109 elevates CYP1A1 mRNA and protein levels in Hepa-1c1c7 cells and that this increase is dependent on both functional AhR and Arnt (Soprano et al., 2001). Because AGN 190730 and AGN 192837 caused a substantial increase in luciferase activity in the H1L1.1c2 cells, we wished to determine whether CYP1A1 protein levels were elevated upon treatment of cells with these two retinoids and whether this elevation in CYP1A1 protein levels was also dependent on functional AhR and Arnt. Figure 3 demonstrates that CYP1A1 protein levels are also elevated in Hepa-1c1c7 cells upon treatment with both AGN 190730 and AGN 192837. Furthermore, this elevation in CYP1A1 protein levels was

not seen in cells that lacked either functional AhR (tao BpRc1) or functional Arnt (BpRc1). Therefore, AGN 190730, AGN 193109, and AGN 192837 all induce CYP1A1 protein levels and this increase in CYP1A1 protein levels requires both AhR and Arnt. Because AGN 190730 caused the greatest increase in luciferase activity in the H1L1.1c2 cells, we chose to use AGN 190730 to study the mechanism of action of this induction of CYP1A1 expression.

Competition of AGN 190730 with the Partial AhR Antagonist α-Naphthoflavone. We next wished to determine whether the AGN 190730-mediated increase in CYP1A1 promoter activity in the H1L1.1c2 cells could be competed by the partial AhR antagonist α-naphthoflavone (Blank et al., 1987; Santostefano et al., 1993; Gasiewicz et al., 1996). As seen in Fig. 4, treatment of H1L1.1c2 cells with increasing concentrations of the α-napthoflavone along with 10^{-5} M AGN 190730 resulted in a concomitant reduction in the fold induction in luciferase activity by AGN 190730. This provides additional evidence that the AGN 190730-dependent increase in CYP1A1 is mediated by AhR.

In Vitro Transformation of AhR into a DNA Recognition form. Activation of AhR into an active transcription factor involves ligand binding and heterodimerization of AhR and Arnt (Gebremichael et al., 1996; Sogawa and Fujii-Kuriyama, 1997; Heid et al., 2000). We next wished to determine whether AGN 190730 could activate AhR and transform AhR into a form that could bind a DRE. As shown in Fig. 5, treatment of in vitro transcribed and translated AhR and Arnt with AGN 190730 results in the formation of an AhR/Arnt complex that can bind specifically to the DRE probe similar to that observed with TCDD treatment. Furthermore, two retinoids (all-*trans*-RA and AGN 191440) that did not cause an increase in transcriptional activation of the CYP1A1 promoter in H1L1.1c2 cells (Fig. 1), were unable to active AhR into a DNA binding form (Fig. 6). Taken together,

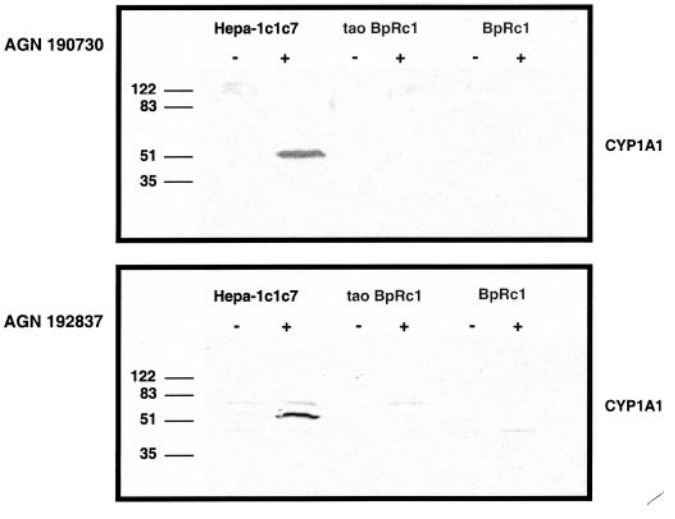


Fig. 3. CYP1A1 protein levels in Hepa-1c1c7, taoBpRc1 and BpRc1 cells after treatment with AGN 190730 and AGN 192837. Hepa-1c1c7, tao BpRc1, and BpRc1 cells were treated with either DMSO (−) or 10^{-5} M of either AGN 190730 (top) or AGN 192837 (bottom) for 4 h. After treatment cells were harvested and cellular protein was isolated. Samples of total cellular protein (40 μg) were separated on a discontinuous SDS-9% polyacrylamide gel and the level of CYP1A1 protein determined by Western blot analysis using goat anti-CYP1A1 polyclonal antibody followed by rabbit anti-goat IgG-horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using enhanced chemiluminescence.

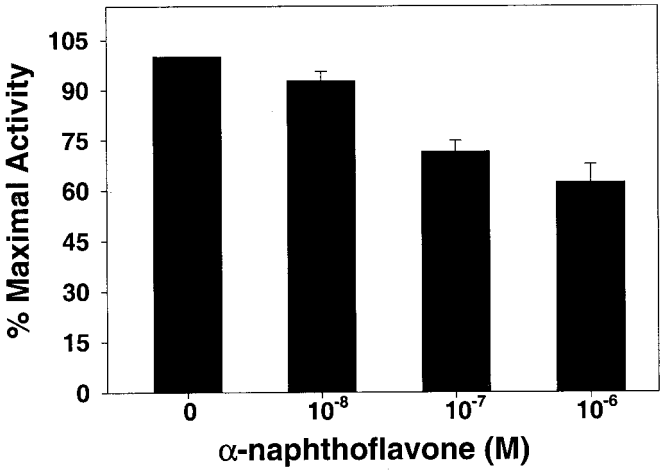


Fig. 4. Competitive inhibition of AGN 190730 induced transcriptional activation of the CYP1A1 promoter by the partial AhR antagonist α-naphthoflavone. HepaH1L1.1c2 cells were treated with 10^{-5} M AGN 193109 along with the partial antagonist α-naphthoflavone at the indicated concentrations for 4 h. After treatment cells were harvested and assayed for luciferase activity. Luciferase units were normalized with protein concentration and fold inductions were calculated by comparing retinoid treated and untreated (DMSO carrier) samples. The percentage maximal activity represents the fold induction for each treatment relative to the fold induction measured when cells were treated with 10^{-5} M AGN 190730 alone. Each data point represents the mean ± S.E.

these data demonstrate that AGN 190730 is capable of activating AhR into a DNA binding form.

Conformational Analysis of AhR. Recently, it has been demonstrated that TCDD treatment of AhR results in the formation of a 35 kDa AhR fragment that is resistant to

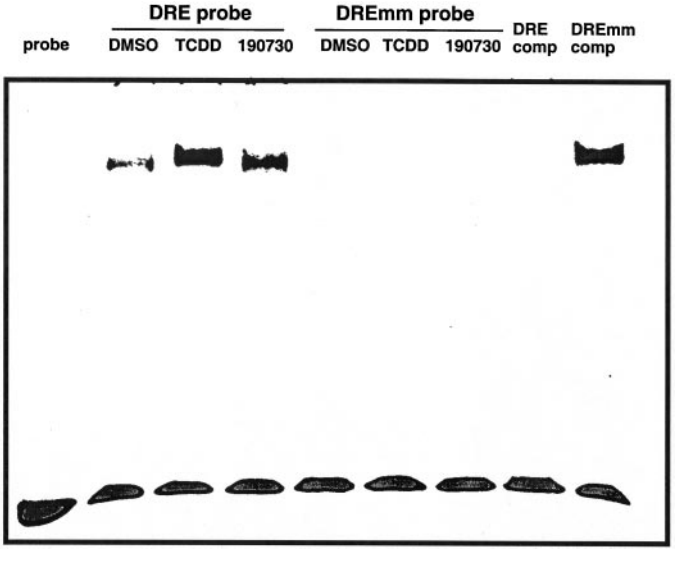


Fig. 5. Transformation of inactive AhR into a DNA recognition form after treatment with AGN 190730. In vitro transcribed/translated AhR and Arnt were mixed in a 1:1 ratio and treated with DMSO, TCDD (10^{-9} M), or AGN 190730 (10^{-5} M) for 2 h. After incubation with the compound, [^{32}P]-labeled DRE or [^{32}P]-labeled DREmm probe were added and the binding reactions were continued for 15 min. In some samples, 100-fold molar excess of unlabeled DRE (DRE comp) or DREmm (DREmm comp) were added to TCDD-treated samples along with the ^{32}P-labeled DRE. After the binding reaction, the protein/DNA mixture was resolved by electrophoresis through a 4.5% polyacrylamide gel containing 2.5% glycerol. The lane-labeled probe contains only the ^{32}P-labeled DRE probe. After electrophoresis, the gel was dried and the radioactive bands detected using a filmless autoradiographic system. Representative gel from four experiments.

trypsin digestion, whereas unliganded AhR does not form the 35-kDa trypsin-resistant fragment upon treatment with trypsin (Kronenberg et al., 2000). We were next interested in determining whether treatment of AhR with AGN 190730 would cause the formation of a similar 35-kDa trypsin-resistant fragment. As shown in Fig. 7, treatment of in vitro transcribed and translated AhR with AGN 190730 results in the formation of a similar 35-kDa trypsin-resistant fragment as that observed with TCDD treatment of AhR. This suggests that the treatment of AhR with AGN 190730 causes a conformational change in AhR similar to that observed upon treatment with TCDD.

AhR Binding. To further demonstrate that AGN 190730 can directly bind to AhR, we have examined the ability of AGN 190730 to competitively inhibit the binding of [^{3}H]TCDD to AhR in cytosolic extracts prepared from both mouse liver and Hepa-1c1c7 cells. As shown in Fig. 8, AGN 190730 inhibited specific [^{3}H]TCDD binding to AhR by approximately 35%, whereas two retinoids (all-*trans*-RA and AGN 191440) that were inactive in the transcriptional activation assay (Fig. 1) and the EMSA (Fig. 6) were unable to inhibit the specific binding of [^{3}H]TCDD to AhR. These data demonstrate that AGN 190730 can directly bind to AhR.

Discussion

The coupling of the discovery of the six different RAR and RXR isotypes and the promise of the pharmacological application of retinoid and rexinoid drugs for cancer chemotherapy, cancer chemoprevention, type II diabetes, atherosclerosis, obesity, and dermatological conditions has resulted in great efforts to develop conformationally restricted retinoid agonists and antagonists that display isotype selectivity. Recently, we reported that the retinoid AGN 193109, in addition to being a potent RAR antagonist (Johnson et al., 1995; Agarwal et al., 1996), also induces CYP1A1 mRNA and protein levels in Hepa-1c1c7 and that this increase was dependent on both functional AhR and Arnt (Soprano et al., 2001).

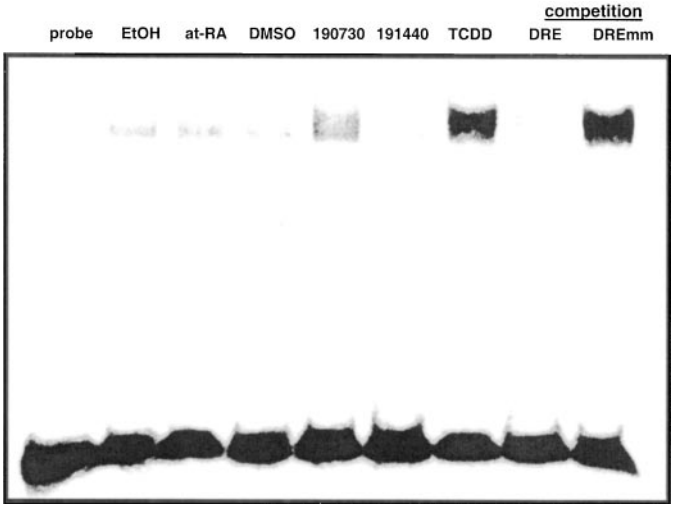


Fig. 6. Analysis of the ability of different retinoids to transform inactive AhR into a DNA recognition form. In vitro transcribed/translated AhR and Arnt were mixed in a 1:1 ratio and treated with ethanol (EtOH), 10^{-5} M all-*trans*-retinoic acid (at-RA), DMSO, 10^{-5} M AGN 190730, 10^{-5} M AGN 191440, or 10^{-9} M TCDD for 2 h. After incubation with the compound, ^{32}P-labeled DRE probe was added and the binding reactions were continued for 15 min. In the competition samples, 100-fold molar excess of unlabeled DRE DNA (DRE) or DREmm DNA (DREmm) were added to TCDD-treated samples along with the ^{32}P-labeled DRE. After the binding reaction, the protein/DNA mixture was resolved by electrophoresis through a 4.5% polyacrylamide gel containing 2.5% glycerol. The lane labeled probe contains only the ^{32}P-labeled DRE probe. After electrophoresis, was the gel was dried and the radioactive bands detected using a filmless autoradiographic system. Representative gel from two experiments.

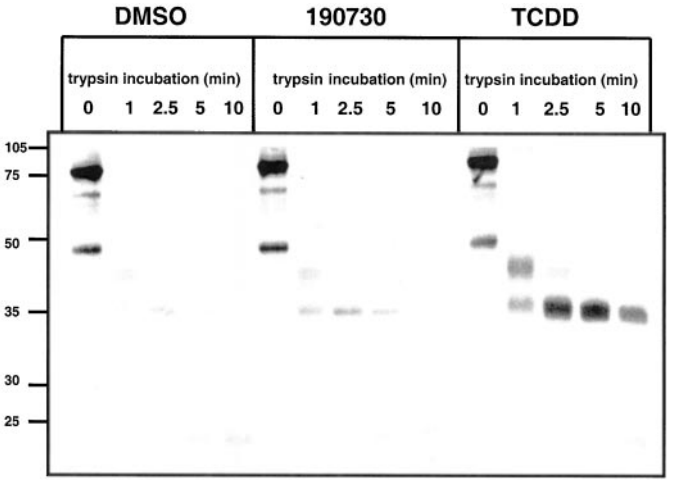


Fig. 7. Conformational analysis of AhR activated by AGN 190730. In vitro transcribed/translated AhR and ARNT were mixed in a 1:1 ratio and activated with DMSO, 10^{-5} M AGN 190730, or 10^{-9} M TCDD in the presence of unlabeled double-stranded DRE for 2 h. After activation, trypsin was added to each sample at a final concentration of 10 μg/ml and incubated for the indicated times (min). The trypsin digestion was stopped by immediately boiling the sample for 5 min after the addition of sample buffer. The protein samples were separated on a SDS-15% polyacrylamide gel, the gel was dried, and the protein bands visualized using a filmless autoradiographic system. The molecular mass markers shown are recombinant protein of the indicated size (Rainbow Markers; Amersham). Representative gel from three experiments.

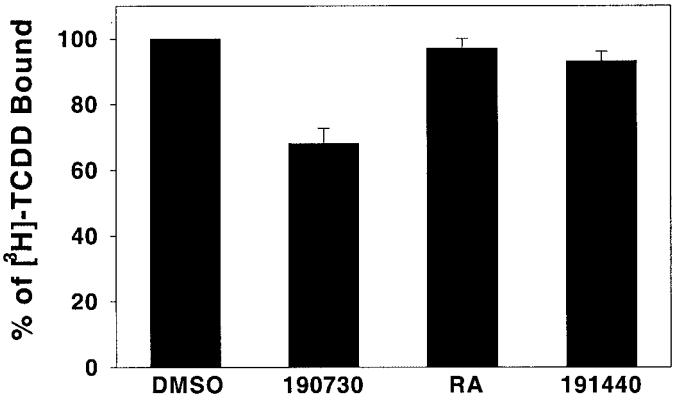


Fig. 8. AGN190730 competes with [^{3}H]TCDD for binding to cytosolic AhR. Cytosolic extracts (3 mg/ml) containing AhR were incubated for 2 h at 20°C with 0.2 nM [^{3}H]TCDD and one of the following: 10^{-5} M AGN190730, 10^{-5} M all-*trans*-RA (RA), 10^{-5} M AGN191440, 50 nM TCDBF, or DMSO carrier (comparable results were obtained with ethanol carrier). Free or loosely bound ligand was separated from bound [^{3}H]TCDD with HAP. Specific binding was calculated by subtracting nonspecific binding (disintegrations per minute of [^{3}H]TCDD plus 50 nM TCDBF) from total [^{3}H]TCDD binding (disintegrations per minute of [^{3}H]TCDD plus DMSO carrier). Specific [^{3}H]TCDDbound was set to 100%. The percentage of [^{3}H]TCDD bound for each retinoid in the competition assay was calculated by dividing the disintegrations per minute of specific [^{3}H]TCDD bound in the retinoid containing sample by the disintegrations per minute of specific [^{3}H]TCDD bound in the DMSO containing sample. Similar results were obtained with both the mouse liver cytosolic extracts and the Hepa-1c1c7 cytosolic extracts. Data represent the mean ± S.E. .

We now report two additional retinoids that elevate CYP1A1 transcriptional activity using a DRE driven luciferase assay and CYP1A1 protein levels in an AhR- and Arnt-dependent fashion. Furthermore, the most potent retinoid AGN 190730 was found to increase AhR-dependent activation of gene expression, induce AhR transformation and DNA binding, cause a conformational change in AhR similar to that induced by TCDD, and to competitively inhibit TCDD binding to AhR. Taken together, these data demonstrate that AGN 190730 induces the transcription of CYP1A1 by a mechanism similar, if not identical, to that of TCDD requiring the activation of the AhR/Arnt signaling pathway.

The structures of the AGN series compounds, and their activity in retinoid binding and transactivation assays, are shown in Table 1. With the exception of AGN 191440, all of these compounds are inactive at the RXRs. With regards to their activity at the RARs, these compounds tend to be selective for the RARβ and RARγ subtypes, with varying degrees of potency and binding affinity. As should be expected with distinct receptors, there is no relationship between these compounds' activity at the RARs and their activity at AhR. Thus, two compounds, AGN 190730 and AGN 192837, which are some of the most efficacious activators of AhR, have relatively weak affinity for the RARs.

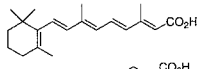
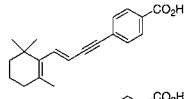
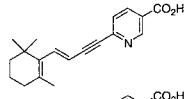
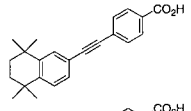
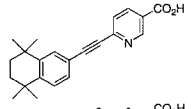
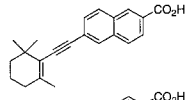
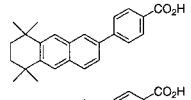
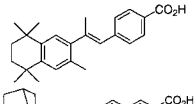
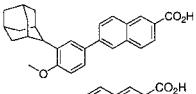
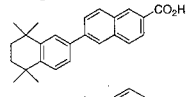
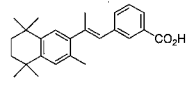
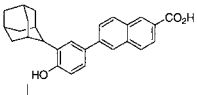
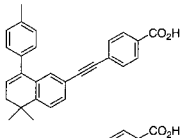
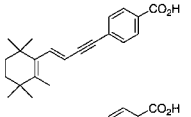
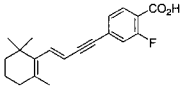
Even with this limited data set, a few comments can be made on the structure of the AGN series retinoids as it relates to their activity at AhR. First, the benzoic acid derivatives, AGN 190121 and AGN 190205, are both weak activators of AhR, whereas the corresponding nicotinic acids, AGN 190186 and AGN 190246, are inactive, indicating that less polar groups are necessary in this position. In fact, the carboxylic acid group, which is required for the retinoid activity, is probably unnecessary for AhR activity, because the highly active compound TCDD does not possess such a group. It also seems that highly rigid structures have higher AhR activity than less rigid structures. Thus, structures with acetylene linked benzoic acid (AGN 190205 and AGN 193109), biphenyl carboxylic acids (AGN 191312), and naphthoic acids (AGN 190730, AGN 191650, and AGN 192837), are activators of AhR. In contrast, compounds having less rigid alkene linking groups, such as AGN 190186, AGN 191440, AGN 192240, AGN 193313, and AGN 193762, are inactive. In addition, the free hydroxyl group in AGN 192837 is important for AhR activity because the methoxy-substituted analog, AGN 191526, is completely inactive. Interestingly, the three compounds having the greatest activity in the induction of CYP1A1, AGN 190730, AGN 193109, and AGN 192837, are structurally distinct lead compounds, each of which may be useful for the design of more potent and selective AhR agonists and antagonists. Such compounds will certainly be useful for elucidating the biology associated with AhR activation and may lead to new therapeutic applications.

To determine whether the induction of CYP1A1 transcription by AGN 190730 was mediated by the AhR/Arnt signal transduction pathway, we have examined the effect of this retinoid on individual steps in this pathway. The first step in the AhR signaling pathway is the binding of ligand by AhR. Studies presented in Fig. 8 demonstrate that AGN 190730 can competitively inhibit the binding of TCDD to AhR in cytosolic extracts and can thus bind to AhR. Upon ligand binding, AhR undergoes a conformational change, possibly exposing a nuclear localization signal to direct its movement from the cytosol to the nucleus. Like TCDD, AGN 190730 was found to induce the formation of a 35-kDa trypsin-resistant fragment upon binding to AhR (Fig. 7) and hence a conformational change in AhR. Once in the nucleus, liganded AhR

TABLE 1

Retinoid receptor transcriptional activation and competitive binding activity for the AGN compounds used in this study

Values represent the mean of three determinations. Errors in this assay are approximately 15% of the mean value.

AGN Compound Number	Structure	RAR Trans. EC_{50} /Bind. K_d			RXR Trans. EC_{50} /Bind. K_d		
		α	β	γ	α	β	γ
ATRA		240 14	38 11	6 16	NA NA	NA NA	NA NA
190121		435 135	7 20	10 190	NA NA	NA NA	NA NA
190186		>1000 1460	25 400	20 240	NA NA	NA NA	NA NA
190205		41 76	0.5 10	0.4 8	NA NA	NA NA	NA NA
190246		56 100	0.3 29	0.4 39	NA NA	NA NA	NA NA
190730		>1000 602	360 234	930 1670	NA NA	NA NA	NA NA
191312		2 5	0.5 5	0.1 10	NA NA	NA NA	NA NA
191440		3160 690	100 950	360 770	450 1870	ND 2350	ND 1990
191526		760 ND	7 ND	7 ND	NA ND	ND ND	ND ND
191650		>1000 600	3 103	19 301	NA NA	NA NA	NA NA
192240		NA NA	NA NA	NA NA	NA NA	NA NA	NA NA
192837		>1000 >1000	300 >1000	210 >1000	NA NA	NA NA	NA NA
193109		NA 22	NA 8	NA 7	NA NA	NA NA	NA NA
193313		>1000 237	3 54	2 42	NA NA	NA NA	NA NA
193762		>1000 119	6 26	4 179	NA NA	NA NA	NA NA

NA, not active (i.e., $>10^4$ nM); ND, not determined.

dimerizes with Arnt to form an active transcription factor that recognizes and binds to DREs in the promoter of target genes. Neither AhR nor Arnt is capable of binding to a DRE as a homodimer. Using EMSA assays, we demonstrate that treatment of in vitro transcribed and translated AhR and Arnt with AGN 190730 results in the formation of a complex that specifically recognizes a DRE (Figs. 5 and 6). Finally, upon binding to the DRE in the promoter of target genes, the liganded AhR/Arnt complex induces transcription of these genes, resulting in an increase in their mRNA and protein levels. Figures 1 to 4 demonstrate the induction of the transcription of a DRE-driven luciferase reporter construct and an AhR- and Arnt-dependent elevation in CYP1A1 protein levels by AGN 190730. Therefore, at each step in the AhR/Arnt signaling pathway, AGN 190730 treatment resulted in a response qualitatively indistinguishable from that of TCDD, strongly supporting the conclusion that some synthetic retinoids can activate the AhR/Arnt signaling pathway and be potent RAR or RXR ligands.

It is most probable that AGN 190730 itself and not a metabolite is responsible for the activation of the AhR/Arnt pathway and the concomitant induction of transcription of the DRE-driven luciferase reporter and CYP1A1. Although these studies were performed using intact cells, where it is not possible to eliminate metabolism of the retinoids, both the protein conformation studies and the EMSAs were performed in vitro using in vitro transcribed and translated proteins. It is unlikely that any significant metabolism of AGN 190730 occurred in these in vitro assays, strongly supporting the conclusion that AGN 190730 directly interacts with AhR and activates the AhR/Arnt signaling pathway.

Although the three retinoids, AGN 190730, AGN 193109, and AGN 192837, are much less active than TCDD, they fall within the same potency range as many other previously reported AhR ligands, including 3-methylcholanthrene, indole [3,2-*b*] carbazole, YH439, omeprazole, and benz[*a*]anthracene (Poland and Glover 1974; Postlind et al., 1993; Quattrochi and Tukey, 1993; Chen et al., 1995; Garrison et al., 1996; Lee et al., 1996). Clearly, the concentrations of these retinoids required to induce CYP1A1 transcriptional activity and activate the AhR/Arnt signal transduction pathway are easily achievable with pharmacological treatments currently in use with retinoid drugs as chemotherapeutic or chemopreventative agents. As new conformationally restricted retinoids and rexinoids that are polycyclic, aromatic, planar, and hydrophobic in nature are developed, attention should be placed on the determination of whether they can activate the AhR/ARNT signaling pathway in addition to their retinoid receptor isotype selectivity.

Whether activation of the AhR/Arnt signaling pathway, including induction of CYP1A1, is harmful to an organism is a complex question, the answer for which depends on many factors, including the nature of the compound, genetic makeup of the organism, and the environment of the organism. Classic AhR ligands, including TCDD, benzo[*a*]pyrene, and 3-methylcholanthrene, have a variety of toxic and carcinogenic effects on animals and humans. On the other hand, other compounds, such as indole-3-carbinol, indole-3-acetonitrile, and YH439, do not seem to cause tissue damage or carcinogenesis in animals despite elevating CYP1A1 levels via the AhR/Arnt signaling pathway (Loub et al., 1975; Babish and Stoewsand, 1978; Lee et al., 1996). Future studies are necessary to determine the consequences, if any, of the activation of AhR/Arnt signaling pathway by pharmacological doses of specific retinoids.

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