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# DIMINISHED TERATOGENICITY OF RETINOID X RECEPTOR-SELECTIVE SYNTHETIC RETINOIDS

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**Abstract**—One feature that contraindicates the wide therapeutic use of retinoids is their teratogenicity. Synthetic retinoids are distinguishable from each other on the basis of their partial or exclusive preference in binding and activation of all-*trans* retinoic acid receptors (RARs) or retinoid X receptors (RXRs). Using mouse embryo limb bud cells in micromass cultures as a bioassay, we examined the inhibitory activities of a number of standard and novel retinoids on chondrogenic cell differentiation. Transient cotransfection of HeLa cells was used to measure the ability of each retinoid to induce transcription of a reporter gene by activating RAR$\alpha$, RAR$\beta$, RAR$\gamma$, or RXR$\alpha$ chimeric constructs. All retinoids in this study that activated RARs to any degree in the cotransfection assay also inhibited chondrogenesis *in vitro*, whereas retinoids that were either specific for RXR or inactive in the cotransfection assay did not. The activity of RAR-selective agonists and the inactivity of RXR-specific agonists in the cotransfection assay correlated well with the relative teratogenicity of six of the representative retinoids studied when orally administered at day 11 to pregnant ICR mice.

*Key words:* RAR; RXR; retinoid receptors; teratogenesis; embryo; transactivation

RA§ and its synthetic analogs are in wide use in experimental and clinical studies as regulators of developmental and oncogenic events [1, 2]. Therapeutic uses of retinoids in dermatology and oncology, however, are accompanied by undesirable side-effects, including teratogenesis [3, 4]. The expectation that some of these side-effects might be obviated is bolstered by recent discoveries on retinoid signal transmission through two classes of nuclear receptor proteins that are not only structurally different but may also be functionally distinct [5].

The two classes of receptors are termed RARs and RXRs. Each class is represented by three genes termed $\alpha$, $\beta$, and $\gamma$. The sequences of RARs $\alpha$, $\beta$, and $\gamma$ are more homologous to each other than to the RXR genes [5, 6]. From cell-free systems and in transfected cells, two distinct pathways for transcriptional activation of responsive genes by retinoid receptors have been identified. In the first pathway, one of the RARs and one of the RXRs bind as heterodimers to specific half-sites in the promoters of the target genes in such a way that the RAR component is located downstream at the binding site. All-*trans* RA and certain synthetic retinoids, e.g. TTNPB, require such heterodimers (RXR/RAR) to activate transcription. The other pathway utilizes a different type of binding site that permits RXRs to bind as homodimers (RXR/RXR); certain synthetic retinoids preferentially activate transcription through such a mechanism. Interestingly, 9-*cis* RA is able to bind and activate both receptor pathways [7–10].

Considerable previous work on structure–activity relationships has established that synthetic retinoids differ from each other by several orders of magnitude in their teratogenic potencies [4, 11, 12]. Efforts are underway in several laboratories to design structurally modified analogs of RA that will retain therapeutic properties and yet be devoid of toxic side-effects [13–16]. This study was undertaken to seek correlations, if any, between teratogenicity and gene transactivational properties of a number of synthetic retinoids that are structural analogs of TTNPB (Fig. 1). Receptor binding and gene transcriptional activation studies have shown recently that TTNPB, a highly teratogenic benzoic acid derivative of retinoic acid, is a high-affinity ligand only for the RAR subtypes with no affinity for the RXRs [13–16].

We employed an *in vitro* screening system to evaluate the retinoids for their teratogenic activities. Mouse embryo limb bud mesenchymal cells spontaneously differentiate into chondrocytes when explanted at high cell density as micromass cultures in droplets of enriched medium [11, 17, 18]. RA consistently inhibits this differentiation process, which can be readily detected and quantified [11, 12, 19]. In this system, TTNPB shows its inhibitory activity to be almost 1000-fold greater than RA, which is similar in magnitude to what has

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§ Abbreviations: RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; TTNPB, 4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)propen-1-yl] benzoic acid; ER, estrogen receptor; ERE, estrogen response element; and CAT, chloramphenicol acetyltransferase.

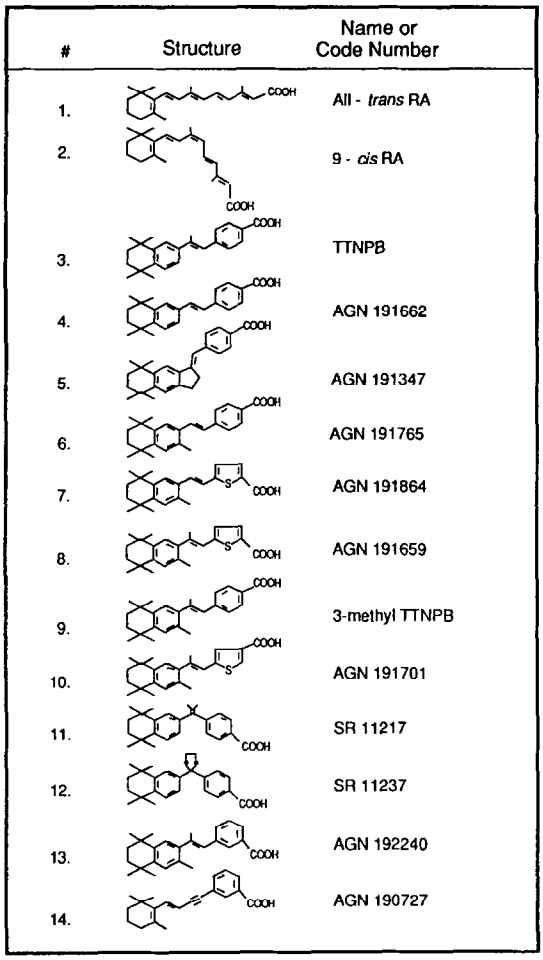


Fig. 1. Structures and names or identifying code numbers of retinoids employed in this study. The structures of compounds 11 and 12 were first reported by Lehmann *et al.* [15].

been reported in the whole animal teratogenesis [11, 12]. We report here that all RAR-specific ligands as well as all retinoids that activated both RARs and RXR$\alpha$ were actively inhibitory in the *in vitro* system. On the other hand, all RXR-specific retinoids were virtually inactive as chondrogenic inhibitors. Only six representative retinoids from the group were examined for teratogenicity in pregnant mice. The results support our suggestion that RXR-specific retinoids have diminished teratogenicity.

## MATERIALS AND METHODS

*Retinoids.* All-*trans* RA was obtained from the Sigma Chemical Co. (St. Louis, MO). 9-*cis* RA was a gift from Ligand Pharmaceuticals (San Diego, CA). All other retinoids used in this study (Fig. 1) were synthesized in the Retinoid Research Department of Chemistry and Biology, Allergan, Inc. (see the review of synthetic methods in Ref. 20 and references therein). All procedures involving manipulation of the retinoids were performed in the dark under dim yellow light to retard photodegradation. The analogs were dissolved in absolute ethanol (10 or 100 mg/mL) from which fresh dilutions were made before use. For oral intubation, the dilutions were made in vegetable (soybean) oil; for tissue culture studies, ethanolic preparations of the retinoids were diluted with the tissue culture medium.

*Receptor transactivation assay.* We determined the transactivation properties of retinoid analogs by measuring their ability to induce transcription in HeLa cells transiently cotransfected with a hybrid receptor gene construct and a reporter gene. The hybrid receptors contained the amino terminus and DNA-binding domains of the human ER and the hormone-binding domain of the human retinoid receptors. These ER-RAR (or ER-RXR) chimeric receptors bind to and activate transcription from promoter sequences recognized by the ER (estrogen response element, ERE), but do so in response to a retinoid ligand [21]. With these constructs we could use an ER-responsive reporter gene that cannot be activated by endogenous retinoid receptors which are present in most mammalian cells. The hybrid receptor gene constructs (ER-RAR$\alpha$, ER-RAR$\beta$, ER-RAR$\gamma$, and ER-RXR$\alpha$) were provided by the laboratory of M. Pfahl at the La Jolla Cancer Research Foundation, La Jolla, CA. HeLa cells were transiently transfected by a method described previously [22] with the expression vectors containing hybrid receptor gene constructs ER-RAR$\alpha$, ER-RAR$\beta$, ER-RAR$\gamma$, or ER-RXR$\alpha$ and a reporter plasmid ERE-tk-CAT [21].

HeLa cells were plated in 12-well dishes at a density of 50,000 cells/well in Dulbecco's Minimum Essential Medium (DMEM; Gibco BRL) supplemented with 10% fetal bovine serum (FBS). The cells were incubated for 20–24 hr at 37° in 5% $CO_2$. For each well, plasmid DNA (0.5 $\mu$g ERE-tk-CAT and 0.1 $\mu$g ER/RAR) was combined and diluted in 25 $\mu$L Opti-MEM (Gibco BRL). Lipofectin, a commercially available liposome mixture (Gibco BRL), was diluted separately to a concentration of 2 $\mu$g/25 $\mu$L in Opti-MEM. The transfection mixture was prepared by swirling the DNA and lipfectin together, waiting 10 min for complexes to form, and further diluting with Opti-MEM to 500 $\mu$L/well. The cells were transfected for 5 hr at 37°, 5% $CO_2$ prior to addition of 500 $\mu$L DMEM supplemented with 20% charcoal-treated FBS. Various concentrations of retinoids were added 18 hr after the start of transfection, and incubation continued for another 20–24 hr at 37°, 5% $CO_2$.

The cells were washed and lyzed directly in the wells for 60 min using 100 $\mu$L of an ice-cold hypotonic buffer containing 1 mM Tris–Cl, pH 7.8, 2 mM EDTA, 1% Triton X-100 and 0.4 mg/mL DNase 1. CAT enzyme activity was determined in 50 $\mu$L of the cell extract using a mixed phase scintillation counting method to measure the amount of $^3$H-acetylated chloramphenicol product that partitioned into the non-polar phase. The substrate, [$^3$H]acetyl CoA, remained in the urea-containing aqueous phase. The extract was assayed in a total volume of 100 $\mu$L containing 27 $\mu$L of Buffer-I (250 mM Tris–

Cl, pH 7.8, 5 mM EDTA), 20 μL of 5 mM chloramphenicol in Buffer-I, 0.75 μL of 4 mM acetyl CoA, 0.8 μL of 2.5 mM [$^3$H]acetyl CoA (0.4 μCi; New England Nuclear) and 1.5 μL of 1 mM HCl. For assay blanks, the chloramphenicol was replaced by Buffer-I. The extract was incubated for 2 hr at 37° and then quenched with 100 μL of 7 M urea. The reaction was transferred to a scintillation vial, and 1 mL of 0.8% 2,5-diphenyloxazole (PPO) in toluene was added. Following vortexing and phase separation for 15 min, cpm in the PPO/toluene phase were counted.

Transcriptional induction of CAT activity is expressed as a percent of maximal induction by all-*trans* RA. Typically, there was a 15-fold induction by ER/RARα, 8-fold by ER/RARβ and 6-fold by ER/RARγ. The $EC_{50}$ values for the retinoid compounds are representative of determinations from two to four independent experiments.

In vitro *chondrogenesis assay*. The *in vitro* bioassay employed high density micromass cultures of day 11 embryonic limb bud cells as described [18]. Briefly, forelimb buds were dissociated in a trypsin-EDTA solution, and the resultant single-cell suspension was plated as 20-μL spots (200,000 cells/spot) on plastic culture dishes. Retinoid concentrations ranging from 0.01 ng/mL to 10 μg/mL (0.03 nM to 30 μM) were added to the culture medium (Eagle's MEM + 10% FBS, GIBCO) 24 hr after initial plating. Control cultures received only the vehicle (ethanol, concentration < 1% by vol.). The cultures were terminated 96 hr after plating, at which time the medium was removed and the cells were fixed for 1 hr in 10% formalin containing 0.5% cetylpyridinium chloride. The cultures were stained with Alcian blue, and dehydrated in ethanol and scored for chondrogenesis under the microscope. An absence or reduction in the number of cartilage nodules in stained cultures as compared with control cultures was taken as a measure of suppression of chondrogenesis. The number of cartilage nodules stained in the whole spot were counted by automated image scan using the N.I.H. Image—1.52 application. The mean number of nodules and standard deviations were calculated for four replicate cultures per concentration. The median concentration of each retinoid causing 50% inhibition of chondrogenesis compared with controls ($IC_{50}$) was calculated by logarithmic curve fitting of the dose–response data.

*Teratogenesis*. Timed pregnant ICR mice were used in the whole animal teratology study essentially as described previously [18]. The day of vaginal plug detection was designated as day 0 of gestation. The animals were intubated once on the morning of day 11 of gestation with the indicated dose in the ethanol/soybean oil vehicle (1:19, v/v). The volume of the vehicle was held constant at 5 mL/kg. The animals in the control group were given an identical volume of the vehicle alone. All animals were killed on day 17 of gestation, and the resultant fetuses were examined for limb and craniofacial defects as described previously [18, 23, 24]. At least five litters were analyzed for each dose.

*Pharmacokinetics of AGN 191701 (compound No. 10)*. A single 10 mg/kg dose was given by gavage to mice on day 11 of gestation, and whole embryos and venous plasma samples were collected hourly from different groups of mice for 6 hr. Three litters were used for each time point. The animals were laparotomized under ether anesthesia; the blood was drawn into heparinized tubes from the inferior vena cava, and embryos were removed from the uterus and divested of their placentae and other membranes. The preparation of the plasma and embryo samples and extraction protocol were the same as described previously [18]. Briefly, the plasma (200 μL) and sonicated embryos (200 μg) were extracted with 2 vol. of ice-cold ethanol by vortexing and centrifugation, and the supernatants were analyzed immediately by injecting a 200-μL aliquot of the extract into the HPLC system. The efficiency of extraction was > 98% in the plasma samples and > 96% in the tissue samples. The HPLC system included two Waters 510 pumps (Waters Associates, Milford, MA), a Rainin Dynamax® chromatography workstation (Rainin Instrument Co., Woburn, MA), and a Waters analytical 5-μm reversed-phase $C_{18}$ column. The elution was done under isocratic conditions using a solvent containing methanol, acetonitrile, and 0.1 M ammonium acetate in a 73:14:13 ratio, respectively, at pH 6.8, at a flow rate of 1 mL/min. The reference retinoids included AGN 191701 [retention time (RT) = 9.4 min], 4-oxo-all-*trans* RA (RT = 4.0 min), 13-*cis* RA (RT = 12.2 min), all-*trans* RA (RT = 14.57 min), and retinol (RT = 19.2 min). Retinoid concentrations were calculated from the integrated HPLC peak areas with reference to standard curves obtained by analyzing known amounts (10–100 ng) of reference retinoids. The standard curves were linear over the range used. The detection limit in this system was 10 ng/mL for AGN 191701, all-*trans* RA, 13-*cis* RA, and retinol.

## RESULTS

*Receptor activation by retinoids*. The molar concentrations at which transactivation in HeLa cells was half-maximal ($EC_{50}$) are summarized in Table 1. As expected from the results of previous investigations [13–16, 25–31], all-*trans* RA (No. 1) and TTNPB (No. 3) were potent activators of all three RAR subtypes but were virtually inactive at RXRα. Compounds 4–7 were also strong RAR activators with no activity at RXRα. The transactivation profile of 3-methyl TTNPB (No. 9) differed considerably from that of TTNPB. 3-Methyl TTNPB and compound 8 were not only able to activate RARβ and RARγ but also RXRα. These two compounds were, therefore, similar to 9-*cis* RA (No. 2) as demonstrated previously [13–16]. The next group of three compounds (No. 10–12) were distinguished by the fact that they were virtually exclusive agonists of RXRα. While compounds 11 and 12 were totally inactive at the RAR subtypes, compound 10 (AGN 191701) still retained weak activity at RARβ and RARγ. The remaining two compounds, 13 and 14, were totally inactive; they activated neither RARs nor RXRα.

In vitro *chondrogenesis*. Table 1 also records the molar concentrations at which these retinoids produced half-maximal ($IC_{50}$) inhibition in the

Table 1. Comparison of molar concentrations of retinoids needed for receptor activation (in transfected HeLa cells) or for chondrogenic inhibition (in limb bud cells)

| Retinoid compounds (No.) | Receptor activation $EC_{50}$* (nM) RAR $\alpha$ | RAR $\beta$ | RAR $\gamma$ | RXR $\alpha$ | *In vitro* chondrogenic inhibition $IC_{50}$† (nM) |
|---|---|---|---|---|---|
| 1 | 5.0 | 1.5 | 0.5 | NA‡ | 31 |
| 2 | 102 | 3.3 | 6.0 | 13.0 | 58 |
| 3 | 21 | 4.0 | 2.4 | NA | 0.06 |
| 4 | 11 | 0.4 | 0.4 | NA | 1.2 |
| 5 | 11 | 0.5 | 0.5 | NA | <0.01 |
| 6 | 14 | 1.0 | 9.2 | NA | 0.8 |
| 7 | 65 | 0.9 | 3.0 | NA | <0.01 |
| 8 | >1000 | 23 | 37 | 11 | 15 |
| 9 | 4580 | 74 | 152 | 385 | 8.0 |
| 10 | >1000 | >1000 | >1000 | 201 | 63 |
| 11 | NA | NA | NA | 11.8 | NA |
| 12 | NA | NA | NA | 16.8 | NA |
| 13 | NA | NA | NA | NA | NA |
| 14 | NA | NA | NA | NA | NA |

* $EC_{50}$ = 50% maximal activation.
† $IC_{50}$ = 50% chondrogenic inhibition.
‡ NA not active (i.e. $EC_{50}$ or $IC_{50} > 10^4$ nM).

differentiation of limb bud mesenchymal cells into chondrogenic nodules in micromass cultures. TTNPB inhibited chondrogenesis with an $IC_{50}$ of 0.06 nM, which confirmed that it had > 500-fold higher potency than all-*trans* RA [11, 12]. The other four exclusive RAR agonists, compounds 4–7, were also very active inhibitors, with $IC_{50}$ values ranging from < 0.01 to 1.2 nM. The chondrogenic inhibitory activities declined drastically as the compounds decreased in their ability to activate RARs even though they simultaneously acquired the ability to activate RXR$\alpha$. Thus, 3-methyl TTNPB and compound 8 were 100- to 200-fold less active as inhibitors of chondrogenesis than TTNPB.

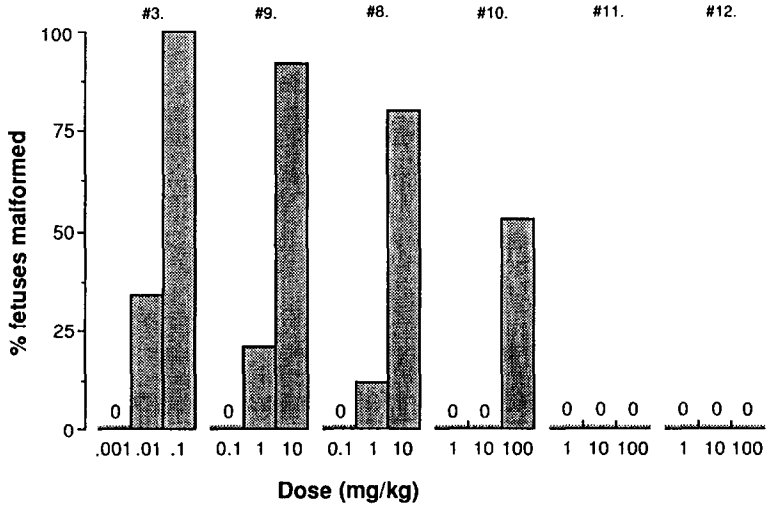


Fig. 2. Teratogenic potencies of the retinoids in ICR mice. Each retinoid is identified by the serial number at the top (referred to in Fig. 1). These are: compound 3, TTNPB; compound 8, AGN 191659; compound 9, 3-methyl TTNPB; compound 10, AGN 191701; compound 11, SR 11217; and compound 12, SR 11237. Ethanol-dissolved retinoids were freshly mixed with soybean oil and given as a single dose by gavage to mice on day 11 of gestation. Seven days later fetuses were removed and examined for limb and craniofacial defects [23, 24]. One-half of every litter was further examined after clearing the soft-tissue in 2% KOH and staining the skeleton with Alizarin red S dye. Percent fetuses malformed equals percent of total number of fetuses affected with any malformation. At least five litters were analyzed for each dose. Each litter yielded an average of ten live fetuses.

Compound 10 (AGN 191701), a strong RXR$\alpha$ activator but a weak RAR activator, was a weak inhibitor of chondrogenesis, being about a 1000-fold less potent inhibitor than TTNPB. The two RXR$\alpha$ specific agonists, compounds 11 and 12, which did not activate any of the RARs, were found to be without any inhibitor activity in the chondrogenesis assay. Compounds 13 and 14 were similarly inactive.

*Teratogenesis.* To assess the teratologic behavior of these retinoids in the whole animal, some of the representative compounds (No. 3 and 8–12) were evaluated in pregnant ICR mice. Retinoids were administered as a single dose by oral intubation on day 11 of gestation. It is known from previous studies that treatment of mice on day 11 with a single oral dose of 100 mg/kg body weight all-*trans* RA results in virtually every fetus showing severe shortening of the limbs as well as craniofacial defects including cleft palate [23, 24]. The results from these studies are summarized in Fig. 2. TTNPB (No. 3) was by far the most teratogenic amongst the compounds studied: all fetuses surviving after a maternal dose of 0.1 mg/kg were severely malformed. These fetuses had cleft palate and severe limb defects of the type documented previously [12, 23, 24]. Interestingly, 3-methyl TTNPB (No. 9) was over 100-fold less teratogenic than TTNPB, consistent with differential effects of the two compounds in RAR transactivation. The teratogenic potency of compound 8 was equal to that of 3-methyl TTNPB just as in the chondrogenesis assay, reflecting similarity in their RAR transactivation profiles. It should be noted that both 3-methyl TTNPB and compound 8 were significantly active at RXR$\alpha$, while TTNPB was essentially inactive at RXR$\alpha$. The teratogenic behavior of compound 10 (AGN191701) is important as a prototype of compounds that preferably activate RXRs over RARs. This retinoid produced no developmental effects at 1 or 10 mg/kg doses, and at 100 mg/kg produced cleft palates in 35% of the exposed fetuses and mild limb defects in 53% of the fetuses. Thus, it was over 1000 times less teratogenic than the structurally related TTNPB. The two specific RXR agonists, compounds 11 and 12, produced no teratogenic effects in mice at 10 or 100 mg/kg doses. Compounds 13 and 14, which were inactive at both RARs and RXR$\alpha$, were also not teratogenic (data not shown).

*Pharmacokinetics of AGN* 191701 *(compound* 10*).* A 10 mg/kg dose given orally in the ethanol/oil vehicle to mice on day 11 of gestation was absorbed and detected in the maternal plasma and the embryo at 1 hr after the oral treatment (Fig. 3). Peak levels ($C_{max}$) in the plasma and the embryo were reached at 2 and 3 hr respectively, and the values were 550 ± 94 ng/mL in the plasma and 284 ± 38 ng/g wet weight in the embryo. The levels in both compartments declined thereafter, but the retinoid was still detectable at 6 hr after the dose (Fig. 3). The retinoid yielded no metabolite at detectable levels in the plasma or the embryo, and the normal amounts of endogenous all-*trans* RA and retinol were not altered by AGN 191701 treatment.

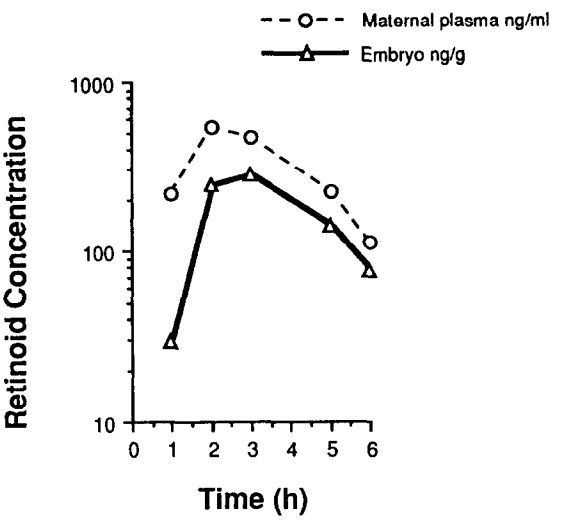


Fig. 3. Pharmacokinetic profile of AGN 191701 (compound 10) in the maternal plasma and embryos. A single 10 mg/kg dose of AGN 191701 was given by gavage to mice on day 11 of gestation, and whole embryos and venous plasma samples were collected hourly from different groups of mice for 6 hr. Each point represents the mean of three litters. AGN 191701 underwent ready placental transfer to the mouse embryo where its peak levels were maintained at 2–3 hr after the dose.

## DISCUSSION

A total of fourteen retinoids including all-*trans* RA and 9-*cis* RA were examined to assess if teratogenicity of each compound was positively or negatively correlated with its receptor activation properties.

The receptor activation assay that we employed involves cotransfection of HeLa cells with a chimeric receptor gene construct and an estrogen receptor-responsive reporter gene that cannot be activated by endogenous retinoid receptors usually present in all mammalian cells. Several investigators have previously reported receptor activation activities of TTNPB and other natural and synthetic retinoids using other cell types as well as different gene constructs and reporter genes [8, 9, 13–16, 25–31]. Given the considerable variations of analytical protocols in other studies, the $EC_{50}$ values of TTNPB in activating individual RAR subtypes are in the same range as reported here. Particularly, our results confirmed previous conclusions that TTNPB and all-*trans* RA bind and transactivate only the RAR subtypes while 3-methyl TTNPB and 9-*cis* RA transactivate both the RAR and RXR subfamilies [15, 16, 30].

From the receptor activation profiles we identified four compounds (No. 4–7) as exclusively RAR agonists with no activity at RXR$\alpha$. All of these RAR agonists actively inhibited chondrogenesis in the limb bud cell micromass cultures, which indicated that these compounds are also potentially strong teratogens. Kistler [11] has reported that teratogenic activities of a number of structural analogs of TTNPB in rats and mice parallel their inhibitory activities in the limb bud cell cultures. As expected, TTNPB was

highly teratogenic in pregnant ICR mice with a potency 500-fold greater than all-*trans* RA.

Recently, we [13] and Boehm *et al.* [16] reported that addition of a methyl group to the 3-position of TTNPB increases activity at RXRs while simultaneously decreasing activity at the RARs. Our results revealed that this compound was 100-fold less active than TTNPB in both teratogenesis bioassays. Compound 8, which like 3-methyl TTNPB can activate both RARs and RXRs, had teratogenic activity similar to that of 3-methyl TTNPB.

Most informative in this study were five compounds (No. 10–14) that demonstrated no teratogenicity at all or at the most were minimally teratogenic in mice or in the chondrogenesis assay. While two of these compounds (13 and 14) activated neither RARs nor RXR$\alpha$, the other two (11 and 12) were exclusive RXR agonists. The remaining member of this group (No. 10, AGN 191701) was similar to the other two RXR agonists in being not teratogenic in mice at lower doses, but at the high dose of 100 mg/kg it produced a teratogenic response. In the chondrogenesis assay, AGN 191701 was a weak inhibitor while the other RXR agonists were completely inactive. Since RAR agonists are strong teratogens, a residual activation of RAR$\beta$ and RAR$\gamma$ by this compound, which was observed in the transactivation assay, may be considered as a possible causative factor in teratogenesis at high doses.

There is previous evidence that RARs are indeed involved in mediating the teratogenic effects of all-*trans* RA, TTNPB, and several other synthetic retinoids including AGN 191701 [32, 33]. Jiang *et al.* [33] have reported that one of the receptors, namely RAR$\beta$2, was exceptionally up-regulated in the susceptible tissues of embryos treated with teratogenic retinoids. There was no RAR$\beta$2 mRNA up-regulation by AGN 191701 after the non-teratogenic 10 mg/kg dose, but a 12-fold enhancement over the basal level was obtained in limb buds of mouse embryos treated with the teratogenic 100 mg/kg dose of this compound.

The receptor activation profile of a given retinoid is by no means the only factor that influences its teratogenicity. It is known that maternal/placental metabolism of natural retinoids and an access to the embryo of the active moieties play a definitive role [34–36]. Arguably, the lack of teratogenicity of RXR$\alpha$ agonists, e.g. compounds 10–12, may simply be due to their inability to find access to the embryo. We have no information at present on the extent of placental transfer of compounds 11 or 12, but the results of a limited pharmacokinetic study on AGN 191701 (compound 10) showed that significant quantities of the intact compound were transferred to the embryo where it could be detected for a period of 6 hr after the 10 mg/kg oral dose. Peak levels of AGN 191701 were 550 ng/mL in the maternal plasma and 284 ng/g wet weight in the embryo (Fig. 3). The fact that no teratogenicity resulted in spite of a significant level of placental transfer of AGN 191701 suggests that this compound has much diminished teratogenic potency compared with all-*trans* RA. A similar pharmacokinetic profile in the maternal plasma and embryo has been reported after a 10 mg/kg dose of all-*trans* RA, which, however, was associated with a fairly high teratogenic response [37]. It should also be noted that the data from the *in vitro* chondrogenesis assay clearly showed that compounds 11 and 12 were, indeed, intrinsically inactive.

It is interesting that although all RAR agonists in our study were active inhibitors in the chondrogenesis assay, their individual potencies varied over a wide range. No obvious correlation is apparent between the inhibitory activity of a retinoid and its transactivation activity at RAR$\alpha$, RAR$\beta$, or RAR$\gamma$. Development of more RAR subtype selective retinoids will be useful to gain further information.

In conclusion, preliminary correlations reported here lend support to the view that RXR agonists elicit a minimal or a much weaker teratogenic response than the RAR agonists. Mechanistic explanations invoking molecular interactions and receptor response pathways may be premature at this stage, since currently most of the information is derived from *in vitro* and cell-free systems. It is established that RAR agonists preferentially act through RAR/RXR heterodimers (see reviews [5, 38, 39]). There is recent evidence that the major role of RXR in transactivation is only to facilitate binding of RAR to the responsive promoters, and that it is RAR which is directly responsible for the transcriptional activity [39, 40]. Although it is not yet known if RXR/RXR homodimers exist in nature, their formation has been shown to be preferentially induced in transfected cells by treatment with RXR-specific ligands [7, 10, 41]. If RXR homodimer formation were also to occur *in vivo*, our results suggest the possibility that response pathways mediated by such homodimers are less likely to lead to teratogenic lesions. It is important to note, however, that RXRs are absolutely essential in normal development, since RXR$\alpha$ null mutant embryos die *in utero* and show heart and eye malformations, presumably due to abrogation of the RAR/RXR function [42, 43].

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## REFERENCES

1. Hofmann C and Eichele G, Retinoids in development. In: *The Retinoids: Biology, Chemistry, and Medicine* (Eds. Sporn MB, Roberts AB and Goodman DS), pp. 387–435. Raven Press, New York, 1994.
2. Hong WK and Itri LM, Retinoids and human cancer. In: *The Retinoids: Biology, Chemistry, and Medicine* (Eds. Sporn MB, Roberts AB and Goodman DS), pp. 597–624. Raven Press, New York, 1994.
3. Kochhar DM and Satre MA, Retinoids and fetal malformations. In: *Dietary Factors and Birth Defects* (Ed. Sharma RP), pp. 134–230. Pacific Division AAAS, San Francisco, CA, 1993.
4. Willhite CC, Molecular correlates in retinoid pharmacology and toxicology. In: *Chemistry and Biology of Synthetic Retinoids* (Eds. Dawson MI and Okamura WH), pp. 539–573. CRC Press, Boca Raton, 1990.
5. Mangelsdorf DJ, Umesono K and Evans RM, The retinoid receptors. In: *The Retinoids: Biology,*

*Chemistry, and Medicine* (Eds. Sporn MB, Roberts AB and Goodman DS), pp. 319–344. Raven Press, New York, 1994.

6. Ruberte E, Kastner P, Dolle P, Krust A, Leroy P, Mendelsohn C, Zelent A and Chambon P, Retinoid acid receptors in the embryo. *Semin Dev Biol* **2**: 153–159, 1991.
7. Zhang XK, Lehmann J, Hoffmann B, Dawson MI, Cameron J, Graupner G, Hermann T, Tran P and Pfahl M, Homodimer formation of retinoid X-receptor induced by 9-*cis* retinoic acid. *Nature* **358**: 587–591, 1992.
8. Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM and Thaller C, 9-cis Retinoic acid is a high affinity ligand for the retinoid X receptor. *Cell* **68**: 397–406, 1992.
9. Levin AA, Sturzenbecker LJ, Kazmer S, Bosakowski T, Huselton C, Allenby G, Speck J, Kratzeisen CL, Rosenberger M, Lovey A and Grippo JF, 9-*cis* Retinoic acid stereoisomer binds and activates the nuclear receptor RXR$\alpha$. *Nature* **355**: 359–361, 1992.
10. Kurokawa R, Direnzo J, Boehm M, Sugarman J, Gloss B, Rosenfeld MG, Heyman RA and Glass CK, Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. *Nature* **371**: 528–531, 1994.
11. Kistler A, Limb bud cell cultures for estimating the teratogenic potential of compounds: validation of the test system with retinoids. *Arch Toxicol* **60**: 403–414, 1987.
12. Kochhar DM, Kraft JC and Nau H, Teratogenicity and disposition of various retinoids *in vivo* and *in vitro*. In: *Pharmacokinetic in Teratogenesis* (Eds. Nau H and Scott WJ), pp. 173–186. CRC Press, Boca Raton, FL, 1987.
13. Beard RL, Gil DW, Marler DK, Henry E, Colon DF, Gillett SJ, Arefieg T, Breen TS, Krauss H, Davies PJA and Chandraratna RAS, Structural basis for the differential RXR and RAR activity of stilbene retinoid analogs. *Bioorg Med Chem Lett* **4**: 1447–1452, 1994.
14. Jong L, Lehmann JM, Hobbs PD, Harlev E, Huffman JC, Pfahl M and Dawson MI, Conformational effects on retinoid receptor selectivity. 1. Effect of 9-double bond geometry on retinoid-X receptor activity. *J Med Chem* **36**: 2605–2613, 1993.
15. Lehmann JM, Jong L, Fanjul A, Cameron JF, Lu XP, Haefner P, Dawson MI and Pfahl M, Retinoids selective for retinoid-X receptor response pathways. *Science* **258**: 1944–1946, 1992.
16. Boehm MF, Zhang L, Badea BA, White SK, Mais DE, Berger E, Suto CM, Goldman ME and Heyman RA, Synthesis and structure–activity relationships of novel retinoid X receptor-selective retinoids. *J Med Chem* **37**: 2930–2941, 1994.
17. Ahrens PB, Solursh M and Reiter RS, Stage-related capacity for limb chondrogenesis in cell culture. *Dev Biol* **60**: 69–82, 1977.
18. Kochhar DM and Penner JD, Analysis of high dysmorphogenic activity of Ro 13-6307, a tetramethylated tetralin analog of retinoic acid. *Teratology* **45**: 637–645, 1992.
19. Kochhar DM, Penner JD and Hickey T, Retinoic acid enhances the displacement of newly synthesized hyaluronate from cell layer to culture medium during early phases of chondrogenesis. *Cell Differ* **14**: 213–221, 1984.
20. Dawson MI and Hobbs PD, The synthetic chemistry of retinoids. In: *The Retinoids: Biology, Chemistry, and Medicine* (Eds. Sporn MB, Roberts AB and Goodman DS), pp. 5–171. Raven Press, New York, 1994.
21. Benbrook D, Lernhardt E and Pfahl M, A new retinoic acid receptor identified from a hepatocellular carcinoma. *Nature* **333**: 669–672, 1988.
22. Husmann M, Lehmann J, Hoffmann B, Hermann T, Tzukerman M and Pfahl M, Antagonism between retinoic acid receptors. *Mol Cell Biol* **11**: 4097–4103, 1991.
23. Kochhar DM, Limb development in mouse embryos. I. Analysis of teratogenic effects of retinoic acid. *Teratology* **7**: 289–298, 1973.
24. Kochhar DM, Penner JD and Tellone CI, Comparative teratogenic activities of two retinoids: Effects on palate and limb development. *Teratogenesis Carcinog Mutagen* **4**: 377–387, 1984.
25. Graupner G, Malle G, Maignan J, Lang G, Prunieras M and Pfahl M, 6′-Substituted naphthalene-2-carboxylic acid analogs, a new class of retinoic acid receptor subtype-specific ligands. *Biochem Biophys Res Commun* **179**: 1554–1561, 1991.
26. Rottman JN, Widom RL, Nadal-Ginard B, Mahdavi V and Karathanasis SK, A retinoic acid-responsive element in the apolipoprotein-AI gene distinguishes between two different retinoic acid response pathways. *Mol Cell Biol* **11**: 3814–3820, 1991.
27. Boehm MF, McClurg MR, Pathirana C, Mangelsdorf D, White SK, Hebert J, Winn D, Goldman ME and Heyman RA, Synthesis of high specific activity [$^3$H]-9-*cis*-retinoic acid and its application for identifying retinoids with unusual binding properties. *J Med Chem* **37**: 408–414, 1994.
28. Lombardo A, Costa E, Chao W-R, Toll L, Hobbs PD, Jong L, Lee M-O, Pfahl M, Ely KR and Dawson MI, Recombinant human retinoic acid receptor $\beta$. Binding of synthetic retinoids and transcriptional activation. *J Biol Chem* **269**: 7297–7303, 1994.
29. Dawson MI, Hobbs PD, Stein RB, Berger TS and Heyman RA, Interaction of retinoids with retinoic acid nuclear receptor isoforms. In: *Retinoids: Progress in Research and Clinical Applications* (Eds. Livrea MA and Packer L), pp. 205–221. Marcel Dekker, New York, 1993.
30. Mangelsdorf DJ, Ong ES, Dyck JA and Evans RM, Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature* **345**: 224–229, 1990.
31. Allegretto EA, McClurg MR, Lazarchik SB, Clemm DL, Kerner SA, Elgort MG, Boehm MF, White SK, Pike JW and Heyman RA, Transactivation properties of retinoic acid and retinoid X receptors in mammalian cells and yeast. Correlation with hormone binding and effects of metabolism. *J Biol Chem* **268**: 26625–26633, 1993.
32. Soprano DR, Tairis N, Gyda M, Harnish DC, Jiang H, Soprano KJ and Kochhar DM, Induction of RAR-$\beta$2 gene expression in embryos and RAR-$\beta$2 transactivation by the synthetic retinoid Ro 13-6307 correlates with its high teratogenic potency. *Toxicol Appl Pharmacol* **122**: 159–163, 1993.
33. Jiang H, Gyda M III, Harnish DC, Chandraratna RA, Soprano KJ, Kochhar DM and Soprano DR, Teratogenesis by retinoic acid analogs positively correlates with elevation of retinoic acid receptor-$\beta$2 mRNA levels in treated embryos. *Teratology* **50**: 38–43, 1994.
34. Creech Kraft J, Kochhar DM, Scott WJ and Nau H, Low teratogenicity of 13-*cis*-retinoic acid (isotretinoin) in the mouse corresponds to low embryo concentrations during organogenesis: Comparison to the all-*trans* isomer. *Toxicol Appl Pharmcol* **87**: 474–482, 1987.
35. Creech Kraft J, Eckhoff Chr, Kochhar DM, Bochert G, Chahoud I and Nau H, Isotretinoin (13-*cis*-retinoic acid) metabolism, *cis-trans* isomerization, glucuronidation, and transfer to the mouse embryo: Consequences for teratogenicity. *Teratogenesis Carcinog Mutagen* **11**: 21–30, 1991.

36. Nau H, Chahoud I, Dencker L, Lammer EJ and Scott WJ, Teratogenicity of vitamin A and retinoids. In: *Vitamin A in Health and Disease* (Ed. Blomhoff R), pp. 615–663. Marcel Dekker, New York, 1994.
37. Creech Kraft J, Löfberg B, Chahoud I, Bochert G and Nau H, Teratogenicity and placental transfer of all-*trans*-, 13-*cis*-, 4-oxo-all-*trans*-, and 4-oxo-13-*cis*-retinoic acid after administration of a low oral dose during organogenesis in mice. *Toxicol Appl Pharmacol* **100**: 162–176, 1989.
38. Chambon P, The retinoid signaling pathway: Molecular and genetic analyses. *Semin Cell Biol* **5**: 115–125, 1994.
39. Stunnenberg HG, Mechanisms of transactivation by retinoic acid receptors. *Bioessays* **15**: 309–315, 1993.
40. Valcarcel R, Holz H, Jimenez CG, Barettino D and Stunnenberg HG, Retinoid-dependent *in vitro* transcription mediated by the RXR/RAR heterodimer. *Genes Dev* **8**: 3068–3079, 1994.
41. Yang Y, Vacchio MS and Ashwell JD, 9-*cis*-Retinoic acid inhibits activation-driven T-cell apoptosis: Implications for retinoid X receptor involvement in thymocyte development. *Proc Natl Acad Sci USA* **90**: 6170–6174, 1993.
42. Sucov HM, Dyson E, Gumeringer CL, Price J, Chien KR and Evans RM, RXR$\alpha$ mutant mice establish a genetic basis for vitamin A signaling in heart morphogenesis. *Genes Dev* **8**: 1007–1018, 1994.
43. Kastner P, Grondona JM, Mark M, Gansmuller A, LeMeur M, Decimo D, Vonesch J-L, Dollé P and Chambon P, Genetic analysis of RXR$\alpha$ developmental function: Convergence of RXR and RAR signaling pathways in heart and eye morphogenesis. *Cell* **78**: 987–1003, 1994.