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SHORT COMMUNICATIONS

Binding affinities of retinoids to fetal cellular retinoic acid-binding protein (CRABP) in relation to their teratogenic potency in hamsters

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Retinoids constitute a large group of synthetic and naturally occurring chemicals similar in structure to retinol (vitamin A). Several retinoids are used clinically for the treatment of recalcitrant cystic and conglobate acne, Darier's disease, pustular psoriasis, lichen ruber planus, basal cell carcinoma, keratoacanthoma and melanoma [1, 2]. A primary limitation to clinical usefulness of retinoids is their teratogenic potential [3]; at therapeutic levels administered during the first trimester of gestation, the developing human embryo can be severely damaged [4]. The malformations include dysmorphia of the face, heart, ears, and central nervous system, and of the axial and appendicular skeleton [4, 5]. The teratogenic effects of retinoids in hamsters have features similar to those observed in humans [4].

Cellular retinoic acid-binding protein (CRABP) is a polypeptide of 136 amino acid residues [6] and has been suggested as a factor involved in cytoplasmic retinoid uptake [7]. By immunohistochemical localization of CRABP, Momoi and co-workers [8] demonstrated that CRABP is expressed in neuroblasts of chick embryo and in the neural tube and notochord of the mouse embryo, and Maden *et al.* [9] established that CRABP forms a gradient across the developing limb which opposes the gradient formed by retinoic acid [10]. Thus, CRABP enhances the concentration gradient of retinoic acid in the limb bud. A family of nuclear retinoic acid receptors has also been described [11–13]. Cellular retinoic acid-binding proteins have been identified in all fetal rat tissues [except serum, where all-*trans*-retinoic acid (all-*trans*-RA) is transported bound to plasma albumin], in the intestine, brain, and kidney of the fetal rabbit, in mouse limb buds, and in embryonic chick skin [14–17]. Additionally, a retinoic acid-binding protein distinct from CRABP has been identified in the lumen of rat epididymis [18]. In the mouse limb bud, all-*trans*-RA has a 315-fold greater affinity for CRABP than 13-*cis*-retinoic acid (13-*cis*-RA) [16]. However, it is unknown if retinoid binding with embryonic CRABP is obligatory for teratogenic activity. In chick embryos, the relative teratogenic potencies of certain retinoids were correlated with affinity for chick limb bud CRABP [19]. Binding to embryonic chick CRABP was specific for retinoids with an acidic polar terminus, although some affinity was demonstrated for retinal.

Retinoids vary over several orders of magnitude in their teratogenic potencies in hamsters [3, 20, 21]. To assess the role of CRABP in retinoid-induced teratogenesis in mammals, the presence and concentrations of CRABP in normal embryonic and fetal hamster tissues were measured, and the ability of retinoid congeners to displace high-specific activity [^{3}H]-all-*trans*-RA from hamster fetal CRABP was investigated. The results are discussed in the context of retinoid teratogenic potency in hamsters.

Materials and Methods

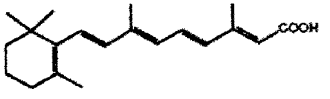
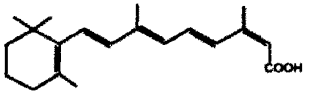
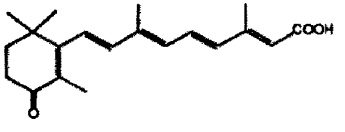
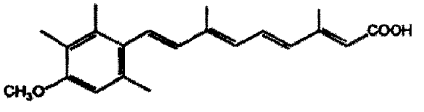
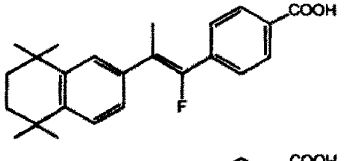
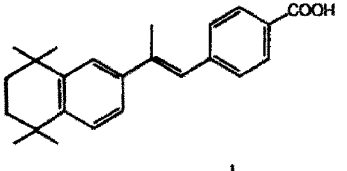
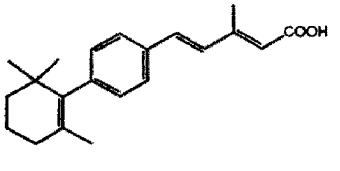
Chemicals. Table 1 lists the retinoids used in this study and their sources. All retinoids were more than 95% pure by reversed-phase HPLC [21]. Bovine serum albumin (BSA), myoglobin and 3-(*N*-morpholino)-propanesulfonic acid (MOPS) were purchased from Sigma (St. Louis, MO). Norit charcoal and HPLC grade water were obtained from Fisher Scientific (Pittsburgh, PA). Dextran T-70 was purchased from Pharmacia (Uppsala, Sweden). Radioactive all-*trans*-RA ([^{3}H]-all-*trans*-RA; [11,12-$^3H_2$]retinoic acid; 52.5 Ci/mmol; 98.8%) was purchased from NEN Research Products (Boston, MA).

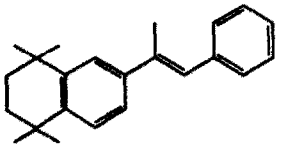
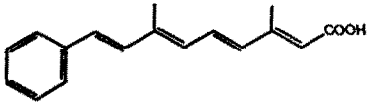
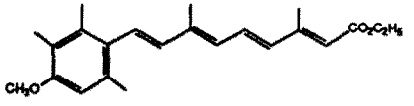
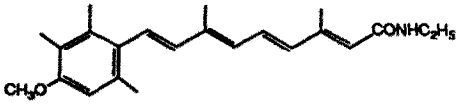
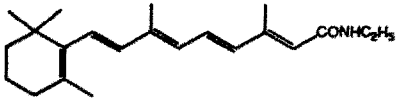
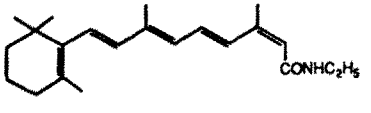
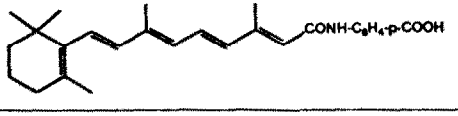
Preparation of cytosol. Timed-pregnant hamsters were purchased from the Charles River Breeding Laboratories (Wilmington, MA). The day following the evening of breeding was designated as day 1 of gestation. Animals were killed by CO_2 asphyxiation on the morning of day 8 or 12 of pregnancy for isolation of embryos or fetuses respectively. Whole embryos or fetuses were collected after laparotomy and placed in a centrifuge tube containing an equal volume (w:v) of ice-cold buffer (10 mM MOPS, 10 mM KCl, 2 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.5). Tissues were homogenized on ice for 1 min using a Tissue Tearor tissue homogenizer (Biospec Products, Bartlesville, OK) on setting 4. The homogenate was centrifuged at 10,000 *g* (4°) for 0.5 hr, and the supernatant fraction was subsequently centrifuged for 1 hr at 105,000 *g* (Beckman SW 41 Ti rotor). The protein concentration of the supernatant fraction was determined using the Pierce protein assay reagent (Pierce, Rockford, IL) with BSA as the standard. Cytosol was frozen at −80° until used.

Ligand binding assay of cellular retinoic acid-binding protein. Frozen cytosol was thawed at 4° overnight in the dark. The cytosol was diluted with fresh MOPS buffer to a final protein concentration of 1.04 mg/mL. An aliquot of 480 μL of dilute cytosol was pipetted into incubation tubes, and 10 μL of 100 nM [^{3}H]-all-*trans*-RA (2.63 μCi in 95% ethanol) was added to each tube. An additional 10 μL of ethanol was added to individual tubes. The retinoid–protein solution was incubated for 12 hr (overnight) at 4° in the dark. The reaction was terminated by the addition of 100 μL of dextran-coated charcoal (2.5% charcoal, 0.25% dextran). The mixture was vortexed immediately and at 2-min intervals for 10 min. The charcoal was sedimented at 1000 *g* for 10 min and the supernatant fraction subsequently filtered through a 0.45 μm filter to remove all traces of charcoal prior to chromatography. Procedures involving all retinoid solutions were performed under yellow light with as little exposure to air as possible to prevent retinoid decomposition.

High-performance size-exclusion chromatography. Methods for high-performance size-exclusion chromatography (HPSEC) were as described by Rainier *et al.* [15]. A Varian model 5000 liquid chromatograph equipped with a SOTAPhase GF-200, 10 μm, 7.1 × 300 mm column and a SOTAPhase GF-200, 7.1 × 75 mm guard column (Rainin, Emeryville, CA) was used. The elution buffer contained 10 mM MOPS, 2 mM 2-mercaptoethanol, 10 mM potassium acetate, and 1 mM EDTA, pH 7.5. The buffer was prepared fresh daily and filtered (0.2 μm) prior to use. The eluent was pumped at a flow rate of 1.0 mL/min and was monitored at 280 nm using a Varian Varichrom UV/Visible spectrophotometer. Filtered supernatant (200 μL) was injected onto the column. Myoglobin and BSA were used as standards. The elution position of the [^{3}H]-all-*trans*-RA–CRABP complex was determined by collecting 0.5-mL fractions of the eluent. Fractions were counted in 4 mL of

Table 1. Retinoid names, structures, binding affinities to CRABP, and teratogenic potency in hamsters

Synonym or code name	Structure	Relative affinity DC_{50}* (M)	Teratogenic dose (ED_{50}) (μmol/kg) [Ref.]
Tretinoin† (Ro1-5488) all-*trans*-RA		4.7×10^{-7}	35 [23]
Isotretinoin† (Ro 4-3780) 13-*cis*-RA		2.6×10^{-6}	74 [23]
Ro 12-4824‡		3.4×10^{-7}	30 [21]
Etretin‡ (Ro 10-1670)		6.4×10^{-6}	Not applicable§
SRI 5631-96‖		2.2×10^{-5}	0.22 (unpublished)¶
TTNPB‡ (Ro 13-7410)		6.2×10^{-7}	<0.06 [25]
SRI 4657-47‖		Slight	Inactive (unpublished)¶

Temarotene‡ (Ro 15-0778)		NC	Inactive (unpublished)¶
Ro 8-8717‡		NC	Inactive (embryolethal) [21]
Etretinate‡ (Ro 10-9359)		NC	19 [24]
Motretinid‡ (Ro 11-1430)		NC	Inactive [21]
All-*trans*-*N*-Ethylretinamide**		NC	Inactive [23]
13-*cis*-*N*-Ethylretinamide**		NC	Inactive [23]
SRI 7167-67‖		NC	Inactive (unpublished)¶

* Concentration of chemical needed to displace 50% of bound all-*trans*-RA. NC = no competition observed.
† Obtained from Sigma (St. Louis, MO).
‡ Obtained from F. Hoffmann-La Roche (Nutley, NJ).
§ No data available in hamsters on free acid of etretinate. The ED_{50} of its ethyl ester (Ro 10-9359) is 19 μmol/kg [24].
‖ Obtained from SRI International (Menlo Park, CA).
¶ Willhite CC, unpublished observation.
** Obtained from Southern Research Institute (Birmingham, AL).

scintillation fluid in a Beckman LS 3801 liquid scintillation counter, and disintegrations per minute were quantified using an external standard.

Saturation analysis. Because of the larger quantities of CRABP that could be isolated from the fetus, day-12 fetal cytosol (500 μg of protein in 480 μL) was incubated in the dark at 4° for 12 hr with 10 μL of [^{3}H]-all-*trans*-RA (1–465 nM in 95% ethanol) and 10 μL of ethanol. The incubation was terminated with the addition of 100 μL of ice-cold dextran-coated charcoal (2.5% charcoal, 0.25% dextran). Samples were centrifuged and filtered, and fractions (0.5 mL) were collected and counted as described above. Specific binding was calculated by the amount of radioactivity in the 10 to 11-mL fraction, and a Scatchard analysis [22] was performed.

Competitive binding assay. Competition studies were performed by incubating fetal protein (500 μg of protein in 480 μL of fresh MOPS buffer) at 4° for 12 hr either with 100 nM [^{3}H]-all-*trans*-RA (2.63 μCi in 10 μL of ethanol) alone, or with 10 μL of ethanol containing from 0.1- to 200-fold molar excess of unlabeled retinoid analog. The incubation mixtures were treated with dextran-coated charcoal, centrifuged, filtered, chromatographed, and counted as described above. Displacement of [^{3}H]-all-*trans*-RA was calculated by comparing the radioactivity in corresponding CRABP fractions with that of a system containing an identical volume of ethanol vehicle only, followed by a probit-maximum likelihood estimation of the retinoid concentration to displace 50% of the labeled ligand.

Results

Presence of CRABP. Discrete radioactivity peaks were observed when samples were incubated with 100 nM [^{3}H]-all-*trans*-RA and separated by HPSEC. The first peak of radioactivity (elution volume 6–8 mL) was not displaced by incubation with 200-fold molar excess of unlabeled all-*trans*-RA. The peak (elution volume, 10–11 mL) that eluted at the same elution volume as myoglobin (molecular mass = 17,000) was abolished by incubating with 200-fold molar excess of all-*trans*-RA (data not shown). This observation provided evidence for the presence of specific binding for retinoic acid. The binding of all-*trans*-RA at the peak corresponding to CRABP was unaltered by a 200-fold excess of all-*trans*-retinol (data not shown). The presence of CRABP was identified initially in day 8 hamster embryos. Based on the assumption that one molecule of all-*trans*-RA binds per CRABP molecule [14], the concentration of CRABP measured in 8-day embryonic tissue was 2.2 pmol/mg protein (data not shown). Day-12 hamster fetal CRABP bound labeled all-*trans*-RA at 8.9 pmol/mg protein.

Saturation analysis. Scatchard plots of data obtained using the fetal CRABP yielded an apparent all-*trans*-RA dissociation constant (K_d) of 12.7 nM. The estimated total specific binding capacity from the Scatchard plot was 11.4 pmol CRABP/mg protein based on the cytosolic fraction (date not shown).

Competitive binding assay. The relative binding affinities of various retinoids to fetal CRABP were determined using an *in vitro* competition protocol. First, the cytosol was incubated with 100 nM [^{3}H]-all-*trans*-RA and 200-fold excess of one of each of the fourteen retinoids (Table 1) to determine if the retinoid displaced [^{3}H]-all-*trans*-RA. Then the amount of free ligand (competitor) was varied in the assay to displace differential amounts of [^{3}H]-all-*trans*-RA. The concentration to displace 50% of the label from the protein (DC_{50}) was calculated (Figs. 1 and 2). The DC_{50} for each retinoid is shown in Table 1, along with the median effective teratogenic dose (ED_{50}) of the retinoids studied here [21, 23–25]. The concentrations required to displace 50% of the bound all-*trans*-RA are not definitive dissociation constants; rather, they express the relative affinity of various retinoids to CRABP.

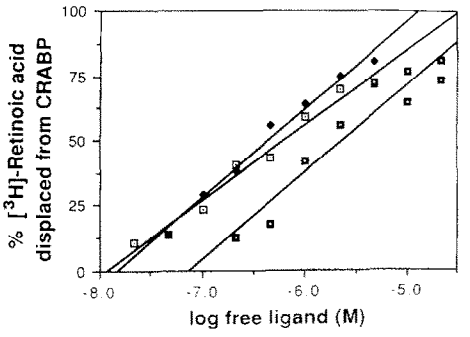


Fig. 1. Competition of retinoid analogs (all-*trans*-RA, Ro 13-7410 and 13-*cis*-RA) with fetal hamster CRABP. The displacement of [^{3}H]all-*trans*-RA occurred with increasing concentrations of all-*trans*-RA (diamonds), Ro 13-7410 (open boxes), or 13-*cis*-RA (solid boxes).

Discussion

Measurable quantities of CRABP were detected in the embryonic, early primitive streak stage (day-8) hamster and in the day-12 hamster fetus. The protein had a high degree of specificity for all-*trans*-RA and possessed a different ligand specificity than the cellular retinol-binding protein found in fetal tissues [15]. The saturation analysis plot for all-*trans*-RA was linear, thus demonstrating a single binding site, which is consistent with the findings of Kwarta *et al.* [16] and Maden and Summerbell [19]. The dissociation constant (K_d) of hamster fetus CRABP of 12.7 mM compares with 2 nM for the mouse limb bud [16], 4.2 nM [26] and 318 nM [27] for rat testis, and 400 nM for human skin [28].

Competition study data showed that binding with CRABP was dependent on a free acid at position C15. Retinoids that lacked a polar terminus (Ro 15-0778) or those retinoids that contained ester (Ro 10-9359) or amide (e.g. Ro 11-1430) substitutions at C15 failed to displace [^{3}H]-all-*trans*-RA. This finding is consistent with CRABP isolated from chick wing buds [19] and hamster tissues [29]. Data from studies with SRI 7167-67 illustrate the importance of the length of the side chain substituent, even with those retinoids containing a carboxyl terminus. Sani *et al.* [30] reported a 15-fold difference in the binding affinities of various retinoids to chick embryo skin CRABP. Our results indicate that the affinity of Ro 13-7410 with CRABP was approximately one-half that of all-*trans*-RA. It should be pointed out, however, that Ro 13-7410 is approximately 750 times as teratogenic in hamsters (based

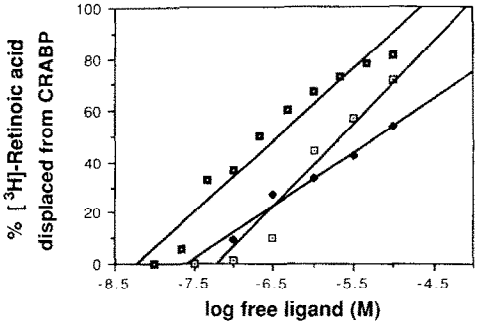


Fig. 2. Competition of retinoid analogs (SRI 5631-96, Ro 12-4824 and Ro 10-1670) with fetal hamster CRABP. The displacement of [^{3}H]all-*trans*-RA occurred with increasing concentrations of SRI 5631-96 (open boxes), Ro 12-4824 (solid boxes), or Ro 10-1670 (diamonds).

on the maternal body weight orally administered dose) as all-*trans*-RA [25]. The ethyl ester congener of etretin (etretinate) is twice as teratogenic in the hamster as all-*trans*-RA [24], though it failed to bind with fetal CRABP. These data support the finding that etretinate is biotransformed to the free acid etretin in hamsters [24]. Etretin was the poorest ligand with an affinity for CRABP that was 18-fold lower than all-*trans*-RA (Table 1). The primary metabolite of all-*trans*-RA in hamsters and humans is all-*trans*-4-oxoretinoic acid (Ro 12-4824) [31], the affinity of which is equal to that of all-*trans*-RA and which is as teratogenic in hamsters as all-*trans*-RA [21]. Isotretinoin (13-*cis*-RA) has approximately one-tenth the affinity for CRABP and one-half the teratogenic potency in hamsters as all-*trans*-RA [23]. Maden and Summerbell [19] found that Ro 13-7410 has twice the affinity with CRABP as all-*trans*-RA in the chick wing bud, while 13-*cis*-RA has six times greater affinity with CRABP than all-*trans*-RA. The present results contrast with those of Kwarta *et al.* [16], who demonstrated that, in the mouse limb bud, all-*trans*-RA is associated with a 315-fold greater binding affinity for CRABP than 13-*cis*-RA. In our experiments, as well as those reported by others [16, 19], the isomerization of 13-*cis*-RA to its all-*trans*-congener cannot be ruled out; therefore, the apparent binding affinity of 13-*cis*-RA depends on the relative event of *cis/trans* isomerization of these polyene retinoids, which, in turn, depends on the conditions of the study.

The present investigation determined that acidic teratogenic retinoids bound with CRABP and that retinoids without appreciable teratogenic activity failed to bind CRABP. The conclusions reached here do not apply to parent drugs (e.g. etretinate) which are metabolized to teratogenically active metabolites (e.g. etretin). However, even in the case of parent acidic congeners, there was no apparent correlation between *in vivo* teratogenic potency and the observed DC_{50}. The present structure–activity patterns suggest that retinoid binding with embryonic CRABP may play a role in retinoid-induced teratogenesis.

Chambon and co-workers [11, 12] and Benbrook *et al.* [13] demonstrated specific retinoic acid nuclear receptors that belong to the steroid hormone family of nuclear receptors. Retinoids modify embryonic gene expression with a mechanism analogous to steroid receptor interactions; all-*trans*-RA binds to CRABP, and the CRABP–all-*trans*-RA complex may act as a shuttle transporting retinoic acid into the nucleus [32, 33] where it can interact with various nuclear receptors [11–13]. Therefore, our conclusion that *in vitro* retinoid affinity for embryonal/fetal CRABP failed to correlate in a quantitative manner with *in vivo* teratogenic potency may reflect biotransformation of retinoids to multiple, teratogenic metabolites, in concert with time-dependent concentrations of each teratogenic entity in the respective embryonic germ layer. Retinoid affinity for retinoic acid nuclear receptors may correlate more closely with their relative biologic activities with an intervening transport function of the retinoid–CRABP complex.

In summary, the binding affinities of fourteen retinoid analogs with fetal hamster CRABP were determined. High-performance size-exclusion chromatography was employed for separation of CRABP in embryonic or fetal tissue homogenates, and displacement of high specific activity [^{3}H]-all-*trans*-retinoic acid from CRABP was evaluated. Teratologically active retinoids bound to CRABP, while congeners that are teratogenically inactive in hamsters (i.e. retinamides, retinoids lacking an acidic polar terminus, retinoids without sufficient ring substituents or elongated side chains) failed to bind with the protein. Of the retinoids that could compete for the CRABP binding site with [^{3}H]all-*trans*-retinoic acid, the following rank order emerged: all-*trans*-retinoic acid = all-*trans*-4-oxoretinoic acid > tetrahydrotetramethylnaphthalenylpropenyl benzoic acid (TTNPB, Ro 13-7410) > (*Z*)-4-[1-fluoro-2(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl] benzoic acid (SRI 5631-96) > 13-*cis*-retinoicacid > etretin(Ro10-1670).

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Effects of *S*-adenosyl-L-methionine on phospholipid methyltransferase activity changes induced by thioacetamide

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Acute administration of thioacetamide (TAA*) induces a maximum degree of hepatic necrosis [1]. Schriewer and Lohman [2] have reported an increase in the microsomal PE/PC ratio by acute effect of thioacetamide. In a more detailed study of microsomal phospholipid composition we have confirmed those results as an acute response of microsomal membranes to the toxic action of thioacetamide [3]. Microsomal PE/PC ratio is the substrate/product ratio of the reaction catalysed by *S*-adenosyl-L-methionine; phospholipid methyltransferase (E.C. 2.1.1.17).

Several pharmacological effects have been reported regarding *S*-adenosyl-L-methionine [4–7], although its biochemical mechanism of action is unknown. *S*-Adenosyl-L-methionine partly recovered the TAA-induced liver necrosis [8]. This observation suggests that *S*-adenosyl-L-methionine could prevent the TAA-induced alteration in PE/PC ratio. SAM:phospholipid methyltransferase as a potential responsible for these microsomal changes induced by TAA is an enzyme whose activity can be modulated by exogenous administration of *S*-adenosyl-L-methionine [9]. In this paper we have studied the role of phospholipid methyltransferase in TAA-induced liver necrosis and the effect of exogenous administration of Ado-met on this activity.

* Abbreviations: Ado-hcy: *S*-adenosyl-L-homocysteine; Ado-met, *S*-adenosyl-L-methionine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAA, thioacetamide.