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Structure-Activity Relationships of Retinoids in Developmental Toxicology

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STRUCTURE-ACTIVITY RELATIONSHIPS OF RETINOIDS IN DEVELOPMENTAL TOXICOLOGY

by

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The following retinoids, (11-^{3}H)-9-cis-retinal, (11-^{3}H)-13-cis-retinoic acid, (11-^{3}H)-all-trans-4-oxo-retinoic acid, (10,11-$^{3}H_2$)-all-trans-retinoic acid, (10,11-$^{3}H_2$)-retinyl acetate, (11-^{3}H)-all-trans-retinamide, (11-^{3}H)-13-cis-retinamide, and (6,7-$^{3}H_2$)-TTNPB, were received from the Biological and Chemical Prevention Program, Chemical and Physical Carcinogenesis Branch, a function

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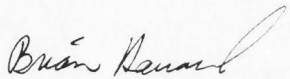

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ABSTRACT

Structure-Activity Relationships of Retinoids

in Developmental Toxicology

by

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The teratogenic potency of retinoid analogs was determined in Syrian hamsters and compared to the teratogenic potency of all-trans-retinoic acid (all-trans-RA, ED_{50} = 10.5 mg/kg). A total of 15 analogs having variations in the cyclohexene ring were evaluated following various amounts of single oral doses on day 8 of gestation. Retinoids containing a five- or six-membered ring were as teratogenic as all-trans-RA, provided they had sufficient lipophilic substituents on the ring. The same pattern emerged for retinoids that had six-membered aromatic ring substitution for the natural cyclohexene ring of vitamin A. Incorporation of a supplementary aromatic ring in the side-chain adjacent to a gem-dimethyl-hexene ring resulted in an increase in teratogenicity by 18-fold compared to all-trans-RA. Major modifications of the cyclohexene ring can be made without altering teratogenic activity. The ring need not be six-membered and can have decreased lipophilicity through the incorporation of polar groups compared to all-trans-RA, but must have sufficient lipophilic substituents to provide the necessary mass for interaction with the retinoid receptor. Incorporation of a supplementary aromatic ring

adjacent to a gem-dimethyl-hexene ring facilitated π-electron delocalization and restricts side-chain flexibility, thereby increasing teratogenic potency.

The pharmacokinetic disposition of 8 retinoids was investigated. Pregnant hamsters were dosed orally with all-trans-RA, 13-cis-retinoic acid, all-trans-4-oxoretinoic acid, 9-cis-retinal, all-trans-retinyl acetate, N-ethyl-all-trans-retinamide, N-ethyl-13-cis-retinamide, and arotinoid. The bioavailability of the retinamides was one-tenth that of the free acid retinoids. The plasma elimination half-life for all-trans-RA was 0.5 h. For 13-cis-retinoic acid and all-trans-4-oxoretinoic acid the elimination half-lives were 4.4 and 5.7 h, respectively.

The binding affinity of various retinoids to cellular retinoic acid-binding protein (cRABP) was determined in day-12 hamster fetuses. Fetal supernatants from the 105,000x g fraction were incubated with high specific-activity [^{3}H]-all-trans-RA in the presence of various concentration of unlabeled retinoids with subsequent isolation of cRABP by size-exclusion HPLC. Teratogenic retinoids, or acidic metabolites of teratogenic retinoids bound to cRABP whereas nonteratogenic retinoids failed to bind.

(181 pages)

CHAPTER I

STATEMENT OF PROBLEM

The retinoids constitute a large group of synthetic and naturally occurring compounds similar in structure to retinol (vitamin A). Several retinoids are used clinically for treatment of recalcitrant cystic and conglobate acne, Darier's disease, pustular psoriasis, lichen ruber planus, basal cell carcinoma, keratoacanthoma and melanoma (Peck, 1982; Orfanos, 1980). In mice certain retinoids exert a therapeutic influence on chemically-induced papillomas and carcinomas of the skin (Bollag, 1975), and in rats certain retinoids prevent cancer of the urinary bladder (Sporn et al., 1977) and mammary gland (Moon and McCormick, 1982; Hartmann and Bollag, 1985).

The toxicology of the retinoids in humans and animals has been reviewed (Howard and Willhite, 1986). Adverse effects of retinoids are usually limited to such dermatologic disorders as alopecia, chelitis, exfoliation, dermatitis, conjunctivitis, paronichia, pruritis, and xerosis. However, when certain retinoids are administered at therapeutic dose levels to women in the first trimester of gestation, the developing embryo can be severely damaged (Willhite et al., 1986). Isotretinoin (Accutane®; 13-cis-retinoic acid; 13-cis-RA) and etretinate (Ro 10-9359, Tigason®) can induce severe malformations in human infants, including dysmorphia of the face, of the central nervous system, of the urogenital system, of the cardiovascular system, and of the axial and appendicular skeleton (Willhite et al., 1986; Happle et al., 1984). The teratogenic effects of retinoids in Syrian golden hamsters have features in common with those observed in humans, who are approximately 16 times more sensitive (on a mg/kg basis) to the teratogenic effects of 13-cis-RA than hamsters (Willhite et al., 1986).

The retinoids exhibit large variations in their teratogenic potencies in animals. Such conformationally restricted retinoids as the retinoidal benzoic acid derivative Ro 13-6298, containing one or two supplementary rings in their side chains, are 10-1,000 times more potent teratogens in hamsters than all-trans-retinoic acid (all-trans-RA) (Flanagan et al., 1987). Ro 13-6298 is 1,000 times more potent at induction of cartilage resorption in rat embryonic limb buds than tetraene retinoids (Kistler, 1985), and it is at least 130 times as embryolethal as all-trans-RA in hamsters (Flanagan et al., 1987). Retinamides and retinylidene 1,3-diketones contain modifications of the polar terminus of the vitamin A molecule, and these retinoids are devoid of teratogenic activity in hamsters (Willhite et al., 1984). These differences may be related to absorption from the gut, biotransformation patterns, pharmacokinetic or disposition differences, differential placental permeabilities, or differential binding affinities with embryonic cellular retinoic acid-binding protein (cRABP).

Proposed mechanisms of retinoid action have included amphipathic membrane alterations via non-specific, detergent-like disruption (Fell and Dingle, 1963), glycosylation of cell surface glycoproteins via formation of retinyl mannose phosphate intermediates (De Luca et al., 1979), and activation and suppression of the genome (Sporn and Roberts, 1983). Retinoid-induced teratogenicity cannot be due to non-specific membrane disruption, because retinoids containing a hydrophilic carboxyl terminus, a 9-carbon flexible side chain, and a β-cyclogeranylidene ring failed to induce terata (Willhite, 1986). Nor can retinoid toxicities be attributed to involvement in sugar transfer reactions, as the retinoidal benzoic acid derivative Ro 13-6298 does not participate in retinyl phosphate mannose pathways (Sporn and Roberts, 1983).

Several research groups have provided biochemical data on retinoid control of cellular proliferation. When retinoic acid was used to induce

differentiation in cultured HL-60 cells, it suppressed expression of the myc oncogene, a nucleotide sequence considered to be involved in excessive proliferation of HL60 cells (Sporn and Roberts, 1983). Omori and Chytil (1982) demonstrated that feeding retinyl acetate, retinoic acid, or 3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (a synthetic retinoid) to retinol-deficient rats suppressed production of a 22 kDa protein; whereas retinoic acid and the synthetic retinoid both induced production of a 55 kDa protein. It appears from these results that retinoids modify cellular gene expression (Sporn and Roberts, 1983; Omori and Chytil, 1982). Chytil and Ong (1979) have put forth the hypothesis that retinoids are bound in the cytosol by either cellular retinol-binding protein (cRBP)(if an alcohol) or cRABP (if an acid) and are subsequently transported into the nucleus where they can modify gene expression.

Cellular retinoic acid-binding protein has been identified in all fetal rat tissues (except in serum, where all-trans-RA is transported bound to plasma albumin), in the intestine, brain, and kidney of the fetal rabbit, in limb buds of the mouse, and in embryonic chick skin (Chytil and Ong, 1983; Rainier et al., 1983; Kwarta et al., 1985). In the mouse limb bud, all-trans-RA has a 315-fold greater affinity for cRABP than 13-cis-RA (Kwarta et al., 1985). Because the teratogenic effective dose 50 (ED_{50}) is 22.3 and 10.5 mg/kg for isotretinoin (13-cis-RA) and all-trans-RA, respectively, and because the dose-response curves are parallel, it can be concluded the 13-cis-RA is approximately one-half as potent a teratogen as all-trans-RA in hamsters (Willhite and Shealy, 1984). And 13-cis-RA is four to eight times less embryopathic than all-trans-RA in the mouse (Kochhar et al., 1984b). However, it is unknown if binding to cRABP is obligatory for teratogenic activity. The dose-response curves for induction of terata by retinoids in the hamster are parallel, suggesting a

common embryotoxic mechanism of action. The structural requirements for teratogenic activity of retinoids in hamsters were primarily dependent upon the presence of, or biotransformation to, a free carboxyl or other moiety with an equivalent pKa at C15 for the polar terminus, not upon the stereochemistry about C13, or C9, nor upon the size of the molecular substituent at C15. For the polyene side-chain, retinoid activity was dependent upon a carbon side-chain of more than 5-carbon atoms, upon conservation of the curved, hydrophobic plane and upon preservation of π-electron delocalization across the polyene chain (Willhite et al., 1984; Willhite, 1986).

SPECIFIC AIMS

This research was designed to determine some of the structural requirements of the vitamin A molecule for induction of terata in hamsters. The project also focused upon the contribution of some of the maternal and embryonic factors (i.e. pharmacokinetics, placental permeability, distribution, and disposition) in retinoid-induced teratogenesis. In addition, a study clarifying the possible role of cRABP as it pertains to the differences in the potencies of retinoid-induced terata is presented.

CHAPTER II

TOXICITY OF RETINOIDS IN HUMANS AND ANIMALS: A LITERATURE REVIEW

INTRODUCTION

As early as ancient Egypt it was recognized that poor nutrition caused night blindness, and as a result vitamin A was the first vitamin identified. Today retinoids are useful in treatment of punctate keratopathy, corneal xerosis, refractory cystic and conglobate acne, xerophthalmia, psoriasis, and keratomalacia (Ubels and Edelhauser, 1982; Ubels et al., 1983). Large scale administration of massive doses (200,000-300,000 I.U.) of vitamin A has been carried out in prophylaxis of nutritional blindness secondary to corneal xerophthalmia and keratomalacia (Vijayaraghaven et al., 1984). The history, industrial synthesis, metabolism, physiologic roles, transport mechanisms, characteristics, functions of extra-and intra-cellular binding proteins, and the possible mechanisms of action of retinol, the retinyl esters, retinoic acid, and their analogs have been the subjects of numerous reviews (Lotan, 1980; Wolf, 1984; Sporn et al., 1984; Goodman, 1984). The present work is a summary of the acute and chronic toxicity of vitamin A and its synthetic congeners, collectively termed the retinoids.

The retinoids comprise a large group of synthetic and naturally occurring compounds, similar in structure to retinol (vitamin A). Retinol is an essential nutrient for visual dark adaptation, bone growth, reproduction, embryonic development, and differentiation of epithelial tissues. The importance of vitamin A in the development of cancer was recognized as early as 1926, when Fujimaki (1926) reported that rats fed a diet deficient in vitamin A developed stomach carcinomas. However, it was not until 1963 that Lasnitzki showed

that vitamin A could prevent or revert the transformation of prostate secretory cylindrical epithelium into dysplastic, hyperplastic, and metaplastic tissues produced by carcinogenic hydrocarbons.

These findings led others to investigate the role of vitamin A in dermatology and oncology (Bollag, 1970; Mayer et al., 1978; Sporn and Newton, 1979). Due to the highly toxic side effects of naturally occurring forms of vitamin A (hypervitaminosis A) at therapeutic dose levels, their clinical use has been limited. In an effort to divorce the toxic side effects from clinical effectiveness, over 1,500 retinoids have been synthesized (Lucek and Colburn, 1985). Some of these are in clinical use for treatment of recalcitrant cystic acne, Darier's disease, pustular psoriasis, lichen ruber planus, basal cell carcinomas, keratoacanthomas, and melanomas. Certain retinoids exert a therapeutic influence on chemically induced papillomas and carcinomas of the skin of mice (Bollag, 1975) and prevent carcinogenesis in the urinary bladder (Sporn et al., 1977) and mammary gland (Moon and McCormick, 1982; Hartman and Bollag, 1985) of rats. The use of retinoids in anticancer and dermatologic therapy has been reviewed (Bollag, 1979; Cunningham and Ehmann, 1983; Hill and Grubbs, 1982; Lauharanta, 1980; 1982; Meyskens, 1983; Orfanos, 1980; Peck, 1981; Peck, 1982; Sporn and Roberts, 1983).

HUMAN TOXICITY

Vitamin A Acute, Subchronic and Chronic

The toxicity of naturally occurring retinoids was reviewed as early as 1953 by Knudson and Rothman. Other than ingestion of bear, halibut, cod liver or their oils, vitamin A toxicity (hypervitaminosis A) due to foodstuffs is rare. However, self medication or consumption of vitamin A supplements can

lead to dizziness, anorexia, headache, weight loss, increased cerebrospinal fluid (CSF) pressure, and skin peeling. The required daily amount is 5,000 I.U. and signs of toxicity occur at 25,000-100,000 I.U./d. Excessive intake also causes gastrointestinal distress, fatigue, insomnia, menstrual disorders, and bone and joint pain. Bone resorption, periosteal calcification with osteocytic osteolysis, and hypermineralization have occurred (Eaton, 1978; Jowsey and Riggs, 1968; Gerber et al., 1954).

The toxicity is dose-related, and chronic toxicity often has more profound effects than a single bolus dose. The toxicity in children is generally more severe than in adults given an equivalent dose. Acute ingestion of 300,000-350,000 I.U. of vitamin A by children induced acute hydrocephalus with obvious bulging of the fontanel within 12 h (Ehrengut, 1955; Marie and See, 1954). Additional signs of intoxication include vomiting, restlessness, diarrhea, and insomnia. Cerebrospinal fluid hypertension may underlie acute hydrocephalus, as removing a small amount of CSF decreased the intensity of the hydrocephalus (Marie and See, 1954). Some cases of idiopathic benign intracranial hypertension (pseudotumor cerebri) have been attributed to retinoid intoxication, especially in children (Farris and Erdman, 1982). Acute intraventricular hemorrhage could be a consequence of retinoid-induced hydrocephalus (Ehrengut, 1955).

In adults, toxicity is most often manifest in dermatologic changes, alopecia, migratory bone and joint pain, headache, and such psychic changes as nervousness and irritability. Symptoms abate as ingestion of the retinoid is reduced or discontinued. Krause (1965) found elevated circulating triglyceride levels, but no signs of overt retinoid poisoning in an elderly male who ingested 50,000 I.U. vitamin A/d for 17 years. At autopsy, the liver contained 5.4 g of retinol. Chronic hypervitaminosis A in humans also causes elevated plasma

alkaline phosphatase activity (Smith and Goodman, 1976), a pathologic condition indicative of bone toxicity. Classic signs of vitamin A intoxication appeared in a 20-year-old woman who ingested 50,000 I.U. vitamin A/d for 2 years. Hepatomegaly, parenchymal disease, portal hypertension, elevated CSF pressure, headache, enhanced alkaline phosphatase and glutamic oxalacetic transaminase, and increased prothrombin time were associated with a circulating retinol concentration of 12.8 μg/ml (normal = 5 ± 1.5 μg/ml). Mucocutaneous side effects included a diffuse scaling erythematous dermatitis and soft palate petechiae (Anonymous, 1982).

Hepatic degeneration and alterations in fat metabolism occur in retinoid intoxication. Hepatic fibrosis and fatty liver accompanied elevations in total serum lipids in women ingesting 100,000-300,000 I.U. vitamin A/d for up to 9 years (Muenter et al., 1971). After 8 days, serum triglyceride concentrations doubled, and these elevations persisted for 9 weeks after cessation of oral 1.0 x 10^6 I.U. vitamin A/d (Dicken, 1981). Retinoids do not appear to elicit systemic toxicity following topical application, but the limiting factor is retinoid-induced skin irritation (Grice et al., 1973; Ashton et al., 1971).

Synthetic Retinoids Acute, Subchronic, and Chronic

The synthetic retinoids share similar side effects with those induced by hypervitaminosis A, and toxicity depends upon the structure of the retinoid and its dose. Acute toxicity is relatively rare; in one instance, a young man (82 kg) ingested 2.04 kg of 13-cis-retinoic acid (13-cis-RA, isotretinoin, Accutane®) over a 2-day period (Sutton, 1983). The only signs of retinoid intoxication reported were dry lips and scattered areas of dry skin. Routine liver function tests, hematology, and urinalysis failed to reveal abnormalities (Sutton, 1983). The most common side effects observed after prolonged

treatment with oral 13-cis-RA were mucocutaneous dryness (chelitis), cutaneous desquamation, alopecia, epistaxis, and conjunctivitis (Pawson et al., 1982). Gastrointestinal distress was reported by 20% of the patients; 15% reported musculoskeletal signs; 10% felt lethargic; and 10% experienced headache. Chronic 13-cis-RA treatment caused elevation of serum triglycerides (very low density lipoprotein; VLDL) (Katz et al., 1980). Elevated serum triglycerides and enhanced sedimentation were found in 25 and 50% of the patients, respectively (Pawson et al., 1982). Ten percent of the patients treated with etretinate developed hypertriglyceridemia (Elias and Williams, 1981).

Although no consistent changes in skin triglyceride concentrations were found in patients treated with oral 13-cis-RA, surface cholesterol increased, and wax esters and squalene decreased, perhaps due to decreased sebum production (Strauss et al., 1980). In a study of 53 patients receiving 1 mg/kg/d 13-cis-RA for treatment of acne, mean triglyceride levels rose from 80 mg/dl to 103 mg/dl in women, and from 82 mg/dl to 129 mg/dl in men after four weeks of treatment. Triglyceride levels remained elevated through the 20th week of therapy. Plasma cholesterol levels also increased in women and men by four weeks and remained elevated through the 20th week of therapy. High density lipoprotein (HDL) cholesterol levels initially decreased in women and men by the fourth week and remained suppressed through the 20th week. Low density lipoprotein (LDL) cholesterol levels rose in women and men by the fourth week. In men, LDL decreased to baseline levels by the eighth week, but the women's LDL cholesterol levels remained elevated. All parameters returned to baseline levels within eight weeks post-treatment. The authors cautioned that treatment which resulted in prolonged changes in plasma lipoproteins could increase the risk of cardiac disease (Bershad et al., 1985).

Pittsley and Yoder (1983) described four cases of skeletal toxicity associated with long-term administration of 13-cis-RA to control ichthyosis. Two of the cases received 3 mg/kg/d, one case 4 mg/kg/d; the other dose was not reported. All four, who had been treated for at least two years, suffered from an ossification disorder similar to idiopathic skeletal hyperostosis. Arthraglias were relieved with discontinuation of the treatment, but ossification did not regress.

Two men with metastatic melanoma were treated with 0.002 mg/kg/d for eight weeks of the aromatic retinoid (arotinoid) p-[(E)-2(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)propenyl]-benzoic acid ethyl ester (Ro 13-6298). The effects of Ro 13-6298 on normal skin after eight weeks of treatment consisted of minor epidermal alterations (Tsambaos and Orfanos, 1982). Tsambaos and Orfanos (1983) described two cases (a woman and a man) of severe psoriasis and psoriatic arthritis. Each case failed to respond to conventional therapy, including etretinate, but responded favorably to 0.002 mg/kg/d of Ro 13-6298. Side effects were limited to dry lips and nasal mucosa, palmarplantar desquamation in both cases, and skin thinning, transient hair loss, and itching in one case. In one case the erythrocyte sedimentation rate (ESR) was slightly elevated. Biochemical and hematological parameters were not altered by arotinoid treatment, serum cholesterol levels decreased, and serum triglycerides remained constant in one case. Subsequent to arotinoid treatment in the other case, pathologically high levels of alkaline phosphatase and γ-glutamyltransferase levels returned to normal. Serum triglycerides and cholesterol levels were unchanged and slightly increased, respectively (Tsamboas and Orfanos, 1983).

Dysmorphogenicity

Most aspects of retinoid toxicity can be controlled by adjusting the dose and/or regimen of ingestion. The teratogenicity of retinoids is the most serious aspect of their toxicity because of the permanency of damage to the human embryo. The susceptible period for teratologic damage occurs prior to the time a woman suspects the pregnancy.

Isotretinoin (13-cis-RA) administered orally during the first trimester of pregnancy has resulted in 34 cases of fetal teratology and 30 cases of spontaneous abortion in humans (Willhite et al., 1986). Lammer et al. (1985) studied 154 cases of embryonic and/or fetal exposure to isotretinoin; exposure was associated with a relative risk of 25.6% (95% confidence interval 11.4 to 57.5). Outcomes of the 154 cases were: 95 elective abortions, 12 spontaneous abortions, 21 malformed infants, and 26 infants without major malformations. The dosage and critical period of exposure during embryogenesis resulting in human terata have not been precisely determined. Willhite et al. (1986) compared the susceptible period of human embryogenesis of a malformed infant whose mother received 1.37 mg/kg isotretinoin per day through the fifth week of gestation (day 42) with the gestational age of isotretinoin-treated hamster embryos. A critical stage of development in the hamster is on day 8, when the hamster embryo has 10-14 somites. The equivalent period of human embryogenesis occurs within stages 8-12, days 18-26. The infant discussed above was treated within the critical period to stage 15 and exhibited a variety of dysmorphia. Figures II.1, II.2, and II.3 are photographs of the infant described above (see figure legends for explanations). The dose, period of exposure, and dysmorphogenicity of observed human terata for infants exposed to isotretinoin are summarized in Table II.1.

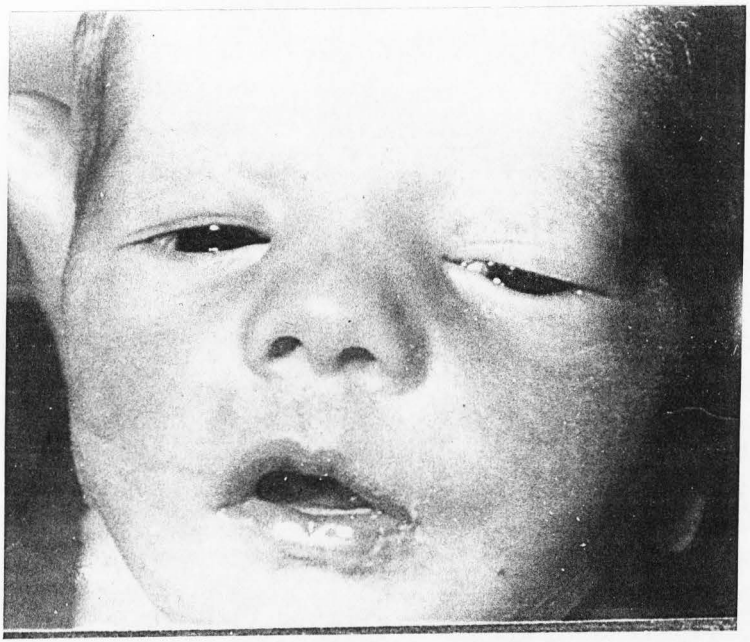


Figure II.1. Male infant whose mother was exposed to 80 mg isotretinoin/d (1.37 mg/kg/d) through day 42 of gestation. Note the depressed nasal bridge, micrognathia, and orbital hypertelorism. The infant also had a cleft of the soft and hard palates. (From Willhite et al., 1986, used with permission.)

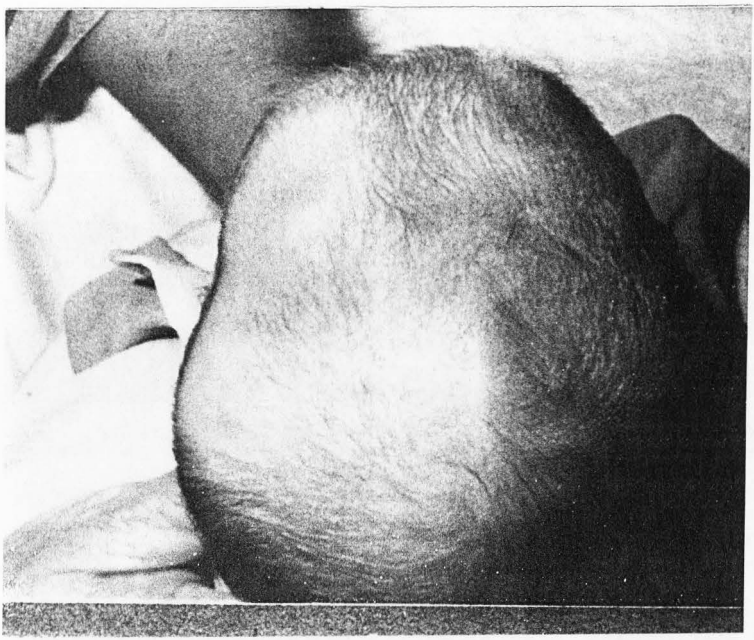


Figure II.2. Superior view of infant in figure II.1. Note the triangular-shaped cranium and the narrow frontal bone. (From Willhite et al., 1986, used with permission.)

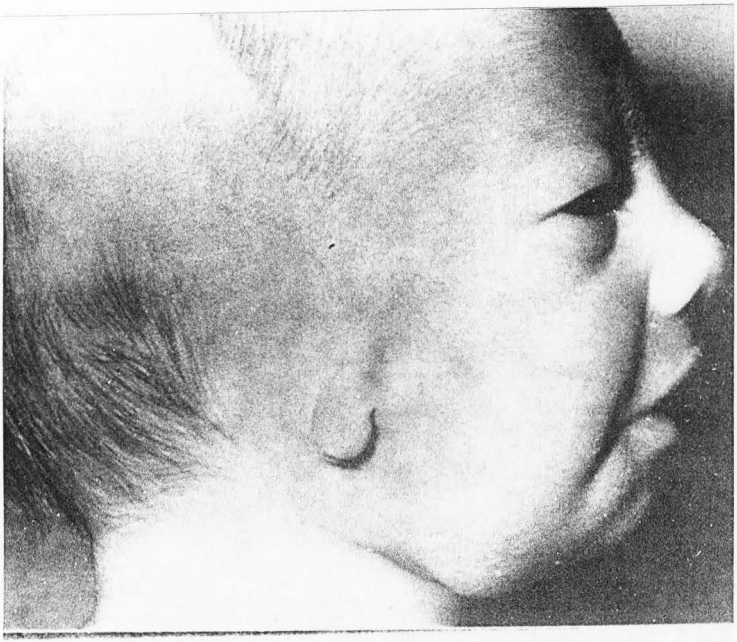


Figure II.3. Side view of infant in figure II.1. Note the micrognathia, absent external auditory meatus, and the low-set rudimentary pinna. (From Willhite et al., 1986, used with permission.)

TABLE II.1

DYSMORPHIA OF HUMAN INFANTS EXPOSED TO ISOTRETINOIN

Exposure	Dysmorphia	Reference
Isotretinoin 40 mg/d 14-65 d	Face: depressed nasal bridge Ears: low set right pinnae, skin tags, atretic canal Skull: low hairline on forehead, bilateral asymmetrical parietal sutures CNS: leptomeningeal cyst, cerebellar vermis absent, cerebellum small, heterotopic neural tissue, hippocampal gyri small, depleted malformed spinal tract, inferior olive malformed, ventricles dilated Cardiovascular system: complete transposition of the great vessels, ventricular septal defect, right subclavian artery distal to the left and right atrium and ventricle distended, superior vena cava congested, Other: skin thick at neck.	Braun et al., 1984
Isotretinoin 80 mg/d 0-42 d	Face: depressed nasal bridge, hypertelorism, micrognathia, left facial paralysis Eyes: did not follow, visual evoked potential abnormal, flat orbital ridges Ears: bilateral ear tags at angle of mandibile, absent external canals, latency in auditory brain stem response Palate: cleft palate, only palatal ridge present Skull: triangular skull, narrow sloping forehead, prominent occiput Muscletone: decreased tone and mass in lower extremities Genitourinary: large scrotal sac Cardiovascular system: ventricular septal defect, aortic stenosis, pulmonary stenosis, dysplastic pulmonary and aortic valves Other: simean crease on left hand, respiratory distress, feeding problems	Hill, 1984

TABLE II.1 (CONTINUED)

Exposure	Dysmorphia	Reference
Isotretinoin 20 mg/d 0-49 d	Face: depressed nasal bridge, micrognathia, anteverted nares Eyes: small palpebral fissures deep orbits, did not follow, microphthalmia Ears: low set, small undifferentiated ear, absent helix, small ear canal Skull: prominent forehead, large anterior occiput Muscletone: hypotonic CNS: hydrocephalus, V-P shunt Cardiovascular system: teratology of Fallot, right vetricular prominence Other: feeding difficulties, meningitis	Benke, 1984
Isotretinoin 50 mg/d 0-49 d	Face: anteverted nares, prominent nose, thin lips, micrognathia, depressed nasal bridge Eyes: small palpebral fissures, deep orbits, hypertelorism, microphthalmia Ears: low set, small undifferentiated, absent helix Palate: hard and soft cleft palate Skull: brachycephaly, low posterior hair line Muscle tone: floppy CNS: Dandy-Walker of posterior fossa, V-P shunt, seizures Other: assisted ventilation, respiratory distress, apnea, repeated infections	Benke, 1984
Isotretinoin 80 mg/d 0-49 d	Face: micrognathia Eyes: antimongoloid slant to fissures, hypertelorism Ears: right ear atretic, small pennae, hypoplastic left external meatus Palate: U-shaped cleft palate Skull: distorted occiput, anterior lateral displacement of pareital whorl Muscle tone: flaccid Pulmonary: incomplete lobulation of left lower and right middle lobes	Fernhoff and Lammer, 1984

TABLE II.1 (CONTINUED)

Exposure	Dysmorphia	Reference
Isotretinoin 80 mg/d 0-49 d	CNS: ventricles mildly dilated, hypoplastic cerebellum with absent vermis, abnormal migration of neural cells in cerebellum Cardiovascular system: large ventricular septal defect, double outlet right ventricle, interrupted aortic arch, truncus arteriosis, patent ductus arteriosis, left and right subclavian arose from descending aorta Other: deep plantar creases 1st, 2nd, and 2nd, 3rd toes.	
Isotretinoin Dose unknown ca. 28-42 d	Eyes: microphthalmia Ears: bilateral rudimentary pinnae, absent 8th nerve function Skull: microcephaly Genitourinary: attenuated nephrogenic zone CNS: seizures, bilateral grade IV intraventricular hemorrhage (IVH) hydrocephalus secondary to IVH, lissencephaly Cardiovascular System: atrial septal defect, ventricular septal defect, aortic stenosis, patent ductus arteriosis, hypoplasia of ascending aorta, interrupted aortic arch Other: hyperbilirubinemia, enlarged liver with cholestasis, webbed neck	De La Cruz et al., 1984
Isotretinoin 40 mg/d 0-105 d	Face: depressed nasal bridge, broad nasal tip, thick alae nasi, downward turned mouth Eyes: hypertelorism, asymmetrical palpebral fissures, left-sided epicanthus, no occular pursuit, visual evoked potential abnormal Ears: low dysplatic, atretic external canals, abnormal helices Skull: prominent occiput, hair whorl on right vertex, high forehead, asymmetrical Muscle tone: hypotonic	Lott et al., 1984

TABLE II.1 (CONTINUED)

Exposure	Dysmorphia	Reference
	CNS: aqueductal stenosis, hydrocephalus V-P shunt, dilated cysterna vena magna and occipital horns, psycomotor retardation. Other: dorsiflexed toes, dimpled elbows	
Isotretinoin 40 mg/d 26-46 d	Other: rudimentary proximal phalanges left hand, no fingers right hand, tibia and fibula tapered at ends of right leg, absent 2nd and 3rd phalanges of 2nd toe of right foot	McBride, 1984
Isotretinoin 80 mg/d 0-84 d	Face: limited facial movement Eyes: microphthalmia, Doll's eye, absent vertical movement Ears: rudimentary pinna, atretic right external auditory canal Palate: large cleft palate Muscle tone: floppy Pulmonary: incomplete lobulation lower left and right middle lobes CNS: absent cerebellar vermis, moderate hydrocephalus Cardiovascular system: ventricular septal defect, interrupted aortic arch, patent ductus arteriosis, truncus arteriosis, double outlet ventricle Other: webbed neck	Hansen and Pearl, 1984
Isotretinoin 1-20 capsules/d 0-84 d	Ears: small and low-set CNS: hydrocephalus with posterior fossa cyst	Rosa, 1984
Isotretinoin 80 mg/d Exposure unknown	Ears: micropinnae, possible occluded external auditory canals CNS: hydrocephalus	Rosa, 1984
Isotretinoin Dose unknown Exposure unknown	Ears: absent external ears, atretic external auditory canals Skull: microcephalus	Rosa, 1984

The pattern of malformations observed in infants included dysmorphia of the face consisting of depressed nasal bridge, micrognathia, small palpebral fissures with deep orbits, and orbital hypertelorism. Anteverted nares, prominent nose, thin lips, broad nasal tip, thick alae nasi, and downward turned mouth have also been reported. Left facial paralysis and limited facial movement were observed in some infants. In several cases the eyes were microphthalmic. The ears were characteristically displaced ventrally and represented by small hypoplastic pinnae or skin tags with an absent helix. The external auditory canal was usually atretic or stenotic. Absent 8th nerve function was observed in one infant. The forehead was often sloping with a narrowing of the biparietal diameter and a prominent occiput resulting in a triangular head. Hair whorls were misplaced and skull sutures may have been asymmetrical. The hairline was low, and the neck contained lateral skin folds. A cleft of the hard and soft palate was usually observed. The auricular and facial dysmorphia share a number of anatomical features with the Treacher-Collins syndrome (mandibulofacial dysostosis) (Poswillo, 1975).

Reported malformations of the central nervous system (CNS) are hydrancephaly, hydrocephaly, microcephaly, and lissencephaly. Various types of obstruction to spinal fluid flow have been reported to cause hydrocephaly (e.g., aquaductal stenosis, Arnold-Chiari malformation, leptomeningeal cyst, and Dandy-Walker syndrome). One infant suffered from hydrocephaly and intracranial hemorrhage (De La Cruz et al., 1984). The most consistent malformation of the CNS was the absence of the cerebellar vermis with abnormal migration of neural cells. Seizures were observed in two cases, but the authors considered seizures secondary to other complications (Benke, 1984; De La Cruz et al., 1984).

Cardiovascular system malformations have generally consisted of overriding aorta, truncus arteriosis, double outlet ventricle, ventricular septal defect, patent ductus arteriosis, dysplastic pulmonary aorta, and the tetralogy of Fallot. Infants with conotruncal heart or aortic arch defects suffered from ectopic, hypoplastic, or aplastic thymic abnormalities (Willhite et al., 1986). The infants were usually flaccid or hypotonic and did not have normal reflexes. They were prone to infection with frequent infections of pneumonia, sepsis, and meningitis, and problems with feeding or nursing occurred often. Micrognathia and cleft palate were accompanied by respiratory obstruction necessitating ventilatory support. In two cases the lungs had incomplete lobulation of the lower left and right middle lobe (Table II.1).

Occasional anomalies of the appendicular skeleton have been observed. Anomalies included rudimentary proximal phalanges with oligodactyly, absent fingers and toes, tapering of the tibia and fibula, unusual plantar creases between the toes, and hypoplastic fingers and nails. Not all pregnant women exposed to isotretinoin have resulted in negative outcomes, however (Kassis et al., 1985).

Etretinate (Tigason®), the trimethylmethoxyphenyl (TMMP) ethyl ester analog of all-trans-retinoic acid (tretinoin, all-trans-RA), is also a human teratogen. Happle et al. (1984) reported 19 cases of embryonic and/or fetal exposure to etretinate. Ten of the infants were normal; 3 abortuses were normal; 3 infants were abnormal; and 1 fetus from a miscarriage and two abortuses were malformed. Craniofacial defects included high arching palate, low-set ears, synostosis of cranial sutures, and other malformations of the calvaria. In one case abnormal cervical vertebrae were observed. Defects of the appendicular skeleton consisted of missing fingers, syndactyly, shortened thumbs, malformed hip and ankle joints, and defective forearm bones.

Meningoencephalocele, meningomyelocele, and anophthalmia were observed in the spontaneous and elective abortuses. The exact doses and duration of exposure associated with specific dysmorphia were not presented. The doses for the affected infants or fetuses ranged from 11.0-55.0 mg/d for days 0-45. Based on a 55 kg female the teratogenic dose can be estimated to be 0.2 mg/kg/d. In general, mothers who bore normal children received lower doses, and two of the exposures occurred after the period of organogenesis. The human teratogenic dose of 0.2 mg/kg/d for etretinate compared to a human teratogenic dose of 1.37 mg/kg/d for 13-cis-RA (Willhite et al., 1986) demonstrates that etretinate is approximately 7x as teratogenic as 13-cis-RA. It is interesting to note that etretinate is at least 4x as teratogenic as 13-cis-RA in the hamster (Willhite and Shealy, 1984; Williams et al., 1984).

Isolated reports of vitamin A-induced human terata have not shown conclusively that vitamin A is a human teratogen. Berhardt and Dorsey (1974) reported an infant born to a mother who had taken 25,000 I.U. vitamin A (from fish liver oils) from conception through the third month of gestation and continued with 50,000 I.U. daily until delivery. The child had a urinary obstruction due to aberrant insertion of the ureters and a ureterocele. A double collecting system of the left kidney, hydronephrosis, and hydroureters were also noted. Another infant whose mother ingested 40,000 I.U. of vitamin A daily between 6 and 10 weeks of gestation was reported by Pilotti and Scorta (1965). The infant suffered from hydronephrosis and absent ureteral orifices with apical vesicle diverticulum. Strange et al. (1978) described a child with hypopolastic kidneys, small adrenals, and dilation of the cerebral ventricle due to stricture of the aqueduct of Sylvius. At autopsy the infant was found to have blood in its skull cavity.

ANIMAL TOXICITY

Acute, Subchronic, and Chronic

The toxicity of retinoids depends upon their route of administration, chemical structure, and dose. Single oral doses of retinoids in common laboratory animals are generally of low toxicity, with the exception of certain conformationally restricted retinoids (arotinoids). There are no marked species differences. The acute oral median lethal dose (LD_{50}) in mice ranged from 6,000 mg/kg for retinyl palmitate, 4,000 mg/kg for retinyl acetate, 3,400 mg/kg for 13-cis-RA to 2,200 mg/kg for all-trans-RA. The oral LD_{50} in mice for the aromatic retinoid, etretinate, was greater than 2,000 mg/kg (Kamm, 1982). Hixson and Denine (1978) reported the LD_{50} for all-trans-RA at 31 mg/kg i.p. and 1,100 mg/kg p.o. after 21 consecutive days of administration to mice. In contrast, the oral LD_{50} for 13-cis-RA was 26,000 mg/kg and the i.p. LD_{50} was 140 mg/kg. The oral LD_{50} of all-trans-RA was approximately 23 times that of 13-cis-RA, and the lethal oral dose of either all-trans-RA or the 13-cis congener was much less after i.p. injection than after oral intubation (36x and 190x, respectively).

Common signs of chronic retinoid toxicity in rats and mice included cartilage and bone resorption, bone fracture, subcutaneous and internal hemorrhage, elevations in CSF pressure, fatty infiltration into hepatic parenchyma, and epithelial drying and scaling (Singh et al., 1968; Mallia et al., 1975). The subchronic and chronic effects of retinoid administration have been studied in rats, mice, and dogs. Similar to the toxicity observed in humans, reductions in body weight, hair thinning, dermatitis, mucocutaneous lesions, elevated alkaline phosphatase, albumin, hemoglobin, erythrocyte count, triglyceride levels, and transaminase activity in animals were common. Hepatic

pathology and testicular atrophy with interruption of spermatogenesis also occurred. Bone fractures, corneal opacity, epiphora, cardiovascular lesions, and erythrophagocytosis in lymph nodes were observed after ingestion of relatively high levels for prolonged periods (Kamm, 1982; Teelmann, 1983). For example, oral administration of 150-250 mg/kg of all-trans-RA induced alopecia, weight loss, and mucocutaneous disorders (Kretzschmar and Leuschner, 1975). Oral or i.p. treatment with all-trans- or 13-cis-RA for 21 days was associated with epidermal and dermal inflammation and hyperkeratosis in male and female mice (Hixson and Denine, 1978). Testicular necrosis and suppression of spermatogenesis occurred in males.

Retinoid-induced hematologic changes included a dose-dependent peripheral anemia, erythrocytopenia, and decreased hemoglobin and packed cell volumes. Orally administered retinoids decreased serum albumin and increased plasma alkaline phosphatase (Sani and Meeks, 1983). The synthetic retinoids (all-trans-N-ethylretinamide, all-trans-2-hydroxyethylretinamide, and all-trans-4-hydroxyphenylretinamide) were, in general, less toxic than all-trans-RA, but the 13-cis analogs were less toxic than any of the all-trans forms. At high doses the retinamides also induced subacute, multifocal hepatic inflammation, mild hepatocellular vacuolization and hepatic necrosis, hypertrophy, and cytomegaly. Testicular toxicity included coagulation necrosis and arrested development of spermatogonia, spermatocytes, and spermatids. Sertoli cells were the last testicular cell population to be affected; thymic and cardiac lesions were reported also (Sani and Meeks, 1983).

Liver and Lipid Disorders

The naturally occurring retinoids are transported from the gut in chylomicrons via the portal system and concentrate in hepatocytes (Lotan,

1980; Goodman, 1984; Pawson et al., 1982). Synthetic retinoids tend to concentrate in the liver, where a number of pathological and biochemical alterations can occur. Treatment of male rats with 100 mg/kg 13-cis-RA in the diet for 11 weeks reduced hepatic microsomal phosphatidylcholine and increased phosphatidylethanolamine concentrations (Alam et al., 1984). The activities of the 9-desaturase and 6-desaturase enzymes in rat liver after retinol treatment were decreased and increased, respectively (Fell and Steele, 1982). The enzymatic changes were consistent with decreased hepatic concentrations of S-adenosylmethionine, a cofactor responsible for phosphatidylcholine synthesis from phosphatidylethanolamine (Fell and Steele, 1982). Radcliffe (1983) reported that feeding all-trans-N-4-hydroxyphenylretinamide at 782 mg/kg to female rats increased the total liver triglyceride and cholesterol concentrations.

Circulating triglyceride and cholesterol concentrations were increased after chronic retinoid treatment as well. Administration of all-trans- or 13-cis-RA to rats induced hypertriglyceridemia (Gerber and Erdman, 1981), in addition to changes in the composition of liver fatty acids (Gerber and Erdman, 1980). When 13-cis-RA was fed to rats at 300 mg/kg of diet for 4 weeks, hepatic phosphatidylcholine concentrations decreased, and phosphatidylethenolamine concentrations increased. The changes in hepatic lipid composition and reduction in the double bond index for total phospholipids suggests decreased conversion of linoleic acid to arachidonic acid, possibly mediated though inhibition of microsomal desaturases and chain-elongating enzymes (Alam and Alam, 1983). Gerber and Erdman (1982) suggested that at least two physiologic mechanisms can account for changes in hepatic lipid composition and accumulation. In hypervitaminosis A, adrenal cortex hypertrophy and enhanced glucocorticoid synthesis can induce hepatic

triglyceride synthesis and decreased fatty acid secretion. The combination of excessive synthesis and decreased secretion leads to fatty livers. Additionally, decreased peripheral tissue activity of lipoprotein lipase may contribute to the elevations in circulating triglycerides and reductions in cholesterol.

At physiologic concentrations, retinoids had direct effects on hepatocytes (Gerber and Erdman, 1982). Hepatic triglyceride synthesis increased, and cholesterol synthesis decreased. Alterations in skin lipids were not considered the result of changes in circulating lipids, but they may have been a direct consequence of retinoid action on sebaceous glands. The effects of retinoids on hepatocytes, however, cannot be attributed to changes in drug-metabolizing enzymes. Intubation of 1-5 mg/kg/d of 13-cis-RA or 0.1-0.5 g/kg/d of the arotinoid ethyl ester (Ro 13-6298) for up to 28 days failed to induce the cytochrome P-450 system in rats (Goerz et al., 1984).

Bone and Connective Tissue

Acute, subchronic, and chronic toxicities of retinoids on bone and other connective tissues in laboratory animals are related to dose and length of administration. Intubation of all-trans-RA and other naturally occurring retinoids induced disturbances in calcium metabolism as evidenced by bone thinning, osteoporosis, and calcification of soft tissues (Leelaprute et al., 1973). For instance, oral administration in rats of up to 10 mg/kg/d of all-trans-RA for 17 weeks induced osteocytic osteolysis, subperiosteal and osteoclastic resorption, and dissolution of bone matrix (Dhem and Goret-Nicaise, 1984). Doses of 0.4-10 mg/kg p.o. in rats for 90 days (Cahn et al., 1975) or dietary administration of 300-1,500 I.U. all-trans-RA for 84 days to rats (Zbinden, 1975) increased the concentrations of serum alkaline phosphatase, a change indicative of increased osteoblast activity. Administration of all-trans- or

13-cis-RA for 21 days induced dose-dependent fractures of the long bones, dermal and epidermal abnormalities, cartilage degeneration, and elevations in plasma alkaline phosphatase (Hixson and Denine, 1978). However, changes in alkaline phosphatase were not always associated with bone fractures. Changes in connective tissues are often observed in concert with hepatic toxicity, such as dose-related decreases in plasma albumin (Hixson et al., 1979).

A number of synthetic retinoids have lower acute and chronic toxicities than all-trans-RA. Treatment with up to 330 mg/kg retinylidene dimedone (all-trans-2-retinylidene-5,5-dimethyl-1,3-cyclohex anedione) failed to induce bone fractures, growth depression, anemia, elevated serum alkaline phosphatase, or testicular degeneration induced by daily oral doses of all-trans-RA (Kurtz et al., 1984). Kistler and coworkers (1984) found that oral administration of the arotinoid ethylsulfone (Ro15-1570; ethyl p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-propenylphenyl sulfone]) to rats failed to cause fractures of the long bones that were induced by oral treatment with etretinate or all-trans-RA (primarily the distal femur or proximal tibia). Skin toxicity (rough coat, erythema, alopecia, and epiphora) and weight loss, induced by subchronic oral doses of the retinoids, were more severe in females than in males. The decreased bone toxicity of the arotinoid compared with etretinate or all-trans-RA was reflected in decreased bone-ash weights for the latter compounds compared with those from animals treated with the sulfur-containing retinoid (Kistler et al., 1984).

The influence of naturally occurring retinoids on the skin has been reviewed (Christophers and Wolff, 1975b; Jarrett, 1975). Topical application of 0.15 mg/d of all-trans-RA to nude mice for one month caused epidermal hyperplasia and acanthosis in 70% of the animals and hyperkeratosis in 25%. Skin toxicity was accompanied by increased alkaline phosphatase activity of

sebaceous glands as well as increased nucleic acid content and changes in the distribution of skin lipids (Cahn et al., 1975). Doses of 0.006 mg/kg/d of the arotinoid Ro 13-6298 administered p.o. to guinea pigs for four and eight weeks resulted in slight epidermal changes similar to those observed in humans (Tsambaos and Orfanos, 1982). Three days after treatment of 0.1 mg/kg/d numerous small vacuoles were observed under an orthokeratotic horny layer. One week after 0.1 mg/kg/d the horny layer was lacking in nearly all animals, and massive and edematuous changes in the remaining epidermis were observed (Tsambaos and Orfanos, 1982). Application of all-trans-RA to rat or human skin increased oxygen consumption and inhibited the pentose phosphate shunt (Raab and Gmeiner, 1976). Addition of all-trans-RA to cultured epidermal cells enhanced their growth and stimulated DNA synthesis (Christophers and Wolff, 1975a). Subcutaneous injection of isotretinoin into male hamsters inhibited sebaceous glands, but similar treatment with etretinate failed to cause involution of these glands (Gomez, 1982). The relative effectiveness of isotretinoin in acne treatment as compared with etretinate may be due to isotretinoin inhibition of sebum production and diminution of the sebaceous gland.

Dysmorphogenicity

Hypervitaminosis A induced teratogenicity has been studied in numerous laboratory animal models: rat (Cohlan, 1953), mouse (Giroud and Martinet, 1959), guinea pig (Giroud and Martinet, 1959; Robens, 1970), hamster (Robens, 1970; Marin-Padilla and Ferm, 1965), rabbit (Giroud and Martinet, 1959), chicken (Cadi et al., 1983; Cadi et al., 1984), pigtail monkey (Fantel et al., 1976), and rhesus monkey (Wilson, 1971; Hendrickx et al., 1980). The teratogenic syndrome induced by administration of retinoid analogs is

essentially that of naturally occurring forms of vitamin A administered during the same period of embryogenesis. Geelen (1979) assembled a detailed review of hypervitaminosis A and teratogenesis induced by naturally occurring retinoids.

In the hamster, malformations induced by a single dose (p.o.) at 10:00 A.M. on day 8 of gestation (the day following the evening of breeding being day 1) are dose-related; as the dose of the active retinoid increased, the severity of malformations and percentage of malformed fetuses increased. Intubation of 50 mg/kg all-trans-retinylidene methyl nitrone (ED_{50} = 39.1 mg/kg) (Willhite and Balogh-Nair, 1984), 25 mg/kg 13-cis-RA (ED_{50} = 22.3 mg/kg) (Willhite and Shealy, 1984), 2.8 mg/kg etretinate (ED_{50} = 5.7 mg/kg) (Williams et al., 1984), 75 mg/kg all-trans-9-(exo-2-bicyclo[2.2.1]heptyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate, 50 mg/kg all-trans-retinoyl fluoride (ED_{50} = 53.8 mg/kg), 81 mg/kg all-trans-5-[2,6-dimethyl-8-(2,6,6-trimethyl-cyclohexen-1-yl)-1,3,5,7-octatetraen-1-yl] tetrazole, 84 mg/kg ethyl all-trans-4-[2-methyl-4-(2,6,6-trimethyl-1-cyclo-hexen-1-yl)-1,3-butadien-1-yl] benzoate (Willhite et al., 1984), 38 mg/kg 7,8-dihydroretinoic acid, 37 mg/kg 7,8-dehydroretinoic acid, 25 mg/kg 9-cis-retinal (ED_{50}=23.5 mg/kg) (Willhite, 1986) increased the number of affected litters (one or more malformed fetuses) compared to vehicle only treatment. The malformations included: unilateral or bilateral palpebral aplasia with subsequent exophthalmia, microphthalmia, and anophthalmia. Micrognathia with primary microglossia, protruding tongue, unilateral or bilateral macrostomia, cleft palate, and agnathia were also observed. An apparent maxillary retrocession was often observed and thought to be secondary to lordosis and reduction of the basichondrocranium. At lower doses palpebral aplasia and macrostomia were the most common malformations. As doses were increased, more severe malformations such as micrognathia, cleft

mandible, and agnathia were observed (Willhite et al., 1984; Willhite, 1986). Mid-sagittal sections of grossly normal and abnormal day-14 fetuses are shown in figures II.4 and II.5, respectively (see figure legend for explanation). The facial skeletal dysmorphia was believed to be the result of delayed and disorganized patterns of cranial neural crest cell migration with death and damage of the crest cells (Wiley et al., 1983).

Malformations of the ears included ventrally displaced imperforate external auditory meatus and hypoplastic auris externa. Microcephalus characterized by anterioposterior reduction and elongation in the superinferior plane with reduction of frontal, parietal, temporal, and occipital bones occurred. Arnold-Chiari malformation types I and II, which are characterized by underdevelopment of the basioccipital, resulted in lordosis of the basichondrocranium, reduction of the pontine flexure, and increased angle of cervical flexure of the hind brain. The cerebellum was displaced caudally with compression of the medulla, and the odontoid process protruded into the cranial cavity (Marin-Padilla and Marin-Padilla, 1981). Cranioschisis occulta with meningoencephalocele or meningocele and cranioschisis aperta with encephaloschisis were also observed (Willhite, 1984).

A caudal regression syndrome characterized by anal atresia, hypoplastic or aplastic tail, sacral rachischisis aperta with myeloschisis or sacral rachischisis occulta with myelo- or meningomyelocele occurred. Infrequently, umbilical hernia or gastroschisis with omphalocele were reported (Willhite et al., 1984; Willhite, 1986). Genitourinary malformations consisted of renal agenesis, bladder exstrophy, and horseshoe kidney.

Intubation of 40-80 mg/kg all-trans-RA induced cardiac malformations, including ventricular septal defect, transposition of the great vessels, double outlet right ventricle, overriding aorta with pulmonary stenosis, hypoplastic

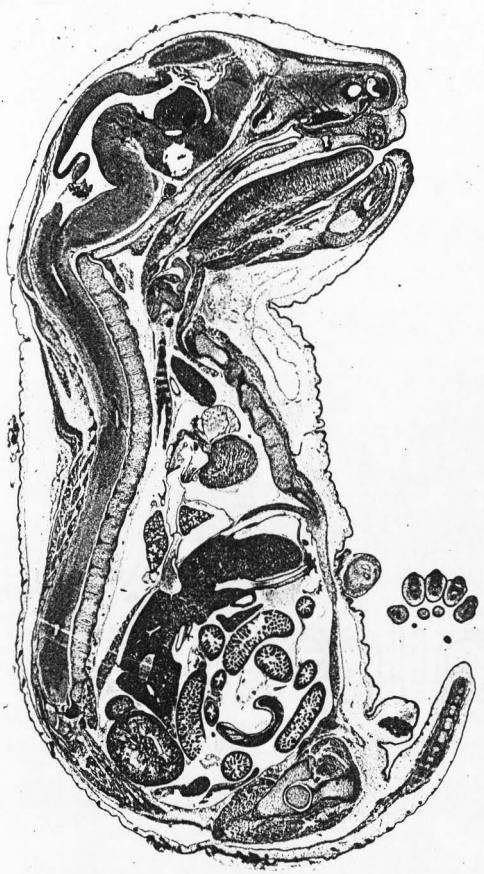


Figure II.4. Mid-sagittal section of grossly normal day-14 hamster fetus recovered from a dam administered Tween 20 on day 8 of gestation. Note the size of brain and calvaria. The basichondrocranium forms a 90^{o} angle with the odontoid process of the second cervical vertibra. (From Willhite and Shealy, 1984, used with permission.)

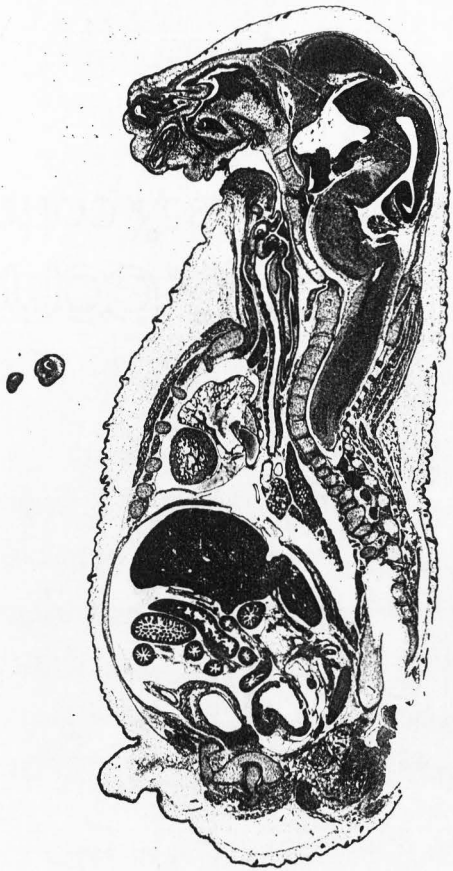


Figure II.5. Mid-sagittal section of a grossly malformed day-14 hamster fetus recovered from a dam treated with 75 mg/kg body weight retinoic acid on day 8 of gestation. Note the agnathia, microcephaly, and microglossia. The basichondrocranium is shorter than that of the control fetus (Figure II.4) and is lordotic to the vertebral axis. It forms nearly a 180^{0} angle with the odontoid process. (From Willhite and Shealy, 1984, used with permission.)

aorta, right-sided aortic arch, atrial septal defect, and other circulatory anomalies (Shenefelt, 1972; Taylor et al., 1980).

Willhite and Balogh-Nair (1985) induced terata in Swiss-Webster mice with 75 mg/kg all-trans-retinylidene methyl nitrone or all-trans-retinol given at 10:00 A.M. on days 7 through 11 (vaginal plug = day 1 for the following mouse studies). Retinol increased the number of malformed litters on days 7, 8, 9, and 11; the nitrone induced significant increases on days 8 and 9. All-trans-retinol was always associated with a higher percentage of malformed fetuses than was the synthetic retinoid. Kochhar et al. (1984b) investigated palate and limb malformations in ICR mice with all-trans-RA and 13-cis-RA. Treatment with 100 mg/kg 13-cis-RA failed to induce terata and 200 mg/kg induced minor limb and some palatal malformations. Four hundred mg/kg 13-cis-RA induced limb and palatal defects similar in severity to a dose of 100 mg/kg all-trans-RA.

Doses of 3 mg/kg etretin (the free acid form of etretinate) administered p.o. to Fu-albino mice on days 7-16 resulted in an elevation in the percentage of malformed fetuses. One mg/kg etretin failed to induce malformations (Kistler and Hummler, 1985). The arotinoid Ro 13-6298 induced malformations in NMRI mice at doses as low as 0.010 mg/kg/d i.p. on days 10-12 or 13-15. A single i.p. injection on days 9 through 15 of 0.20 mg/kg Ro 13-6298 also induced malformations similar to those induced by all-trans-RA (Zimmermann et al., 1985).

A single oral dose of 200 mg/kg 13-cis-RA administered to C57Bl/6J mice induced ear, thymic, great vessel, and facial abnormalities (Johnston et al., 1985; Jarvis et al., 1985). Histological changes were specific for neural crest cells. Kochhar and coworkers (1985) induced teratogenesis in ICR mice with all-trans-RA, 13-cis-RA, etretinate, and etretin and tested these retinoids for

their ability to suppress chondrogenesis in limb bud mesenchymal cell cultures. Etretinate was the most potent teratogen, but failed to suppress chondrogenesis in vitro. All-trans-RA was a more potent teratogen in vivo than 13-cis-RA, but both had equivalent in vitro activity.

In vitro studies with mouse limb buds have been employed. Forelimb buds of 10 to 12 day ICR/JLC mice embryos were exposed to 10 I.U./ml retinol for 24 h. Abnormal limb development was observed (Nakamura, 1977). Kwasigroch et al. (1984) treated pregnant ICR mice on day 11 with all-trans-RA and subsequently cultured the embryonic limb buds for 3 days. When compared to limbs of 17 day in utero fetuses, the limb culture effects were qualitatively similar with micromelia and digital defects. Mouse limb bud mesenchymal cells cultured with 1 μg/ml all-trans-RA had a 10% inhibition of growth and a 24% decrease in [^{3}H]glucosamine-labeled glycosaminoglycan synthesis. Retinoic acid treated-cells released twice as much hyaluronic acid into the medium as did control cells (Kochhar et al., 1984a). Zimmerman and Tsambaos (1985) cultured NMRI mouse limb buds and embryonic-mouse mesenchymal cells in high-density cultures with all-trans-RA, 13-cis-RA, etretin, or motretinid (the ethyl amide analog of etretinate) at 0.1, 1.0, and 10.0 ug/ml. All of the retinoids initiated dose-dependent inhibition of differentiation of limb buds from the blastoma stage and of chondrogenic differentiation in organ culture and high-density culture. The efficacy of the retinoids varied: all-trans-RA > 13-cis-RA > etretinate > motretinid.

Steele et al. (1982) employed whole embryo culture techniques to investigate the teratogenic effects of vitamin A (purchased in soluble unspecified form). Rat embryos were cultured with serum drawn from rats or humans previous to or after ingestion of 100,000 I.U. vitamin A. Somite number and protein content in the post-vitamin A serum cultured embryos were

reduced after 48 h. Pratt et al. (1985) cultured day 8 CD-1 mouse embryos for 48 h with 13-cis-RA. Defects of the first and second visceral arches and their derivatives were observed at 0.6 μg/ml. Using day 10 mouse embryos, 6 μg/ml 13-cis-RA induced limb and palate defects.

Intubation of 25 mg/kg all-trans-RA to day-nine Sprague-Dawley rats was embryolethal by full term. Lethality was attributed to agenesis or hypogenesis of the chorioallantoic placenta (Vickers, 1985). Etretinate, etretin, and 13-cis-RA are also teratogenic in the rat. Two, 4, or 8 mg/kg etretinate or 15 or 30 mg/kg etretin administered orally on days 7-16 induced a variety of dysmorphia similar to those described for the hamster. Additionally, etretinate, etretin, all-trans-RA, and 13-cis-RA are teratogenic in the rabbit (Kamm, 1982; Kistler and Hummler, 1985; Hummler and Schupback, 1981).

Hendrickx et al. (1980) administered all-trans-RA orally to rhesus monkeys, 20 or 40 mg/kg, between days 19-45 or 17-45, respectively. Of 15 animals dosed, 10 fetuses were malformed; 4 were normal; and 1 was resorbed. Malformations were similar to those previously described for humans. Fantel et al. (1976) obtained similar results with oral all-trans-RA in the pigtail monkey.

In bone cultures with fetal rat humeri, all-trans-RA induced cartilage resorption. Inhibitors of DNA synthesis did not affect the resorption process, indicating that cell division is not a necessary component of the resorption process. Resorption was dependent on RNA, protein, and glycoprotein synthesis. These data were interpreted as an indication that retinoic acid altered gene expression (Kistler, 1982). Arotinoid Ro 13-7410 (the free acid form of arotinoid Ro 13-6298), Ro 13-6307 (the tetramethylatedtetralin derivative of retinoic acid), all-trans-RA, 13-cis-RA, etretin, or etretinate (if activated, e.g. de-esterified) induced cartilage resportion when added to day 13

rat limb bud cultures. Arotinoid Ro 13-7410, the most effective retinoid, was ca. 1,000 times more potent than all-trans-RA. Ro 13-6307 was followed by that of etretin, etretinate and all-trans-RA in their efficacy to induce resorption. The retinoid Ro 15-0778, the same molecule as Ro13-7410 without a carboxyl terminal moiety, lacked activity (Kistler, 1985).

The embryonic chick has also been employed to assess retinoid ability to adversely affect development. A single injection of 125 g all-trans-RA or other active synthetic retinoids into the amniotic cavity of day 10 chick embryos induced ptilopody (feathers on normally scaly feet), crossed beak, acropodial truncature, and club-shaped feather filaments (Cadi et al., 1984; Dhouailly and Hardy, 1978). The retinoids which exhibited the most toxic effects (e.g., 100% mortality or more severe malformations) were those with one or two supplementary side-chain rings: Ro 13-6307, its ethyl ester (Ro 13-2389) and the arotinoid acid (Ro 13-7410) and its ethyl ester (Ro 13-6298).

Recently Kistler (1986b) demonstrated that retinoic acid-induced teratogenicity could be suppressed in a dose-dependent fashion by simultaneously dosing rats p.o. with the cyclohexanetrione Ro31-0521 (3,3,5,5-tetraallyl-2-hydroxy-N-(5-methyl-3-isoxazolyl)-4,6-dioxo-1-cyclo hexene-1-carboxamide sodium salt), which stimulates prostaglandin synthesis, and retinoic acid. In vitro, Ro 31-0521 suppressed retinoic acid-induced cartilage resorption (Dhouailly and Hardy, 1978).

The dose-response curves for induction of terata by retinoids in the hamster are significantly parallel suggesting a common embryotoxic mechanism. The structural requirements for induction of terata by retinoids in hamsters are primarily dependent upon the presence of, or biotransformation to, a free carboxyl or other moiety with an equivalent pKa at C15, not upon the stereochemistry about C13 nor the size of the molecular substituent at C15. In

addition they are dependent upon a carbon side-chain of more than 5 carbon atoms and upon conservation of the curved hydrophobic plane of the polyene chain. All of the retinamides and retinylidene 1,3- diketones tested in hamsters were devoid of teratogenic activity, except when administered at dose levels associated with maternal toxicity (Willhite and Shealy, 1984; Willhite et al., 1984; Willhite, 1986). A free carboxyl group at C15 is required for binding of retinoids to cellular retinoic acid-binding protein (cRABP) (Chytil and Ong, 1983). Cellular retinoic acid-binding protein (cRABP) has been identified in all fetal rat tissues, in the intestine, brain, and kidney of the fetal rabbit, and in limb buds of the mouse (Chytil and Ong, 1983; Rainier et al., 1983; Kwarta et al., 1985). In mouse limb buds, all-trans-RA has a 315-fold greater affinity for cRABP than 13-cis-RA (Kwarta et al., 1985). This parallels in vivo data: 13-cis-RA is one-half as teratogenic as all-trans-RA in the hamster (ED_{50} = 22.3 mg/kg for 13-cis-RA; ED_{50} = 10.5 mg/kg for all-trans-RA, 66) and four to eight times less embryopathic than all-trans-RA in the mouse (Kochhar et al., 1984b). However, it is still unknown if binding of retinoids to cRABP is imperative for teratogenic activity. Additional studies of the β-cyclogeranylidene ring and side chain modifications will provide more information on the exacting requirements of the retinoid structure for teratogenic activity.

Hamsters are apparently more sensitive to the teratogenic effects of retinoids than mice. When treated with retinylidene methyl nitrone, hamsters were afflicted with a more severe syndrome of malformation than mice (Willhite and Balogh-Nair, 1984; Willhite and Balogh-Nair, 1985). A dose of 100 mg/kg 13-cis-RA failed to induce limb malformations in ICR mice on day 11 (Kochhar et al., 1984b), but 25 mg/kg 13-cis-RA significantly increased the number of affected litters in hamsters on day 8 (Willhite and Balogh-Nair,

1984). The species differences may be related to differential absorption from the gut, species-specific biotransformation patterns, pharmacokinetic or disposition differences, or to differential levels of or binding affinity for embryonic cRABP.

Mutagenicity and Carcinogenicity

The mutagenic and carcinogenic potentials of the retinoids have been summarized by Hummler and Schupback (1981) and Kamm (1982). Retinal, etretinate, 13-cis-RA, and all-trans-RA were not mutagenic for Salmonella typhimurium (Kamm, 1982). Etretinate was not mutagenic for Escherichia coli WP2, nor was it mutagenic in the mouse host-mediated S. typhimurium assay at up to an oral dose of 1 mg/kg. Etretinate showed no clastogenic activity in mice (Hummler and Schupback, 1981).

Carcinogenesis bioassay of rats orally exposed to 13-cis-RA at up to 32 mg/kg/d found an increased incidence of pheochromocytoma, possibly related to retinoid-induced adrenal hypertrophy (Kamm, 1982). The incidence of hepatic adenomas and angiomas and leukemia decreased relative to controls. Oral isotretinoin doses of 3, 20, 60, or 120 mg/kg/d in dogs for up to one year caused a dose-related toxicity of the cardiovascular system (fibrosis, focal calcification, and elastic fiber degeneration), testicular degeneration, fatty liver, and lymph node edema, but no evidence for a significant carcinogenic response was reported. Chronic toxicity of etretinate in dogs was confined to the usual hypervitaminosis A syndrome (Teelmann, 1981), but no evidence for a carcinogenic response was reported. No increase in tumor incidence was observed in rats fed up to 3 mg/kg/d etretinate for two years. Dietary administration of 1 or 3 mg/kg/d was associated with a decreased number of

rats bearing spontaneous multiple tumors, and at 3 mg/kg/d the number of mammary tumors in female rats decreased (Kamm, 1982).

In sharp contrast to these studies, Ohshima et al. (1985) found an increase in pancreatic islet cell adenomas in aged male ACI/segHapBR rats when all-trans-N-(4-pivaloyloxyphenyl) retinamide or all-trans-4-N-(2-hydroxyethyl) retinamide were administered at 951 mg/kg and 687 mg/kg diet, respectively, for up to 54 weeks. Female Fisher 344 rats dosed with 600 mg N-butyl-N-(4-hydroxybutyl) nitrosamine over a 6 week period and fed 515 mg/kg diet all-trans-2-hydroxyethyl retinamide had an increase in the incidence of bladder tumors after one year compared with animals given the nitrosamine alone. Two years subsequent to nitrosamine and retinoid administration, the retinoid treatment group's tumors had progressed farther than the group administered nitrosamine alone (Quander et al., 1985).

STRUCTURE-TOXICITY RELATIONSHIPS AND POSTULATED MECHANISMS OF ACTION

Conformationally restricted retinoids, sometimes referred to as arotinoids, are held in a fixed cisoid arrangement (Loeliger et al., 1980; Dawson et al., 1984). These multi-ring retinoids contain two aromatic rings, such that the retinoid resembles structurally the 10,11-cisoid and/or the 12,13-cisoid rotameric forms of retinoic acid. The para-substitutions on the phenyl ring act as spacers between C11 and the carboxyl terminus to maintain conjugation; the rings are held away from one another forming a 71^{o} angle. The multi-ring retinoids are 3-1,600 times more toxic to mice than all-trans-RA. These retinoids are also more active than all-trans- or 13-cis-RA in hamster tracheal explant cultures (Newton et al., 1980), have activities similar to that of all-trans-RA for inhibition of tumor promoter-induced mouse epidermal

ornithine decarboxylase (Dawson et al., 1984), and are more potent teratogens in hamsters than all-trans-RA, etretinate, or other synthetic retinoids (Flanagan et al., 1987). The tetramethylated indanes and tetralins of the E-configuration were the most active in the mouse papilloma assay. To date, the most toxic retinoid reported has been the aldehyde-containing arotinoid, inducing hypervitaminosis A in mice at 0.0125 mg/kg (Loeliger et al., 1980). Substitution of a sulfur-containing group at the polar terminus failed to block retinoid toxicity, but the Z-isomers of carbon (Loeliger et al., 1980) or sulfur-containing (Klaus et al., 1983) restricted retinoids were devoid of pharmacologic activity or toxicity in mice at up to 400 mg/kg. The E-configuration appears to be an obligatory condition for biologic activity. The sulfide derivative of the arotinoid structure was the least toxic compared with the sulfoxide and sulfone; all three retinoids may serve as parent compounds for sulfinic and sulfonic acid metabolites (Klaus et al., 1983). Binding of conformationally restricted retinoids to cRABP correlates with their biological activity (Sani et al., 1984). However, some retinoids, such as the ethyl sulfone arotinoid, do not bind to the protein and are still highly active (Klaus et al., 1983). Such retinoids may serve as "pro-drugs", whose acidic metabolites may be responsible for in vivo pharmacological activity.

The mechanism of retinoid action has been attributed to their amphipathic characteristics causing membrane alterations via detergent disruption (Fell and Dingle, 1963), glycosylation of cell surface glycoproteins via formation of retinyl mannose phosphate intermediates (De Luca et al., 1979), and activation and suppression of the genome (Sporn and Roberts, 1983). Retinoid-induced teratogenicity cannot be due to non-specific membrane disruption, because certain retinoids containing the hydrophilic carboxyl terminus, a 9-carbon flexible side-chain, and the β-cyclogeranylidene ring were ineffective at

inducing terata (Willhite, 1986). Nor can the toxicity be attributed to involvement of retinoids in sugar transfer reactions, as the retinoidal benzoic acid derivative Ro 13-6298 does not participate in retinyl phosphate mannose pathways (Sporn and Roberts, 1983).

Several groups have provided biochemical data on retinoid control of cellular proliferation. When retinoic acid was used to induce differentiation in HL-60 cells, it suppressed expression of the myc oncogene, a nucleotide sequence considered to be involved in excessive proliferation of HL-60 cells (Sporn and Roberts, 1983). Omori and Chytil (1982) demonstrated that feeding retinyl acetate, retinoic acid, or 3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (a synthetic retinoid) to retinol-deficient rats suppressed production of a 22 kDa protein; whereas retinoic acid and the synthetic retinoid also induced a 55 kDa protein. Sporn and Roberts (1983) and Omori and Chytil (1982) suggested that retinoids are modifiers of gene expression . Chytil and Ong (1979) suggested that retinoids are bound in the cytosol by either cellular retinol-binding protein (cRBP) (if an alcohol) or cellular retinoic acid-binding protein (cRABP) (if an acid) and transported into the nucleus where they can modify gene expression. An alternative to the steroid-like mechanism for retinoids has been proposed by Sporn and Roberts (1983). They suggested that retinoids "control gene expression via interaction with protein kinases both cyclic adenosine 3',5'-monophosphate [cAMP] dependent and [cAMP] independent" (Sporn and Roberts, 1983, p.3037).

CHAPTER III

STRUCTURE-TOXICITY RELATIONSHIPS OF THE TETRAMETHYLATED TETRALIN AND INDANE ANALOGS OF RETINOIC ACID

INTRODUCTION

The retinoids, some of which exert prophylactic effects against cancer in laboratory animals (Moon and McCormick, 1982; Hartmann and Bollag, 1985), are employed clinically to treat certain dermatologic disorders (Ward et al., 1983; Orfanos, 1985; Cunliffe et al., 1985). Certain retinoids are potent teratogens in humans (Fehr and Koch-Weser, 1983; Ruther and Kietzmann, 1984; Grote et al., 1985; Willhite et al., 1986; Happle et al., 1984; Lammer et al., 1985; Rosa et al., 1986) and hamsters (Willhite and Shealy, 1984; Williams et al., 1984). The toxicology of retinoids in humans and animals has been summarized (Howard and Willhite, 1986; Geelen, 1979).

Retinoids that contain one or two aromatic rings in their side chain are more potent inhibitors of fetal rat bone chondrogenesis (Kistler, 1981) and of chick skin differentiation (Kistler, 1984) in vitro than such tetraene retinoids as all-trans-retinoic acid (all-trans-RA). These multi-ring retinoids are 10-1,000 times more effective in the mouse antipapilloma assay (Loeliger et al., 1980) and are more effective towards induction of differentiation of human promyelocytic HL-60 cells (Bollag, 1985) than tetraene retinoids. In this chapter the effects of modifications of the ring and side-chain on the teratogenic potency of retinoids in hamsters are reported. The results are compared with established structural relationships for retinoid teratogenic activity in hamsters.

MATERIALS AND METHODS

Chemicals

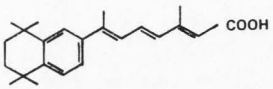
The retinoids all-trans-3,7-dimethyl-9-(1,1,3,3-tetramethylindanyl)-2,4,6,8-nonatetraenoic acid (Ro 13-4306), (E)-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-methyl-2,4,6-octratrienoic acid (Ro 13-6307), and ethyl (E)-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-methyl-2,4,6-octratrienoate (Ro 13-2389) (Figure III.1) were gifts from Hoffmann-La Roche, Inc. (Nutley, NJ). The retinoids were stored under desiccant at -80°C. All retinoids had a purity of greater than 98 percent when assayed at 340 and 254 nm with acetonitrile-1 percent aqueous ammonium acetate 85:15 and 93:7 as the mobile phase using a Spherisorb ODS 5-μm column (Universal Scientific, Atlanta, GA) and a flow rate of 1.0 ml/min. Retinoids were dissolved in a small volume of reagent grade acetone and solubilized in Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma, St. Louis, MO).

Animals

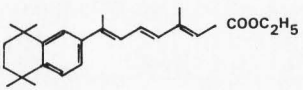
Virgin female Syrian Golden hamsters [LAK:LVG (SYR)] were purchased from the Charles River Breeding Laboratories, Inc., Wilmington, MA. Hamsters were mated by the method described by Ferm (1967). The day following the evening of breeding was considered day one of gestation. Male and female animals were allowed free access to tap water and laboratory stock diet (Wayne F6 Lab Blox® #8664-00) and were housed separately in polycarbonate cages with pine shavings as bedding. The light cycle was maintained at 14 h light/10 h dark. Animals were housed in an AAALAC- accredited facility.

A single oral dose was administered by gavage at 10:00 A.M. on day 8 of gestation at a rate of 5 ml/kg of body weight. Retinoid doses were selected

(E)-7-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-methyl-2,4,6-octatrienoic acid



Ethyl(E)-7-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-3-methyl-2,4,6-octatrienoate



all-trans-3,7-Dimethyl-9-(1,1,3,3-tetramethylindanyl)-2,4,6,8-nonatetraenoic acid

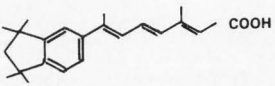


Figure III.1. Molecular structures of selected retinoids.

by inspection as calculated from their molar concentrations relative to that of all-trans-RA. When a limited amount of retinoid was available, a dose equimolar with that of 75 mg/kg of all-trans-RA was administered to the pregnant dam. Animals were killed in a saturated CO_2 environment on day 14 and the pregnant uteri collected by laparotomy. Fetuses were blotted dry on absorbent paper and weighed. The numbers of resorptions, number of dead, and number of living fetuses were recorded. All live fetuses were examined with a binocular dissecting microscope for the presence of gross congenital malformations. One-third of each litter was fixed in Bouin's solution, sectioned in the midsagittal plane, and examined for internal abnormalities (Willhite and Shealy, 1984). The remaining two-thirds were fixed in 95% ethanol, cleared in 2% KOH, stained with alizarin red S, and examined for confirmation of skeletal anomalies.

Statistics

The litter was considered the experimental unit for statistical procedures. The maternal weight change was calculated by subtracting the total litter weight (including dead, autolyzed fetuses) from the dam's weight change between days 8 and 14. Univariate analysis of variance was used to analyze maternal weight change and fetal weights (Sokal and Rohlf, 1981). The Newman-Keuls test was used to test differences between the vehicle-treated control and treatment group means, subsequent to a significant F-test (Sokal and Rohlf, 1981). The number of resorptions for each retinoid dose was compared to the vehicle-treated control value using the nonparametric Mann-Whitney test (Sokal and Rohlf, 1981). A litter was considered affected, if it contained one or more malformed or dead fetuses or three or more resorptions. Fisher's exact test for a 2 x 2 contingency table (Sokal and Rohlf, 1981) was

used to test the statistical significance of the affected litters at each retinoid dose. All statistical procedures were considered significant at the p=0.05 level.

The dose-response curves, the median effective dose for induction of terata (ED_{50}), and the embryonic LD_{50} were generated using the log-probit model for those retinoids associated with a significant teratogenic response (Schoofs and Willhite, 1984). The numbers of malformed fetuses at each dose were used to calculate the parameters of the dose-response curve. To determine parallelism and potency ratio, the slopes of the dose-response curves with their confidence limits for each retinoid associated with a significant teratogenic response were compared to the slope of the dose-response curve of an identical population of hamsters treated with the reference retinoid, all-trans-RA (Willhite and Shealy, 1984).

RESULTS

Three of the 28 dams treated with the Tween 20: acetone vehicle produced affected litters (Table III.1). The single malformation was limited to a rib fusion. One litter contained a dead fetus, and the third litter contained three resorptions. At no point in the study were signs of maternal retinoid toxicity observed (e.g., maternal weight loss, alopecia, exfoliation, dermatitis, conjunctivitis). Oral intubation of 6.7 or 13.5 mg/kg Ro 13-4603 increased significantly the numbers of affected litters compared to control litters. Doses of 6.7 or 13.5 mg/kg Ro 13-4603 (equimolar doses corresponding to 6.25 and 12.5 mg/kg all-trans-RA) induced malformations in 73.4 and 100 percent of the recovered fetuses, respectively (Table III.1). The median effective teratogenic dose (ED_{50}) for Ro 13-4306 was 4.7 mg/kg (4.2-5.2, 95 percent confidence interval).

TABLE III.1

TERATOGENIC ACTIVITY OF Ro 13-4306 ON HAMSTER EMBRYONIC DEVELOPMENT

	Dose (mg/kg)				
	Tween 20 0.5 mg/100g	0.67	2.14	6.7	13.5
No. animals treated	30	7	10	11	9
No. litters	28	3	10	9	7
No. affected litters	3	0	3	9*	7*
No. implantations	288	37	119	98	78
No. resorbed (%)	8(2.7)	0	4(3.4)	3(3.1)	2(2.6)
No. dead (%)	1(0.35)	0	0	1(1.0)	0
Fetuses examined	279	37	115	94	76
No. abnormal fetuses (%)	1(0.36)	0	8(7.0)	69(73.4)	76(100)
Mean litter frequency of malformed fetuses	0.036	0	0.80	7.7	10.9
Mean fetal body wt. (g±SD)	1.24±0.14	1.37±0.04	1.29±0.05	1.16±0.15	1.18±0.09
Mean maternal wt. change (g±SD)	13.9±6.0	17.8±4.8	15.9±3.2	17.4±4.0	16.3±3.0
Malformations					
Microcephaly	0	0	0	0	48
Arnold-Chiari malformation	0	0	0	2	3
Encephalocele	0	0	0	1	3
Exencephaly	0	0	0	3	4
Cystic rachischisis	0	0	0	0	1
Microstomia	0	0	0	1	48
Macrostomia	0	0	7	54	17
Maxillary retrocession	0	0	0	13	31
Rib fusion	1	0	0	8	39
Palpebral aplasia with exophthalmia	0	0	0	32	61
Anophthalmia	0	0	0	1	0
Protruding tongue	0	0	0	0	47
Hypoplastic tail	0	0	0	0	3
Lordotic skull base	0	0	0	2	3
Atretic or stenotic external auditory meatus	0	0	0	1	61
Lowset, hypoplastic, malformed, or absent pinnae	0	0	1	33	76

* $p<0.05$ compared to vehicle control.

Doses of 0.71 mg/kg or higher Ro 13-6307 increased significantly the number of affected litters compared to control values (Table III.2). Doses of 0.35, 0.71, or 2.2 mg/kg of Ro 13-6307 (doses equimolar to 0.31, 0.63, and 2.0 mg/kg all-trans-RA, respectively) induced malformations in 12.1, 53.3, and 98.2 percent of the recovered fetuses, respectively (Table III.2). Doses of Ro 13-6307 at 7.1, 14.2, or 28.4 mg/kg induced malformations in all recovered fetuses. The mean fetal body weights of fetuses recovered from dams treated with 14.2 or 28.4 mg/kg Ro 13-6307 were depressed significantly compared with those from vehicle-treated dams (Tables III.1 and III.2). As the dose increased, both the numbers of malformed fetuses and numbers of malformations per fetus increased (Table III.2). The ED_{50} for Ro 13-6307 was 0.66 mg/kg (0.60-0.72).

Doses of 0.75 mg/kg or higher Ro 13-2389 increased significantly the number of affected litters above control values (Table III.3). Fetuses recovered from dams treated with 0.38, 0.75, or 2.4 mg/kg Ro 13-2389 had corresponding malformation rates of 2.4, 64.0, and 98.5 percent. The total numbers of resorptions and malformed fetuses were dose-dependent; all implantation sites were resorbed in the highest (30.0 mg/kg) dose group (Table III.3). The mean fetal body weight was depressed significantly in the 7.5 and 15.0 mg/kg dose groups. The teratogenic ED_{50} for Ro 13-2389 was 0.71 mg/kg (0.66-0.78), and the embryonic LD_{50} was 8.65 mg/kg (7.50-10.15).

The syndrome of malformations induced by these three retinoids was identical to that observed after treatment with a teratogenic dose of all-trans-RA during an identical gestational stage (Willhite and Shealy, 1984). Prominent dysmorphia common at the higher doses included craniofacial and axial skeletal defects, as well as a caudal regression syndrome consisting of a hypoplastic or aplastic tail, anal atresia, and sacral cystic rachischisis. Macrostomia and microstomia dominated the lower dose ranges (approximately 1 mg/kg Ro 13-

TABLE III.2

TERATOGENIC ACTIVITY OF Ro 13-6307 ON HAMSTER EMBRYONIC DEVELOPMENT

	Dose (mg/kg)					
	0.35	0.71	2.2	7.1	14.2	28.4
No. animals treated	11	12	12	9	11	11
No. litters	8	9	10	8	7	7
No. affected litter	5	8*	10*	8*	7*	7*
No. implantations	101	92	117	89	85	71
No. resorbed (%)	2(2.0)	2(2.1)	3(2.5)	2(2.2)	28(32.9)*	7(7.9)
No. dead (%)	0	0	0	0	3(3.5)	4(5.6)
Fetuses examined	99	92	114	87	54	60
No. abnormal fetuses (%)	12(12.1)	49(53.3)	112(98.2)	87(100)	54(100)	60(100)
Mean litter frequency of malformed fetuses	1.5	5.4	11.2	10.8	7.7	8.6
Mean fetal body wt. (G±SD)	1.25±2.9	1.29±0.09	1.17±0.11	1.13±0.07	0.95±0.15*	1.0±0.11*
Mean maternal wt. change (g±SD)	16.8±2.9	13.9±2.1	13.4±5.1	11.2±5.9	14.3±4.3	8.2±10.2
Malformations						
Micrognathia/agnathia[a]	0	0	0	17	41	45
Microcephaly	0	0	40	55	31	30
Arnold-Chiari malformation	0	0	4	4	12	7
Encephalacele	0	0	14	4	1	1
Exencephaly	0	1	6	6	23	29
Cystic rachischisis	0	0	0	1	33	56
Microstomia	0	0	67	52	10	15
Macrostomia	9	47	26	17	0	0
Maxillary retrocession	1	7	18	15	0	0
Rib fusion	0	1	8	11	14	32
Palpebral aplasia with exophthalmia	0	28	86	78	42	60
Anophthalmia	0	0	0	1	0	0
Protruding tongue	0	0	55	67	17	12
Microglossia	0	0	11	0	4	10
Hypoplastic tail	0	0	0	9	24	52
Lordotic skull base	0	0	4	4	12	7
Absent or hypoplastic kidney	0	0	0	1	7	8
Acystia	0	0	0	0	0	2
Anal atresia	0	0	0	3	14	49
Atretic or stenotic external auditory meatus	0	0	23	73	53	59
Lowset, hypoplastic, malformed, or absent pinnae	1	0	82	88	54	59

* $p<0.05$ compared to vehicle control.

[a] Includes cleft mandible.

TABLE III.3

TERATOGENIC ACTIVITY OF Ro 13-2389 ON HAMSTER EMBRYONIC DEVELOPMENT

	DOSE (mg/kg)					
	0.38	0.75	2.4	7.5	15.0	30.0
No. animals treated	10	11	13	12	10	8
No. litters	10	9	11	11	10	7
No. affected litters	2	9*	11*	11*	10*	7*
No. implantations	128	107	149	129	114	78
No. resorbed (%)	4(3.1)	1(.9)	10(6.7)	33(25.5)*	103(90)*	78(100)*
No. dead (%)	0	0	0	2(1.6)	0	0
Fetuses examined	124	106	139	94	11	0
No. abnormal fetuses (%)	3(2.4)	68(64)	137(98.5)	94(100)	11(100)	0
Mean litter frequency of malformed fetuses	0.3	7.6	11.6	8.5	3.7	0
Mean fetal body wt. (g±SD)	1.27±0.07	1.31±0.09	1.23±0.08	1.0±0.08*	0.90±0.3*	0
Mean maternal wt. change (g±SD)	18.6±4.3	13.1±0.1	12.9±3.8	19.5±6.8	17.3±8.9	11.9±4.0
Malformations						
Micrognathia/agnathia[a]	0	0	0	79	11	0
Microcephaly	0	0	32	79	11	0
Arnold-Chiari malformation	0	0	0	6	0	0
Encephalacele	1	0	2	6	0	0
Exencephaly	0	0	2	17	0	0
Cystic rachischisis	0	0	0	20	8	0
Microstomia	0	0	67	12	0	0
Macrostomia	0	65	54	3	0	0
Maxillary retrocession	1	0	31	3	0	0
Cleft palate[b]	0	0	0	3	0	0
Rib fusion	0	1	8	35	7	0
Palpebral aplasia with exophthalmia	0	2	124	89	11	0
Anophthalmia	0	0	0	4	0	0
Protruding tongue	0	0	61	15	0	0
Microglossia	0	0	0	14	3	0
Hypoplastic tail	1	0	5	24	11	0
Lordotic skull base	0	0	0	6	0	0
Anal atresia	0	0	0	24	11	0
Renal agenesis	0	0	0	2	1	0
Acystia	0	0	0	1	0	0
Atretic or stenotic external auditory meatus	0	0	3	90	11	0
Lowset, hypoplastic, malformed, or absent pinnae	0	0	82	93	11	0

* $p<0.05$ compared to vehicle control.
[a] Includes cleft mandible.
[b] Includes cleft lip.

6307 or 13-2389) with the more severe malformations of the face and jaw (micrognathia and agnathia) prevailing at higher doses (Tables III.1, III.2, and III.3). Frequently a fused amorphous boney mass and mandibular ankylosis were observed after alizarin straining of fetuses that, upon initial gross examination, were classified as agnathic or micrognathic. Ossification of the lower spine was decreased markedly in fetuses afflicted with cystic rachischisis (data not shown).

The dose-response curves for Ro 13-4306, Ro 13-6307, and Ro 13-2389 were significantly parallel with that of all-trans-RA ($p=0.00007$, $p=0.000001$ and $p=0.004$, respectively). The slopes of the retinoid Ro 13-4306, Ro 13-6307, and Ro 13-2389 dose-response curves were 5.8 (4.8-6.7), 5.6 (4.6-6.6), and 6.7 (5.6-7.9, respectively. On a mg/kg basis, Ro 13-4306, Ro 13-6307, and Ro 13-2389 were 2.3 (2.0-2.5,) 16.1 (14.4-18.0), and 15.1 (13.6-16.6) times more potent teratogens than all-trans-RA, respectively. On a molar basis, Ro 13-4306 was 2.4 times as potent as all-trans-RA. The retinoids Ro 13-6307 and Ro 13-2389 were 18.0 times more potent teratogens in hamsters than all-trans-RA.

DISCUSSION

The retinoids studied here contain either a 5,5,8,8-tetramethylated tetralin (Ro 13-2389 and Ro 13-6307) or 1,1,3,3-tetramethylated indane (Ro 13-4306) ring system. This double-ring system contrasts with the simple β-cyclogeranylidene ring of such tetraene retinoids as retinol and retinoic acid. The present results show not only that these synthetic multi-ring retinoids are more potent teratogens than all-trans-RA, but that retinoids containing the tetramethylated tetralin system were 7 times more potent teratogens than retinoids with the tetramethylated indane system.

This same relationship was true for other in vivo and in vitro assays; the tetramethylated teralin retinoids were more active than tetramethylated indanes. Cadi et al. (1984) reported that Ro 13-2389 and Ro 13-6307 were substantially more effective at modification of chick embryo tarsometatarsal morphogenesis than all-trans-RA, but Ro 13-6307 and Ro 13-2389 were only 0.1 times as potent as the cisoid free acid retinoidal benzoic acid (arotinoid) congener, Ro 13-7410. In the mouse antipapilloma assay, Ro 13-2389 was more effective at decreasing papilloma diameter than either Ro 13-4306 or all-trans-RA (Loeliger et al., 1980). Ro 13-2389 was approximately 100 times as potent as all-trans-RA and was 17 times more potent than Ro 13-4306 at induction of hypervitaminosis A in mice. However, the therapeutic ratio of Ro 13-2389 was 2.5 times more favorable than all-trans-RA (Loeliger et al., 1980). Table III.4 contains data summarized from the literature on the relative biologic potency of the retinoids studied here. The tetralin and the indane congeners were consistently more biologically active than all-trans-RA - between 10- to 1,000-fold more active - across a multitude of in vitro and in vivo structure-activity studies (Table III.4).

In previous studies (Willhite et al., 1984) tetraene retinoid teratogenic activity in hamsters was dependent upon the presence of or biotransformation to a free carboxylic acid or other moiety with an equivalent pKa at C15. The teratogenic activity was dependent upon a side-chain of more than 5-carbon length, conservation of the curved, hydrophobic plane and preservation of electron delocalization across the polyene chain (Willhite, 1986). Reduction of in situ production of the acidic congener with an amide (Howard et al., 1986) or dimedone (Willhite et al., 1984) substitution rendered the retinoid teratogenically inactive in hamsters. It is reasonable to assume that the similar teratogenic profile of the ethyl ester Ro 13-2389 can be attributed to

TABLE III.4

RETINOID ACTIVITY IN VITRO AND IN VIVO

RETINOID	ED_{50} HAMSTER TERATOGENICITY (μMol/kg)	FETAL RAT BONE CULTURE[b] (nM)	MOUSE MAMMARY GLAND CULTURE[c] (nM)	LIMB BUD CHONDROGENESIS CULTURE[d] (nM)	CHICK FOOT SKIN CULTURE[e] (nM)	PROMYELOCYTIC HL-60 CELL CULTURE[f] (nM)
all-trans-RA	35.0[a]	1,200	1,000	100	10,000	100
13-6307	1.95	35	1.0	0.60	10	8.0
13-2389	1.95	400	- [g]	-	-	-
13-4306	14.5	200	100	-	100	80

[a] ED_{50} data from Willhite and Shealy (1984)
[b] ED_{40} data summarized from Kistler (1981)
[c] Lowest inhibiting concentration data summarized from Kistler (1986a)
[d] IC_{50} data summarized from Kistler (1985)
[e] Complete inhibition data summarized from Kistler (1984)
[f] ED_{50} data summarized from Bollag (1985)
[g] No data available

in situ generation of the free acid, just as with etretinate (Ro 10-9359) (Williams et al., 1984) or arotinoid ethyl ester (Ro 13-6298) (Flannagan et al.,1987), where the ester underwent hydrolysis to the free acid congeners etretin (Ro 10-1670) and Ro 13-7410, respectively. It is the free acid form of the retinoid that binds to cellular retinoic acid-binding protein (cRABP) in hamster tissue (Brandes et al., 1983). This binding protein has also been detected in a number of human tumors including breast, lung, prostate, cervix, and colon, in addition to several animal tumor models. In animals, however, cRABP occurs at higher concentrations and in more fetal tissues than in adult tissues. There appears to be a mechanistic link between the toxicity of retinoids in embryonic development and intrinsic pharmacologic activity in carcinogenesis.

The retinoidal benzoic acid derivatives such as (E)-4-(2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propen-1-yl) benzoic acid (Ro 13-7410) are the most potent retinoid teratogens yet reported (Flanagan et al., 1987). These retinoids are at least 750 times more teratogenic than all-trans-RA in hamsters. The tetramethylated tetralin analogs are the next most potent retinoidal teratogens studied to date, followed by the tetramethylated indane (Ro 13-4306), etretinate (ED_{50}= 5.7 mg/kg) (Williams et al., 1984), all-trans-RA (ED_{50}= 10.5 mg/kg) and 13-cis-retinoic acid (ED_{50}= 22.3 mg/kg) (Willhite and Shealy, 1984). The teratogenic activity of the retinoid increased with the degree of conformational restriction, provided an acidic moiety was present in either the parent retinoid or in a metabolite of the parent compound. Willhite (1986) found that the no modification of the side-chain per se enhanced embryotoxicity. Introduction of the planar aromatic ring in lieu of the chair or boat natural β-cyclogeranylidene ring increased embryotoxic activity (Williams et al., 1984). Introduction of an aromatic ring in the side-chain as

in Ro 13-4306 or Ro 13-6307, corresponding to the 7-8 region of all-trans-RA, along with gem dimethyl modification of the natural cyclohexene ring, not only restricted side-chain flexibility and preserved the hydrophobic plane of the chain, but facilitated π-electron delocalization across the retinoid. Facilitation of charge-transfer across the retinoid molecule (Leavitt and Mass, 1985) appears to be an important aspect of retinoid structure-teratogenicity relationships.

The increased teratogenic activity of the arotinoids and of the multi-ring retinoids studied here, as contrasted with such tetraene retinoids as all-trans- and 13-cis-retinoic acid, cannot be ascribed completely to maternal pharmacokinetic or placental permeability factors (Creech-Kraft et al., 1987). There are several orders of magnitude difference in teratogenic potency among the retinoids. It is unlikely that a 1,000-fold lower maternal oral dose of a highly toxic retinoid (e.g., arotinoid) would result in greater retinoid concentrations in the embryo. Binding affinities for cRABP correlate with the biological activity of retinoids in hamster tissues (Sani et al., 1984). Binding affinities for cRABP also correlate with the ability of retinoids to induce limb bud malformations in chicks (e.g., Ro 13-7410 was the most potent inducer of limb malformations and also the most effective competitor for cRABP) (Maden and Summerbell, 1986). The relative biological potencies of these retinoids are consistent across a multitude of in vivo and in vitro assays suggesting the existence of specific cellular receptor(s) responsible for pharmacologic and toxicologic activities. Increased binding affinities for embryonic cRABP of the retinoids studied here, as contrasted to the simple tetraene retinoids, may account for the marked differences in teratogenic activity.

CHAPTER IV

STRUCTURE-ACTIVITY RELATIONSHIPS OF RETINOIDS IN DEVELOPMENTAL TOXICOLOGY: CONTRIBUTION OF THE VITAMIN A β-CYCLOGERANYLIDENE RING

INTRODUCTION

Retinoids are employed clinically to treat actinic keratosis, bronchial metaplasia, laryngeal papillomatosis, cervical dysplasia, and myelodysplastic syndromes, and for treatment of such dermatologic conditions as psoriasis, Darier's disease, and cystic acne. The use of retinoids as preventive and therapeutic anticancer agents has been reviewed (Lippman et al., 1987a, 1987b). The toxicity of retinoids in humans and animals has been reviewed (Howard and Willhite, 1986).

Oral retinoid treatment in pregnant hamsters produced a syndrome of malformations strikingly similar to those observed in human infants exposed prenatally to retinoids (Willhite et al., 1986). Previous structure-activity investigations have considered the contribution of the polar terminus (Willhite et al., 1984; Willhite and Balogh-Nair, 1984; Willhite and Shealy, 1984) and the polyene side-chain (Willhite, 1986) in retinoid teratogenicity. The present work focuses on the influence of the β-cyclogeranylidene ring of the vitamin A molecule on teratogenicity in hamsters.

MATERIALS AND METHODS

Chemicals

Hoffmann-La Roche, Inc., Nutley, NJ, supplied the retinoids Ro 12-4824 (all-trans-4-oxoretinoic acid), Ro 12-4825, Ro 8-9750, Ro 10-1770, Ro 8-7699 (acetyldimethylcyclopentenyl retinoic acid), Ro 8-8717, Ro 11-1430 (motretinid;

Tasmaderm®), Ro 11-4768, the 2-chloro analog of etretinate Ro 12-0995, and Ro 21-6667 (Table IV.1). The congener juvenile hormone III (Table IV.1) was purchased from Calbiochem, La Jolla, CA. SRI 2712-24 (Table IV.1) was synthesized as described by Dawson and associates (1981). All retinoids were stored under inert gas at -80°C.

Retinoids were analyzed by isocratic high performance liquid chromatography (HPLC) using a Varian 5000 chromatograph with a Spherisorb ODS 5-μm column (Universal Scientific, Atlanta, GA) and a Spherex SC18 5-cm x 4.6-cm pre-column (Phenomonex, Rancho Palos Verdes, CA). The retinoids were assayed at 340 and 254 nm with acetonitrile-1% aqueous ammonium acetate (85:15 or 93:7) as the eluent. Retinoids were monitored with a Varian Vari-Chrom UV/Visible spectrophotometer, and the peak area was integrated with a Hewlett-Packard model 3390 A integrator. HPLC grade acetonitrile and water (J.T. Baker, Jackson, TN) were filtered and vacuum-degassed prior to use. Injection samples (2 μl) were prepared by dissolving each retinoid (1.0 mg/ml) in either acetonitrile or methanol. Retinoid purity was estimated as shown in Table IV.2. Juvenile hormone III (Calbiochem, La Jolla, CA) was purchased as not less than 96 percent pure. Ro 21-6667 was administered as supplied by Hoffmann-La Roche.

All retinoids, except juvenile hormone III, were dissolved in a small quantity of reagent grade acetone and solubilized in Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma Chemical Co., St. Louis, MO), such that the final concentration of acetone did not exceed 5%. Juvenile hormone III was purchased dissolved in hexane, and the hexane was evaporated under nitrogen to near dryness in a volume of not more than 150 μl. The hormone was then dissolved in Tween 20 so that the hexane concentration was less than

TABLE IV.1

MOLECULAR STRUCTURE OF RETINOIDS

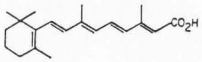
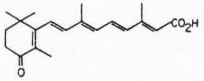
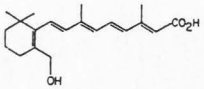
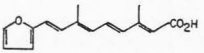
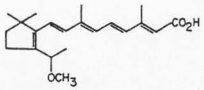
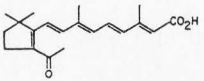
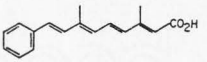
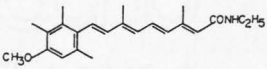
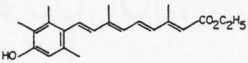
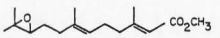
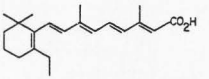
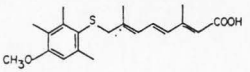
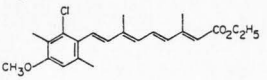
COMPOUND NAME	CODE NUMBER	STRUCTURE
all-trans-3,7-dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid (all-trans-retinoic acid)		
all-trans-3,7-dimethyl-9-(2,6,6-trimethyl-3-oxo-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid (all-trans-4-oxoretinoic acid)	Ro 12-4824	
all-trans-9-(2-hydroxymethyl-6,6-dimethyl-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid	Ro 12-4825	
all-trans-9-(2-furyl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid	Ro 8-9750	
all-trans-9-[2-(1-methoxyethyl)-5,5-dimethyl-1-cyclopenten-1-yl]-3,7-dimethyl-2,4,6,8-nonatetraenoic acid	Ro 10-1770	
all-trans-9-(2-acetyl-5,5-dimethyl-1-cyclopenten-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid	Ro 8-7699	
all-trans-3,7-dimethyl-9-phenyl-2,4,6,8-nonatetraenoic acid	Ro 8-8717	
N-ethyl all-trans-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoamide (Motretinid)	Ro 11-1430	
ethyl all-trans-9-(4-hydroxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate	Ro 11-4768	
methyl all-trans-10,11-epoxy-3,7,11-trimethyl-2,6-dodecadienoate (juvenile hormone III)		
all-trans-9-(2-ethyl-6,6-dimethyl-1-cyclohexen-1-yl)-3,7-dimethyl-2,4,6,8-nonatetraenoic acid	SRI 2712-24	
all-trans-8-(4-methoxy-2,3,6-trimethylphenylthio)-3,7-dimethyl-2,4,6-octatrienoic acid	Ro 21-6667	
ethyl all-trans-9-(2-chloro-3,6-dimethyl-4-methoxyphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate	Ro 12-0995	

TABLE IV.2

PURITY OF RETINOIDS

Retinoid	Solvent system[a]	254 nm Purity[b] (%)	254 nm t_r[c] (min)	340 nm Purity (%)	340 nm t_r (min)
Ro 12-4824	A	100	6.8	99.8	6.8
	B	100	13.8	99.1	13.8
Ro 12-4825	A	100	6.9	100	6.9
	B	100	14.6	100	14.6
Ro 8-9750	A	100	2.9	99.4	2.9
	B	100	5.3	99.6	5.3
Ro 10-1770	C	100	4.9	100	4.9
Ro 8-7699	C	86.1	4.9	95	4.9
Ro 8-8717	A	100	2.9	99.4	2.9
	B	100	5.3	99.6	5.3
Ro 11-1430	B	100	5.8	99.9	5.8
Ro 11-4768	A	98.8	6.0	98.1	6.0
	B	99.7	6.8	99.7	6.8
Ro 12-0995	A	98.7	16.2	-	-
SRI 2712-24	A	98.4	9.0	95.5	9.0

[a]Solvent system: A [acetonitrile/1% ammonium acetate (85:15); B [acetonitrile/1% ammonium acetate (93:7)]; C [acetonitrile/1% ammonium acetate/tetrahydrofuran (76:16:8)].

[b]Purity determined by peak area compared to total area under peaks.

[c]t_r: retention time.

0.05 percent. All retinoids and solutions were handled and administered under yellow light with as little exposure to air as possible.

Animals

Animals were housed, bred, and treated as described in chapter III.

Statistics

Statistics were performed as described in chapter III.

RESULTS

Neither signs of the hypervitaminosis A syndrome nor other evidence of maternal intoxication were observed in any of the dams during the experimental period (including maternal weight loss, chelitis, stomatitis, conjunctivitis, erythema, desquamation, alopecia, xerosis, pruritus, or exfoliation). Three of the thirty-nine litters recovered from Tween 20: acetone (95:5, v/v)-treated control dams were considered affected. One litter contained a dead fetus; the second contained one malformed fetus (a fused rib); and the third had three resorptions (Table IV.3).

Treatment with 4.9 mg/kg or more of all-trans-4-oxoretinoic acid (Ro 12-4824) increased significantly the number of affected litters compared to the vehicle-treated control (Table IV.3). Doses of 19.6 and 39.2 mg/kg increased significantly the number of resorptions compared to the control. The teratogenic ED_{50} for Ro 12-4824 was 9.3 mg/kg (8.6-10.0, 95% confidence interval). The dose-response curve was significantly parallel ($p=0.0029$) with that of all-trans-RA with a slope of 6.4 (5.3-7.4), and 4-oxoretinoic acid was 1.1 (1.0-1.3) times more potent a teratogen than all-trans-RA on a mg/kg basis.

TABLE IV.3

TERATOGENIC ACTIVITY OF Ro 12-4824

		Dose (mg/kg)				
	Tween 20	4.9	9.8	20	39	78
No. animals treated	42	9	10	8	6	6
No. litters	39	8	7	8	6	4
No. affected litters	3	5*	7*	8*	6*	4*
No. implants	437	107	92	115	95	46
No. resorbed (%)	8(1.8)	1 (0.93)	3 (3.3)	9* (7.8)	16* (16.8)	4 (8.7)
No. dead fetuses (%)	1(0.2)	0 (0)	1 (1.1)	1 (0.9)	3 (3.2)	1 (2.2)
No. fetuses examined	428	106	88	105	76	41
No. abnormal fetuses (%)	1(0.2)	12 (11.3)	35 (39.8)	105 (100)	76 (100)	41 (100)
Mean litter frequency of malformed fetuses	0.026	1.5	5.0	13.1	12.7	10.3
Mean fetal body weight (g±SD)	1.26±0.14	1.22±0.13	1.26±0.06	1.11±0.11	0.94±0.18*	0.97±0.14*
Mean maternal weight change (g±SD)	14.4±5.4	16.7±6.5	15.2±3.3	20.2±4.4	22.8±4.5*	27.3±2.9*
Malformations						
Macrostomia	0	10	31	16	4	0
Microcephaly	0	0	0	63	58	32
Encephalocele	0	0	0	11	9	3
Exencephaly	0	0	0	16	17	9
Rib fusion	1	2	4	75	69	39
Arnold-Chiari malformation types I or II	0	0	0	11	17	11
Cystic rachischisis	0	0	0	83	60	29
Rachischisis aperta	0	0	0	3	16	11

TABLE IV.3 (CON'T)

TERATOGENIC ACTIVITY OF Ro 12-4824

		Dose (mg/kg)				
	Tween 20	4.9	9.8	20	39	78
Microstomia	0	0	0	73	39	1
Maxillary retrocession	0	0	0	12	0	0
Palpebral aplasia with exophthalmia	0	0	0	86	73	40
Protruding tongue	0	0	0	60	31	0
Aplastic or hypoplastic tail	0	0	0	87	76	41
Anal atresia	0	0	0	93	76	41
Lordotic skull base	0	0	0	11	17	11
Low-set, hypoplastic, or absent pinnae	0	0	0	105	75	41
Renal agenesis	0	0	0	9	19	13
Microglossia	0	0	0	0	16	9
Micrognathia or agnathia[a]	0	0	0	0	36	40
General edema	0	0	0	0	4	2
Gastroschisis	0	0	0	0	3	1

[a] Includes cleft mandible
* Significantly different ($p<0.05$) compared to vehicle control.

Oral intubation of 9.8 or 20 mg/kg of the C18 hydroxylated congener of all-trans-RA (Ro 12-4825) increased significantly the number of affected litters and decreased significantly the mean fetal body weight (Table IV.4). The mean litter frequency of malformed fetuses increased as the dose increased (Table IV.4). The ED_{50} for Ro 12-4825 was 9.1 mg/kg (8.5-9.7). The dose-response curve slope was 7.2 (5.9-8.5), and the probability that the curve was parallel with that for all-trans-RA was p=0.054. The potency ratio for the teratogenic dose-response curve of Ro 12-4825 to that of all-trans-RA was 1.2 (1.1-1.3).

Intubation of the retinoid SRI 2712-24 was associated with a significant teratogenic response in dams treated with 5.25 mg/kg or greater (Table IV.5). The highest dose of SRI 2712-24 (21 mg/kg) also induced a significant increase in the number of resorbed conception sites (Table IV.5). The mean litter frequency of malformed fetuses increased with increasing doses of SRI 2712-24 (Table IV.5). The teratogenic ED_{50} for SRI 2712-24 was 6.1 mg/kg (5.7-6.5). The slope of significantly parallel dose-response curve (p=0.00011) was 6.1 (5.7 - 6.5). The potency ratio for induction of terata by SRI 2712-24 versus that by all-trans-RA was 1.7 (1.6-1.9).

Intubation of 0.53 mg/kg or higher of Ro 10-1770 increased significantly the number of affected litters compared to vehicle-treated animals (Table IV.6). The mean fetal body weight of fetuses recovered from 1.1 and 2.7 mg/kg-treated dams was decreased significantly (Table IV.6). The mean litter frequency of malformed fetuses increased in a dose-dependent fashion (Table IV.6). The teratogenic ED_{50} for Ro 10-1770 was 0.55 mg/kg (0.51-0.58); Ro 10-1770 was 19.3 times (17.6-21.1) as potent a teratogen as all-trans-RA. The slope of the significantly parallel dose-response curve (p=0.0036) was 6.5 (5.5-7.4). Again, as with other retinoids in this series, although there were severe congenital malformations at doses of 1.1 or 2.7 mg/kg, the exposure to the

TABLE IV.4

TERATOGENIC ACTIVITY OF Ro 12-4825

	Dose (mg/kg)		
	4.9	9.8	20
No. animals treated	7	10	6
No. litters	7	8	6
No. affected litters	3	7*	6*
No. implants	107	127	87
No. resorbed (%)	1 (0.9)	4 (3.1)	4 (4.6)
No. dead fetuses (%)	0 (0)	0 (0)	1 (1.1)
No. fetuses examined	106	123	82
No. abnormal fetuses (%)	6 (5.7)	67 (54.5)	82 (100)
Mean litter frequency of malformed fetuses	0.86	8.9	13.7
Mean fetal body weight (g±SD)	1.14±0.09	1.09±0.05*	1.05±0.07*
Mean maternal weight change (g±SD)	14.7±3.3	17.9±3.3	16.2±5.3
Malformations			
Exencephaly	0	0	6
Microcephaly	0	1	34
Encephalocele	0	1	6
Arnold-Chiari malformation types I or II	0	0	6
Microstomia	0	8	30
Macrostomia	3	34	29
Palpebral aplasia with exophthalmia	0	18	54
Rib fusion	3	16	27
Protruding tongue	0	5	33
Maxillary retrocession	0	9	24
Cystic rachischisis	0	0	15
Low-set, hypoplastic, malformed, or absent pinnae	0	30	81
Hypoplastic tail	0	0	1
Anal atresia	0	0	5
Lordotic skull base	0	0	6
Hypoplastic kidney	0	0	2

*Significantly different ($p<0.05$) compared to vehicle control.

TABLE IV.5

TERATOGENIC ACTIVITY OF SRI 2712-24

	Dose (mg/kg)			
	2.6	5.3	10.5	21
No. animals treated	10	10	11	11
No. litters	10	10	11	10
No. affected litters	4	10*	11*	10*
No. implants	119	107	147	129
No. resorbed (%)	2 (1.7)	0 (0)	6 (4.1)	10* (7.8)
No. dead fetuses (%)	1 (0.8)	1 (0.9)	0 (0)	0 (0)
No. fetuses examined	116	106	141	119
No. abnormal fetuses (%)	3 (2.6)	43 (40.6)	128 (90.8)	116 (97.5)
Mean litter frequency of malformed fetuses	0.30	4.3	11.6	11.6
Mean fetal body weight (g±SD)	1.22±0.11	1.25±0.11	1.29±0.08	1.15±0.08
Mean maternal weight change (g±SD)	13.2±4.2	15.8±5.2	15.8±8.2	20.2±7.7
Malformations				
Arnold-Chiari malformation types I or II	0	0	0	4
Microcephaly	0	0	0	21
Exencephaly	0	0	1	12
Encephalocele	0	0	1	9
Macrostomia	1	38	128	43
Maxillary retrocession	1	3	10	33
Rib fusion	1	3	11	49
Microstomia	0	0	0	47
Protruding tongue	0	0	0	36
Cystic rachischisis	0	0	0	2
Lordotic skull base	0	0	0	4
Hypoplastic kidney	0	0	0	2
Low-set, hypoplastic, or absent pinnae	0	5	65	106
Palpebral aplasia with exophthalmia	0	0	0	71
Anophthalmia	0	0	0	2
Hypoplastic tail	0	0	0	3
Gastroschisis	0	0	0	4

*Significantly different ($p<0.05$) compared to vehicle control.

TABLE IV.6

TERATOGENIC ACTIVITY OF Ro 10-1770

	Dose (mg/kg)			
	0.3	0.5	1.1	2.7
No. animals treated	10	10	12	10
No. litters	9	10	12	10
No. affected litters	3	9*	12*	10*
No. implants	115	120	161	128
No. resorbed (%)	3 (2.6)	5 (4.2)	5 (3.1)	4 (3.1)
No. dead fetuses (%)	0 (0)	2 (1.7)	2 (1.2)	0 (0)
No. fetuses examined	113	113	154	124
No. abnormal fetuses (%)	12 (10.6)	33 (29.2)	153 (99.3)	124(100)
Mean litter frequency of malformed fetuses	1.33	3.2	12.7	12.4
Mean fetal body weight (g±SD)	1.32±0.05	1.26±0.11	1.13±0.09*	1.09±0.09*
Mean maternal weight change (g±SD)	12.4±3.4	13.0±6.3	13.6±5.5	19.6±3.4
Malformations				
Micrognathia or agnathia[a]	0	0	0	38
Microcephaly	0	0	8	71
Exencephaly	0	0	5	14
Encephalocele	0	0	18	9
Arnold-Chiari malformation types I or II	0	0	0	5
Cystic rachischisis	0	0	21	86
Macrostomia	8	17	115	32
Maxillary retrocession	0	3	63	32
Rachischisis aperta	0	0	2	1
Rib fusion	4	12	56	66
Microstomia	0	0	11	54
Protruding tongue	0	0	14	70
Lordotic skull base	0	0	0	5
Low-set, hypoplastic, or absent pinnae	0	2	130	121
Palpebral aplasia with exophthalmia	0	0	77	115
Anophthalmia	0	0	1	1
Anal atresia	0	0	0	109
Gastroschisis or omphalocele	0	0	0	6
General Edema	0	0	0	1
Aplastic or hypoplastic tail	0	0	8	102
Hypoplastic or aplastic kidney	0	0	1	13

*Significantly different ($p<0.05$) compared to vehicle control.
[a]Includes cleft mandible.

highly teratogenic retinoid failed to cause a marked increase in embryonic or fetal deaths as compared to the control.

Intubation of Ro 8-7699 induced a significant increase in the number of affected litters at doses of 11 mg/kg or higher (Table IV.7). The mean fetal body weight in the 5.5 mg/kg dose group was elevated significantly compared with vehicle-treated controls, whereas treatment with 52 mg/kg resulted in a significant decrease. As the dose of Ro 8-7699 was increased from 5.5 to 39 mg/kg, there was an increase in the percentage of total fetuses considered malformed. The percentage of malformed fetuses remained at 99-100 at the higher dose levels. Mean maternal body weight change was increased significantly in the 26 and 52 mg/kg-treatment group (Table IV.7). The ED50 for Ro 8-7699 was 11.0 mg/kg (10.2-12.0). The slope of the dose-response curve was 6.9 (5.7-8.2) and was significantly parallel (p=0.0085) with that for all-trans-RA. Ro 8-7699 was 1.2 (1.1-1.3) times less potent a teratogen than all-trans-RA. At the doses studied, there was an inconsistent rate of embryonic death. As the dose was increased from 5.5 to 52 mg/kg, the resorption percentage increased, but at 79 mg/kg the percentage of resorbed embryos decreased to values nearly equivalent to that of the vehicle control.

Juvenile hormone III was teratogenically inactive at doses of 33 and 67 mg/kg (Table IV.8), doses equimolar to 39 and 75 mg/kg of all-trans-RA, respectively. None of the recovered litters exposed to juvenile hormone III was affected, with only one resorption in one litter occurring in the 33 mg/kg-dose group (Table IV.8).

Intubation of 1.6 mg/kg or more of the chlorodimethylmethoxyphenyl analog (Ro 12-0995) increased significantly the number of affected litters (Table IV.9). Litters recovered from dams treated with 1.6, 3.1 and 6.2 mg/kg of Ro 12-0995 had corresponding malformation rates of 40.2, 88.5, and 98.6

TABLE IV.7

TERATOGENIC ACTIVITY OF Ro 8-7699

	Dose (mg/kg)					
	5.5	11	26	39	52	79
No. animals treated	9	8	9	11	9	9
No. litters	7	7	7	9	8	9
No. affected litters	1	7*	7*	9*	8*	9*
No. implants	83	94	80	98	96	109
No. resorbed (%)	1 (1.2)	6 (6.5)	6 (7.5)	8* (8.16)	32* (33.0)	3 (2.8)
No. dead fetuses (%)	1 (1.2)	1 (1.1)	2 (2.5)	5 (5.1)	5 (5.2)	1 (0.9)
No. fetuses examined	81	87	72	85	59	105
No. abnormal fetuses (%)	1 (2.3)	49 (56.3)	69 (96.0)	85 (100)	59 (100)	104 (99)
Mean litter frequency of malformed fetuses	0.14	7.0	9.9	9.4	7.4	11.8
Mean fetal body weight (g±SD)	1.42±0.11*	1.21±0.07	1.19±0.13	1.14±0.12	0.99±0.22*	1.20±0.18
Mean maternal weight change (g±SD)	14.3±4.0	19.6±4.0	22.4±2.8*	18.6±4.4	23.7±5.1*	15.6±5.4
Malformations						
Micrognathia or agnathia[a]	0	0	15	22	40	0
Microcephaly	0	0	16	57	48	68
Arnold-Chiari malformation types I or II	0	0	3	18	9	24
Encephalocele	0	0	2	9	1	2
Exencephaly	0	2	2	3	10	6
Cystic rachischisis	0	2	33	78	47	63
Rachischisis aperta	0	0	1	5	0	0
Microstomia	0	0	2	24	13	50
Macrostomia	0	32	18	22	6	13
Maxillary retrocession	0	18	38	14	4	47
Gastroschisis or omphalocele	0	0	0	5	4	3
Cleft palate	0	0	0	0	0	2
Rib fusion	1	10	26	42	33	48
Palpebral aplasia with exophthalmia	0	8	49	76	56	83

TABLE IV.7 (CON'T)

TERATOGENIC ACTIVITY OF Ro 8-7699

	Dose (mg/kg)					
	5.5	11	26	39	52	79
Anophthalmia or microphthalmia	0	0	0	0	1	0
Microglossia	0	0	0	0	9	0
Protruding tongue	0	1	8	20	10	68
Aplastic or hypoplastic tail	0	0	18	75	52	82
Anal atresia	0	0	21	79	50	90
Lordotic skull base	0	0	3	18	9	24
Horseshoe kidney	0	0	0	0	0	3
Hypoplastic kidney	0	0	0	7	0	9
Renal agenesis	0	0	4	9	11	6
Low-set, hypoplastic, or absent pinnae	0	6	43	82	57	58

*Significantly different ($p<0.05$) compared to vehicle control.
[a]Includes cleft mandible.

TABLE IV.8

TERATOGENIC ACTIVITY OF JUVENILE HORMONE III

	Dose (mg/kg)	
	33	67
No. animals treated	4	6
No. litters	4	6
No. affected litters	0	0
No. implants	54	77
No. resorbed (%)	1 (1.8)	0 (0)
No. dead fetuses (%)	0 (0)	0 (0)
No. fetuses examined	53	77
No. abnormal fetuses (%)	0 (0)	0 (0)
Mean litter frequency of malformed fetuses	0	0
Mean fetal body weight (g±SD)	1.36±0.04	1.31±0.06
Mean maternal weight change (g±SD)	11.5±3.3	12.9±1.4

TABLE IV.9

TERATOGENIC ACTIVITY OF Ro 12-0995

	Dose (mg/kg)			
	0.8	1.6	3.1	6.2
No. animals treated	12	12	10	10
No. litters	8	8	9	6
No. affected litters	3	7*	9*	6*
No. implants	100	101	132	75
No. resorbed (%)	2 (2.0)	4 (4.0)	1 (0.8)	1 (1.3)
No. dead fetuses (%)	1 (1.0)	0 (0)	0 (0)	0 (0)
No. fetuses examined	97	97	131	74
No. abnormal fetuses (%)	4 (4.1)	39 (40.2)	116 (88.5)	73 (98.6)
Mean litter frequency of malformed fetuses	0.50	4.8	12.9	12.2
Mean fetal body weight (g±SD)	1.32±0.10	1.29±0.08	1.21±0.10	1.22±0.10
Mean maternal weight change (g±SD)	18.1±7.9	16.1±3.9	18.1±2.6	15.9±5.4
Malformations				
Microcephaly	0	0	0	21
Microstomia	0	6	0	5
Macrostomia	0	26	108	67
Maxillary retrocession	3	5	9	62
Palpebral aplasia with exophthalmia	0	0	41	67
Rib fusion	1	1	7	2
Low-set, hypoplastic, or absent pinnae	0	0	1	33

*Significantly different ($p<0.05$) compared to vehicle control.

percent (Table IV.9). The ED50 for Ro 12-0995 was 1.8 mg/kg (1.7-1.9), and the slope of the dose response curve was 5.7 (4.9-6.6). The dose-response curve for Ro 12-0995 was significantly parallel with the of all-trans-RA (p=0.0001). Ro 12-0995 was 5.9 (5.3-6.5) times more potent a teratogen than all-trans-RA on a mg/kg basis. Intubation of Ro 12-0995 was, however, not embryolethal; even at the highest dose studied wherein nearly 99 percent of the fetuses were afflicted with obvious, severe malformations, only a 1.3 percent resorption rate was observed. This was nearly identical to the embryonic death rate of 1.8 percent in the vehicle control group (Table IV.3).

Doses of 85 mg/kg or more of the 4-hydroxy congener of etretinate (Ro 11-4768) increased significantly the number of affected litters (Table IV.10). The numbers of resorptions were elevated significantly at the 113 and 170 mg/kg-dose levels. Fetuses recovered from dams treated with 113 or 170 mg/kg of Ro 11-4768 had corresponding malformation rates of 85.7 and 95.0 percent (Table IV.10). Because the mean litter frequency of malformed fetuses was very similar at the 57 and 85 mg/kg-doses (Table IV.10), the 85 mg/kg data were omitted, and the ED50 of Ro 11-4768 was calculated as 87.3 mg/kg (80.8-93.9). Because of the similar teratogenic response rate at the 57 and 85 mg/kg-dose levels, the dose-response curve of Ro 11-4768 was not considered as significantly parallel (p=0.068) with the dose-response curve for all-trans-RA. The slope of the dose-response curve for Ro 11-4769 was 7.3 (6.1-8.5). On a mg/kg basis Ro 11-4768 was 8.2 times (7.5-9.1) less potent than all-trans-RA.

Oral intubation of 32 mg/kg or more of the phenyl analog Ro 8-8717 elevated significantly the number of affected litters and the number of resorptions. Doses of 32, 64, and 128 mg/kg were associated with resorption rates of 38, 79, and 100 percent, respectively (Table IV.11). However, at doses

TABLE IV.10

TERATOGENIC ACTIVITY OF Ro 11-4768

	Dose (mg/kg)				
	28	57	85	113	170
No. animals treated	8	8	11	9	9
No. litters	8	8	7	8	9
No. affected litters	1	3	5*	8*	9*
No. implants	73	87	79	74	98
No. resorbed (%)	0 (0)	4 (4.6)	3 (3.9)	11* (14.9)	8* (8.2)
No. dead fetuses (%)	0 (0)	0 (0)	0 (0)	0 (0)	2 (2.0)
No. fetuses examined	73	83	76	63	88
No. abnormal fetuses (%)	1 (1.3)	5 (6.0)	4 (5.2)	54 (85.7)	84 (95.0)
Mean litter frequency of malformed fetuses	0.13	0.63	0.57	6.4	9.3
Mean fetal body weight (g±SD)	1.24±0.09	1.26±0.13	1.14±0.01	1.20±0.11	1.20±0.21
Mean maternal weight change (g±SD)	15.1±2.5	14.5±5.3	16.3±2.7	16.2±2.2	18.7±5.4
Malformations					
Micrognathia or agnathia	0	0	0	3	27
Microcephaly	0	0	0	9	34
Arnold-Chiari malformation types I or II	0	0	0	3	3
Encephalocele	0	0	0	2	1
Exencephaly	0	0	0	0	7
Cystic rachischisis	0	0	0	0	44

TABLE IV.10 (CON'T)

TERATOGENIC ACTIVITY OF Ro 11-4768

	Dose (mg/kg)				
	28	57	85	113	170
Rachischisis aperta	0	0	0	10	3
Microstomia	0	0	0	3	12
Macrostomia	0	3	1	31	34
Maxillary retrocession	1	1	1	30	34
Gastroschisis or omphalocele	0	0	0	0	2
Rib fusion	0	2	3	11	27
Palpebral aplasia with exophthalmia	0	1	0	49	58
Anophthalmia/microphthalmia	0	0	0	1	1
Protruding tongue	0	0	0	7	9
Microglossia	0	0	0	0	3
Aplastic or hypoplastic tail	0	0	0	0	39
Anal atresia	0	0	0	0	40
Lordotic skull base	0	0	0	3	3
Renal agenesis	0	0	0	0	12
Hypoplastic kidney	0	0	0	0	1
Low-set, hypoplastic or absent pinnae	0	0	0	5	41

*Significantly different ($p<0.05$) compared to vehicle control.

TABLE IV.11

TERATOGENIC ACTIVITY OF Ro 8-8717

	Dose (mg/kg)		
	32	64	128
No. animals treated	14	7	6
No. litters	12	6	5
No. affected litters	8*	6*	5*
No. implants	171	67	59
No. resorbed (%)	65* (38.0)	53* (79.1)	59* (100)
No. dead fetuses (%)	2 (1.2)	0 (0)	0 (0)
No. fetuses examined	104	14	0
No. abnormal fetuses (%)	5 (4.8)	1 (7.1)	0 (0)
Mean litter frequency of malformed fetuses	0.42	0.17	0
Mean fetal body weight (g±SD)	1.15±0.11	1.03±0.12*	-
Mean maternal weight change (g±SD)	21.1±6.0*	18.7±1.9	25.2±4.1*
Malformations			
Omphalocele	0	1	-
Rib fusion	4	0	-
Anophthalmia	1	0	-

*Significantly different ($p<0.05$) compared to vehicle control.

of 32 and 64 mg/kg only 4.8 and 7.1 percent, respectively, of the recovered fetuses were malformed. The mean fetal body weight of fetuses recovered from dams treated with 64 mg/kg of Ro 8-8717 was decreased significantly. The mean maternal weight change of dams treated with 32 or 128 mg/kg of Ro 8-8717 was increased significantly (Table IV.11). The embryonic LD50 for Ro 8-8717 was 36.7 mg/kg (35.6-41.0; 95% confidence interval). The slope of the dose-response curve was 2.6 (2.1 - 3.0). Ro 8-8717 was 1.5 (1.3 - 1.8) times as embryolethal as all-trans-RA. The dose-response curve for embryolethality of Ro 8-8717 was significantly parallel with the embryonic LD50 curve of all-trans-RA (p=0.001).

A dose of 61 mg/kg of the furyl analog Ro 8-9750 (equimolar to 75 mg/kg all-trans-RA) neither induced the characteristic syndrome of malformations observed with retinoid treatment nor induced a significant increase in the number of litters containing abnormal fetuses (Table IV.12). All of the recovered fetuses were considered normal. Low rates of embryonic death were associated with intubation of the furyl retinoid analog.

Intubation of 83 or 166 mg/kg of Ro 21-6667, doses respectively equimolar to 75 and 150 mg/kg of all-trans-RA, failed to induce a teratogenic response (Table IV.13). All of the recovered fetuses from both dose groups were normal. The number of resorptions observed was near that observed in the control group with one and two resorptions being recorded in the 83 and 166 mg/kg-dose groups, respectively (Table IV.13).

Intubation of up to 177 mg/kg of motretinid (Ro 11-1430), the ethyl amide congener of etretinate (Ro 10-9359), failed to induce a significant teratogenic response, however at a dose of 350 mg/kg such a response did occur with five of the seven litters being affected (Table IV.14). The

TABLE IV.12

TERATOGENIC ACTIVITY OF Ro 8-9750

	Dose (mg/kg)
	61
No. animals treated	7
No. litters	4
No. affected litters	0
No. implants	42
No. resorbed (%)	2 (4.8)
No. fetuses examined	40
No. abnormal fetuses (%)	0 (0)
Mean litter frequency of malformed fetuses	0
Mean fetal body weight (g±SD)	1.25±0.08
Mean maternal weight change (g±SD)	14.9±5.1

TABLE IV.13

TERATOGENIC ACTIVITY OF Ro 21-6667

	Dose (mg/kg)	
	83	166
No. animals treated	8	3
No. litters	7	3
No. affected litters	0	0
No. implants	73	46
No. resorbed (%)	1 (1.4)	2 (4.3)
No. dead fetuses (%)	0 (0)	0 (0)
No. fetuses examined	72	44
No. abnormal fetuses (%)	0 (0)	0
Mean litter frequency of malformed fetuses	0	0
Mean fetal body weight (g±SD)	1.27±0.10	1.19±0.16
Mean maternal weight change (g±SD)	17.6±3.8	14.5±3.1

TABLE IV.14

TERATOGENIC ACTIVITY OF Ro 11-1430

	Dose (mg/kg)		
	88	177	350
No. animals treated	7	5	7
No. litters	6	3	7
No. affected litters	0	1	5*
No. implants	55	27	76
No. resorbed (%)	0 (0)	0 (0)	2 (2.6)
No. dead fetuses (%)	0 (0)	0 (0)	0 (0)
No. fetuses examined	55	22	74
No. abnormal fetuses (%)	0 (0)	1 (4.5)	14 (18.4)
Mean litter frequency of malformed fetuses	0	0.25	2.0
Mean fetal body weight (g±SD)	1.17±0.07	1.09±0.15	1.11±0.11
Mean maternal weight change (g±SD)	14.2±9.6	12.8±1.8	10.5±6.2
Malformations			
Encephalocele	0	1	0
Macrostomia	0	0	9
Maxillary retrocession	0	0	6
Rib fusion	0	0	5

*Significantly different ($p<0.05$) compared to vehicle control.

malformations included macrostomia, maxillary retrocession, and fused ribs (Table IV.14).

Each retinoid associated with a significant teratogenic response induced an identical syndrome of malformations that were characteristic of and indistinguishable from that induced by oral intubation of all-trans-RA. At lower doses (i.e., doses that induce ca. 50 percent malformed fetuses) these malformations consisted of macro- or microstomia, palpebral aplasia with exophthalmia and fused ribs. In general, as the retinoid dose was increased, the number of malformed fetuses, number of malformations per fetus, and the severity of malformations increased (e.g., Table IV.7). At higher doses (i.e., doses that induce 100 percent malformed fetuses) the more severe craniofacial malformations and malformations of the central nervous system predominated, such as microcephaly, exencephaly, and the Arnold-Chiari malformation. At these high doses the ears were frequently low-set, malformed, or absent with an atretic or stenotic external auditory meatus. The characteristic caudal regression syndrome composed of cystic rachischisis, rachischisis aperta, a hypoplastic or aplastic tail, and anal atresia was routinely observed in fetuses recovered from dams treated with retinoid doses that induced a high percentage of malformed fetuses.

DISCUSSION

The present results demonstrate that major modifications of the natural β-cyclogeranylidene (2,6,6-trimethyl-1-cyclohexenyl) ring of the retinoid skeleton can be made without eliminating teratogenic potency, as was previously found on studies with the norbornyl and cyclohexyl ring analogs (Willhite et al., 1984). In the present study, two different classes of ring modifications, polar substituent changes on the ring of all-trans-RA (Ro 12-

4824, Ro 12-4825, Ro 10-1770, Ro 8-7699, Ro 8-9750, SRI 2712-24, and juvenile hormone III) and modifications of the trimethylmethoxyphenyl ring of etretinate (Ro 8-8717, Ro 11-1430, Ro 11-4768, Ro 21-6667, Ro 12-0995), were investigated. Major perturbations in ring structure do not have the corresponding effect on teratogenic potency that similar modifications in the retinoid polar terminus (Willhite et al., 1984) or polyene chain (Willhite, 1986) have.

all-trans-4-Oxoretinoic acid (Ro 12-4824), a metabolite of orally administered all-trans-RA in hamsters (Frolik et al., 1979;1980; Frolik, 1981), exhibited teratogenic potency on a molar basis (ED50= 30 μmole/kg) that was essentially equivalent to that of all-trans-RA (ED50= 35 μmole/kg) (Willhite and Shealy, 1984). This ring modification, which is the same as that found in that natural carotenoid cathaxanthin, is indicative of metabolic deactivation (Frolik et al., 1979;1980; Frolik, 1981), according to the present results the metabolite is equitoxic to the parent acid. At present it is unknown to what extent the oxidized, teratogenic metabolites contribute to the embryotoxicity of all-trans-RA and 13-cis-retinoic acid (13-cis-RA, isotretinoin). These observations are consistent with those of Goulding and Pratt (1986), Webster et al. (1986), and Kochhar and Penner (1987), who found the 4-oxo metabolite of 13-cis-RA equipotent to 13-cis-RA in intact pregnant ICR or C57 B1/6J mice, cultured CD-1 mouse embryos, cultured CFHB rat embryos, and cultured ICR mouse forelimb buds. 4-Oxoretinoic acid was nearly as active as all-trans-RA in the hamster tracheal organ culture assay (4-oxoretinoic acid ED50 = 700 pM; all-trans-RA ED50 = 30 pM) (Newton et al., 1980). Introduction of either a hydroxyl group (Ro 12-4825) or a methyl group (SRI 2712-24) on the methyl group at the 2-position of the trimethylcyclohexenyl ring failed to markedly alter teratogenic potency as compared to all-trans-RA. Replacement of

trimethylcyclohexenyl ring with a 5,5-dimethyl-1-cyclopentenyl ring having a 1-methoxyethyl group (Ro 10-1770) or an acetyl group (Ro 8-7699) at the 2-position was associated with significant teratogenic activity. In fact, Ro 10-1770 was 19 times more potent a teratogen than all-trans-RA, and Ro 8-7699 had activity comparable to all-trans-RA. Ro 10-1770 was almost as active as all-trans-RA in the hamster tracheal organ culture assay (Sporn et al., 1975; Newton et al., 1980) and was at least as effective in binding to chick embryo skin cellular retinoic acid-binding protein (cRABP) (Sani et al., 1978). Ro 8-7699 induced embryonic chick tarsometatarsal ptilopody at the same concentrations as all-trans-RA (Cadi et al., 1984), was more potent than all-trans-RA in the fetal rat bone culture (Kistler, 1981), and possessed greater affinity for chick skin cRABP (Sani and Hill, 1974;1976). Its ester derivative induced regression of mouse skin papillomas and had a more favorable therapeutic ratio than that of all-trans-RA (Mayer et al., 1978). Therefore, polar groups can be introduced at positions corresponding to the 2- and 3-positions of the cyclohexenyl ring without loss of teratogenic potency.

The last compound in this series that was examined was juvenile hormone III, which only retains three carbons of the cyclohexene ring and the methyl at the 2-position. In addition, the ring double bond has been replaced by a oxyrane ring and the bonds corresponding to the 7,8- and 9,10-double bonds of the retinoid side chain have been saturated. This compound was not teratogenic in hamsters, just as it was inactive in the hamster tracheal organ culture assay (Newton et al., 1978).

As early as 1960, Morton suggested that retinoid analogs having a hydroxylated polyene chain or ring would be useful compounds. Replacement of the trimethylcyclohexenyl ring of ethyl all-trans-retinoate with a 2,3,6-trimethyl-4-methoxyphenyl ring affords the dermatological agent etretinate (Ro

10-9359), a human (Happle et al., 1984; Grote et al., 1985) and hamster (Williams et al., 1984) teratogen that is twice as potent as all-trans-RA. Replacement of the 2-methyl group with a smaller, electron-withdrawing chloro group afforded Ro 12-0995, which was six times more potent as a teratogen in hamsters than all-trans-RA and at least three times more potent than etretinate. In contrast, Ro 12-0995 had low activity in inducing differentiation of HL-60 leukemia and RDFD myeloblast cells, was only a poor inducer of cAMP-dependent protein kinase activity (Fontana et al., 1986), and was relatively inactive in the hamster tracheal organ culture assay (Newton et al., 1978). It was only somewhat active in causing the regression of mouse epidermal papillomas and had a therapeutic ratio in mice that was ten-fold lower than that of all-trans-RA (Mayer et al., 1978). Replacement of the methoxy group on the ring with the more polar hydroxyl (Ro 11-4768) decreased teratogenic potency 15-fold compared with etretinate (Williams et al., 1984). This phenol was inactive in the hamster tracheal organ culture assay (Newton et al., 1980).

Removal of the substituents from the phenyl ring afforded Ro 8-8717, which was embryolethal, being approximately 1.5 times as toxic as all-trans-RA, but did not induce the characteristic teratogenic syndrome in fetal hamsters observed for teratogenic retinoids (Table IV.11). Ro 8-8717 was a poor ligand for chick embryo skin cRABP (Sani and Hill, 1976), was not a ligand for embryonal carcinoma cells (Jetten et al., 1982), failed to induce differentiation of embryonal carcinoma cells, and had only marginal activity in the hamster tracheal organ culture allay (Newton et al., 1980). The related furan (Ro 8-9750) was not teratogenic (Table IV.12). It was also inactive in the hamster tracheal organ culture (Newton et al., 1980), showed 10 percent of the activity of all-trans-RA in embryonal chick skin (Wilkoff et al., 1976),

but could compete effectively for the retinoic acid-binding site of chick embryo skin cRABP (Sani and Hill, 1976). The results on Ro 8-8717 and Ro 8-9750 indicate that the substituents on the aromatic ring are required for activity.

Two other retinoids in this series were also examined. The analog of etretin having a thiomethylene group on the side chain in place of the trans 8,9-double bond (Ro 21-6667) did not show teratogenic effects up to a dose of 166 mg/kg. In contrast, 7,8-dihydroretinoic acid exhibited significant teratogenic activity (Willhite, 1986). Therefore, the double bond adjacent to the aromatic ring does not appear necessary for activity because saturation, which increases rotation about the bond and disrupts π-electron delocalization across the retinoid, did not abolish activity. It is more likely that rapid metabolism of the thio group afford more polar, inactive species. This compound was also inactive in the hamster tracheal organ culture assay (Newton et al., 1978) and was a poor ligand for chick embryo cRABP (Sani et al., 1978).

The N-ethyl amide (Ro 11-1430, motretinid) of etretin, which is the major teratogenic metabolite of etretinate (Kistler and Hummler, 1985), was inactive as a teratogen at a dose of 177 mg/kg, however an extremely large dose (350 mg/kg) was teratogenic. This same paradigm was observed for other retinamides including the N-ethyl and N-(2-hydroxyethyl) amides of all-trans-RA and 13-cis-RA (Willhite and Shealy, 1984). The significant teratogenic response of motretinid (Table IV.14) at the large dose is consistent with the suggestion (Kochhar et al., 1987) that after a very large dose the limited in situ biotransformation of the amide to the acid assumes toxicological significance. These results contrast with those of Kochhar et al. (1987), who reported that a single oral 100 mg/kg dose of motretinid to pregnant ICR and

NMRI mice induced a 35% and 70% frequency, respectively, of cleft palate. The present results in hamsters agree with other findings of low activity or inactivity for the amide such as those of Kistler (1981) of inactivity in fetal rat bone culture and of Zimmerman and Tsambaos (1985) in embryonic mouse limb bud mesenchymal cell cultures. The amide and its ethyl ester congener did not suppress chondrogenesis in cultured ICR mouse limb buds, whereas the free acid, as well as all-trans-RA and 13-cis-RA, at a dose of 50 ng/ml did (Kochhar et al., 1987). The amide was ineffective against experimental mammary or urinary cancer in rats (Moon et al., 1983). In other assay systems, motretinid displayed biological activity and low toxicity. It inhibited radiation-induced oncogenic transformation (Harisiadis et al., 1978), enhanced cell-mediated immunity (Athanassiades, 1981), and induced the regression of carcinomas and established papillomas in mice (Bollag, 1975; Bollag and Matter, 1981). It was reported to have a more favorable therapeutic ratio than all-trans-RA (Mayer et al., 1978), was as active as etretinate in reversing epithelial cell keratinization in hamster tracheal organ culture (Newton et al., 1978), and was active in the embryonal chick skin bioassay (Wilkoff et al., 1976).

Motretinid is clinically available in Europe (Cunningham and Ehmann, 1983) for the control of cystic acne as an alternative to systemic administration of the human teratogen 13-cis-RA (Lammer et al., 1985). It is effective when applied topically against ichthyosis vulgaris (Berger and Tsambaos, 1981) and against papular and pustular acne vulgaris as a 0.1% solution having less skin irritation than topical all-trans-RA (Scherrer and Ott, 1976). This ethyl amide congener may present a larger, and hence more favorable, therapeutic ratio for dermatologic purposes than other currently

available retinoids when the high risk of toxicity in women of childbearing age is considered (Lammer et al., 1985).

When the present results in hamsters are compared with those in intact chick embryos, cultured embryonal cells, the hamster tracheal organ culture assay, and cell-free systems with their known or anticipated metabolic products, consistent patterns emerged. The hydrophobic head of the retinoid skeleton can assume either a twist chair or aromatic planar conformation and retain teratogenic activity providing sufficient hydrophobic substituents are present on the ring. These substituents may reside either out of the plane of the ring as in the case of the cyclohexenyl ring-bearing retinoid or in the plane of the aromatic ring as in the case of the etretin congeners. Polar groups such as methoxy, hydroxy, and keto may be substituents on either the ring or on carbons adjacent to the ring without loss of teratogenic activity. Thus, retinoid teratogenicity is dependent upon not only a polar terminal group having an acidic pK_a and uninterrupted π-electron delocalization across a molecule of sufficient length but also on the hydrophobicity of the head. The three-dimensional structure and charge-transfer properties of retinoids have roles in their teratogenic activities (Flanagan et al., 1987). Studies on the contributions of retinoid conformation and the roll of the cellular binding proteins in retinoid teratogenesis are necessary to formulate more accurate structure-activity predictions.

CHAPTER V

COMPARATIVE DISTRIBUTION, PHARMACOKINETICS, AND PLACENTAL PERMEABILITIES OF ALL-TRANS-RETINOIC ACID, 13-CIS-RETINOIC ACID, ALL-TRANS-4-OXO-RETINOIC ACID, 9-CIS-RETINAL, AND RETINYL ACETATE

INTRODUCTION

Certain retinoids are potent human (Lammer et al., 1985; Willhite et al., 1986; Happle et al., 1984) and animal (Howard and Willhite, 1986) teratogens. Various investigators have examined the effect of polar terminus (Willhite et al., 1984; Willhite and Shealy, 1984), side-chain (Willhite, 1986), and cyclohexenyl ring (chapter IV) modifications on teratogenic potency. The differences in teratogenic potency may be related to differences in absorption, elimination, distribution, placental permeability, or to differential binding affinity(ies) with (a) putative intracellular receptor(s).

The pharmacokinetics and distribution of five retinoid analogs in pregnant hamsters were studied here. The retinoids included the reference compound all-trans-retinoic acid (all-trans-RA), the side-chain modified 13-cis-retinoic acid (13-cis-RA), the polar end group modified retinyl acetate, the ring modified all-trans-4-oxoretinoic acid (all-trans-4-oxo-RA), and the side-chain and polar terminus modified 9-cis-retinaldehyde (9-cis-retinal).

MATERIALS AND METHODS

Chemicals

The tritiated retinoids all-trans-[10,11-$^{3}H_{2}$]-retinoic acid (95%, 3.39 Ci/mmole in toluene), 13-cis-[11-^{3}H]-retinoic acid (95.9%; 3.21 Ci/mmole in toluene), all-trans-4-oxo-[11-^{3}H]-retinoic acid (95.2%; 3.1 Ci/mmole in toluene),

9-cis-[11-^{3}H]-retinaldehyde (95.0%; 1.97 Ci/mmol in toluene), and all-trans-[10,11-$^{3}H_2$]-retinal acetate (95.2%; 3.39 Ci/mmol in toluene) were obtained under the courtesy of the Biological and Chemical Prevention Program, Chemical and Physical Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute, NIH, Bethesda, MD. Nonlabeled all-trans-retinoic acid (all-trans-RA), 13-cis-retinoic acid (13-cis-RA), and retinyl acetate were purchased from Kodak, Rochester, NY. all-trans-4-Oxoretinoic acid (Ro12-4824; all-trans-4-oxo-RA), 9-cis-retinaldehyde (9-cis-retinal) and the metabolite of 13-cis-RA, 13-cis-4-oxoretinoic acid (13-cis-4-oxo-RA) were gifts of Hoffmann-La Roche, Inc., Nutley, NJ. Nonradioactive compounds were chromatographed by a previously described system (Willhite and Shealy, 1984); all were greater than 95% pure.

Aliquots of radioactive retinoids (in toluene) were pipetted into a volumetric flask, and the cold retinoid was added and dissolved. The solution was then diluted in Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma Chemical Co., St. Louis, MO), such that the final toluene concentration was 20%. Nonlabeled retinoids were dissolved in a small volume of reagent grade acetone (Fisher Scientific, Pittsburgh, PA) and subsequently dissolved in Tween 20. The final acetone concentration did not exceed five percent.

Animals

Animals were purchased, bred, and housed, as described in chapter III.

Treatment

Two studies were performed. The first study investigated the comparative distribution and placental permeabilities, and the second evaluated comparative pharmacokinetic parameters of the parent retinoids. In the first study (Phase I), six pregnant hamsters were dosed with a single oral intubation of [^{3}H]-all-

trans-RA, and six additional pregnant hamsters were dosed with [^{3}H]-13-cis-RA. Five pregnant hamsters were given [^{3}H]-all-trans-4-oxo-RA, and five received [^{3}H]-retinyl acetate. Four pregnant hamsters received [^{3}H]-9-cis-retinal. Administered doses were equivalent to 10.5 mg/kg all-trans-RA (35 μmol/kg; 35 μCi/animal) in Tween 20 on day eight of pregnancy. The plasma was collected at prespecified intervals (see below) and the radioactivity counted. The dams were killed 96 h after dosing. Tissues were collected, digested, and radioactivity was counted. In the second study (Phase II) nonradiolabeled compound was administered to three animals for each chemical, and plasma was collected up to 24 h after dosing. Plasma was assayed by high performance liquid chromatography to quantify retinoid levels.

Blood and Tissue Collection Schedule

Blood was collected from the post-orbital plexus in heparinized glass hematocrit tubes at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h after administration of the radiolabeled retinoids. The heparinized blood was centrifuged immediately after collection in a microhematocrit centrifuge for 10 min to obtain plasma. The plasma was weighed, placed into scintillation vials, and mixed with 200 μl of 70% aqueous perchloric acid (Fisher Scientific, Pittsburgh, PA). The plasma solution was incubated at 60^{o}C for 4 h. The incubated solution was cooled to room temperature and mixed with 500 μl of 30% hydrogen peroxide (Fisher Scientific). The samples were subsequently incubated at 60^{o}C for 4 h to decolorize. Samples were cooled to room temperature, and 15 ml of scintillation fluid were added (7:3 toluene-scintillation surfactant with 0.28 percent Preblend 2a70, Fisher Scientific, Pittsburgh, PA and Research Products International Corp., Mount Prospect, IL, respectively). Tissues were homogenized with two volumes of distilled water,

and a 150 µl aliquot was taken to be processed as described above. The residual radioactivity was counted in a Beckman LS 3801 scintillation counter. Tritium activity was quantified by using an external standard quench correction mode.

High Performance Liquid Chromatography

In the second phase of the study, whole blood was collected, centrifuged, and weighed as described above. Three hundred ul of HPLC grade methanol (Fisher Scientific) were added to each plasma sample (90-150 µg), the samples purged with argon and stored at -80°C, until chromatographic separations could be performed. Samples were thawed in a dark room at room temperature and then centrifuged at 10,000 x g for 30 min. The supernatant was decanted and filtered through 0.2 µm microfilter (Bioanalytical Systems, West Lafayette, IN) at 1,000 x g for 5 min. The methanol extracted samples were analyzed by HPLC using a Varian Model 5000 chromatograph equipped with a Spherisorb ODS 5-µm column (Universal Scientific, Atlanta, GA) and a Spherex C18 5-cm X 4.6-cm pre-column (Phenomonex, Rancho Palos Verdes, CA). The HPLC grade acetonitrile and water (J. T. Baker, Jackson, TN) were filtered (0.2 µm) and vacuum-degassed prior to use. Two hundred µl of extracted plasma were injected onto the column. The retinoids all-trans-RA, 13-cis-RA, and all-trans-4-oxo-RA were monitored at 365 nm with a flow rate of 1.5 ml/min. The 9-cis-retinal was chromatographed using a flow rate of 1.0 ml/min and monitored at 365 nm. Retinyl acetate was chromatographed using a flow rate of 1.5 ml/min and monitored at 340 nm. Under these respective conditions all-trans-RA, 13-cis-RA, all-trans-4-oxo-RA, and 13-cis-4-oxo-RA had respective retention times of 8.86, 5.91, 3.87, and 3.70 min. Handling of retinoid solutions was done under yellow light. Retinoid levels were quantified using a Hewlett-

Packard model 3390A (Hewlett Packard, Rockville, MD) integrator in peak height mode with an external standard.

Plasma concentration values were fitted to a curve with a nonlinear regression program (Metzler et al., 1974; Sedman and Wagner, 1977) Area under the curve (AUC) was calculated using the trapezoidal method of Giabaldi and Perrier (1982).

RESULTS

Distribution

Ninety-six h after administration of 10.5 mg/kg [^{3}H]-all-trans-RA, radioactivity was distributed equally to all sampled tissues, except perirenal fat (Figure V.1). The perirenal fat contained 0.9 nmol eq./g tissue; whereas, other sampled tissues contained approximately 2.5-3.0 nmol eq./g tissue. The liver had the most radioactivity (3.5 nmol eq./g tissue, Fig. V.1).

The pattern of radioactive distribution 96 h following an oral dose of 10.5 mg/kg [^{3}H]-13-cis-RA closely resembled that of [^{3}H]-all-trans-RA (Fig. V.1). As was the case for [^{3}H]-all-trans-RA, the perirenal fat contained approximately three times less radioactivity than other tissues; the liver contained the most (7.1 nmol eq./g tissue) (Fig. V.1). The amount of radioactivity in tissues, except fat, was greater for ^{3}H-13-cis-RA than for [^{3}H]-all-trans-RA (Fig. V.1).

Radioactive tissue distribution after an equivalent dose (11 mg/kg) of [^{3}H]-all-trans-4-oxo-RA was very similar to the radioactive distribution of the free acids ^{3}H-all-trans-RA and [^{3}H]-13-cis-RA (Fig. V.1). Again, as with the first two free acids, perirenal fat contained the least amount of radioactivity 96 h after dosing. The amount of radioactivity found in tissues after oral

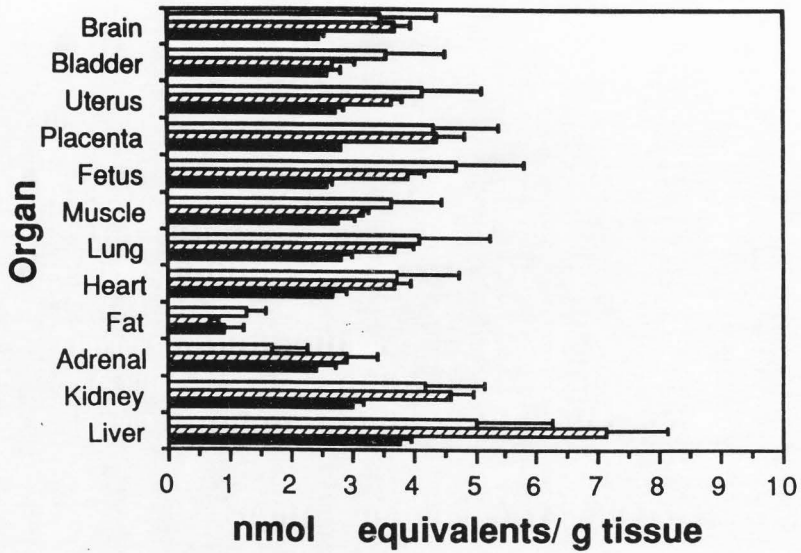


Figure V.1. Distribution of radioactivity in pregnant hamsters following oral administration of [^{3}H]-all-trans-RA, [^{3}H]-13-cis-RA, or [^{3}H]-all-trans-4-oxo-RA. Tissues were sampled 96 h after doses of 10.5 mg/kg 13-cis-RA (▮), 11.0 mg/kg all-trans-4-oxo-RA (▨), or 10.5 mg/kg all-trans-RA (▭). Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue. Bars represent S.E.M.

administration of [^{3}H]-all-trans-RA was consistently lower after administration of an equimolar amount of [^{3}H]-all-trans-4-oxo-RA (Fig. V.1).

In sharp contrast to the distribution of the free acids, [^{3}H]-9-cis-retinal and [^{3}H]-retinyl acetate were distributed in a dissimilar manner. Radioactivity accumulated in the lungs and liver of animals treated with 10.5 mg/kg [^{3}H]-9-cis-retinal; the organs contained 118 and 96.8 nmol eq./g tissue, respectively (Fig. V.2). Remaining tissues also contained radioactivity, but at levels less than 5 nmol eq./g tissue (Fig. V.2). Treatment with 12.1 mg/kg [^{3}H]-retinyl acetate also resulted in the bulk of radioactivity accumulating in the lungs and liver with lesser amounts being distributed to other tissues (Fig. V.3). The lungs and liver of animals treated with [^{3}H]-retinyl acetate had corresponding concentrations of 17.2 and 16.5 nmol eq./g tissue.

Pharmacokinetics

The level of all-trans-RA peaked at 1 h after oral administration with a rapid decline between 2 and 4 h sampling. (Fig. V.4). The apparent elimination half-life for all-trans-RA was estimated to be 0.5 h. The metabolites 13-cis-RA and all-trans-4-oxo-RA were present at 0.5 h after dosing with 10.5 mg/kg all-trans-RA. These metabolites persisted for 12 and 24 h, respectively. Peak plasma concentrations of both metabolites occurred 2 h after dosing. Detectable amounts of all-trans-RA were absent from the plasma after 4 h (limit of detection = 0.16 nmol/g plasma).

The pharmacokinetic parameters of 13-cis-RA are presented in table V.1. The parameters were obtained from nonlinear regression fittings of animal plasma concentration-time values. The $t_{1/2}$ for 13-cis-RA was 4.41 h, and the AUC was 22.38 nmol.h/g. Isotretinoin (13-cis-RA) reached peak plasma concentrations 1 h after dosing (Fig. V.5), followed by exponential elimination.

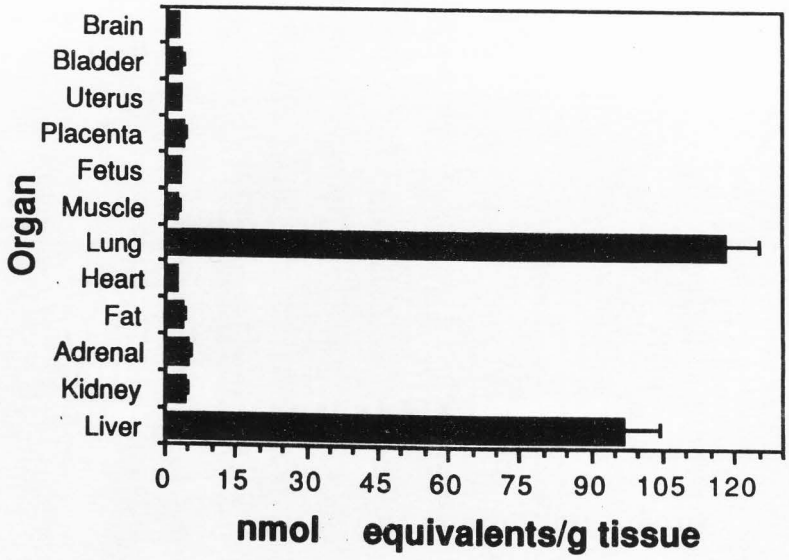


Figure V.2. Distribution of radioactivity 96 h after a single oral dose of 10.5 mg/kg [^{3}H]-9-cis-retinal to day-eight pregnant hamsters. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue. Bars represent S.E.M.

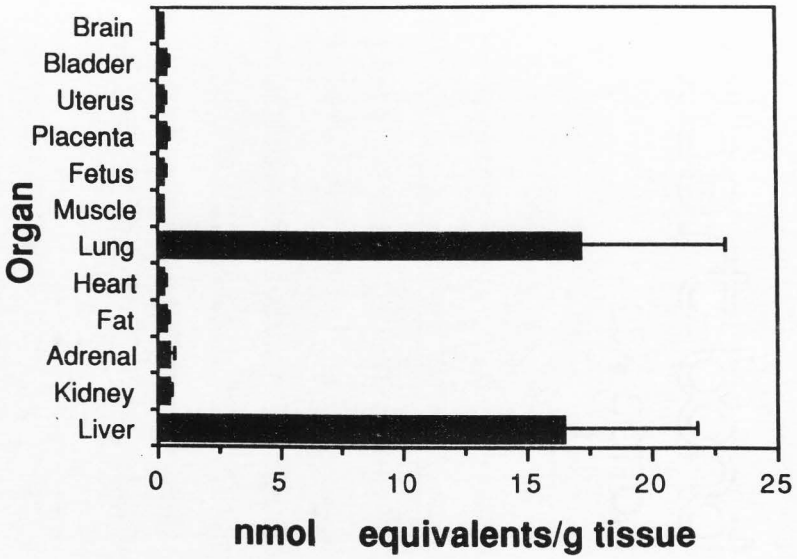


Figure V.3. Distribution of radioactivity 96 h after a single oral dose of 12.1 mg/kg [^{3}H]-retinyl acetate to day-eight pregnant hamsters. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue. Bars represent S.E.M.

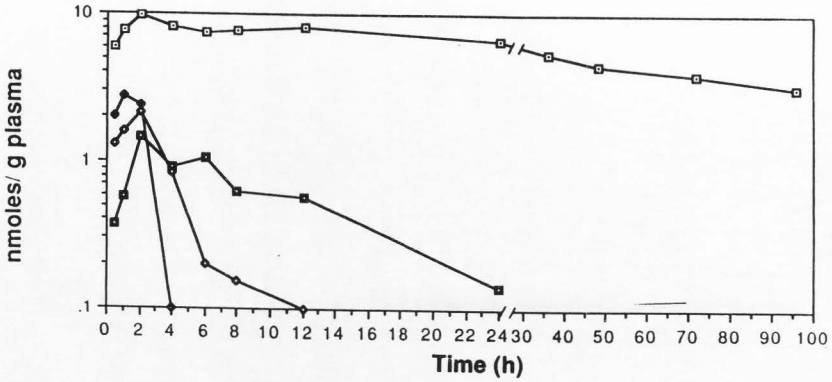


Figure V.4. Plasma concentrations of retinoids following administration of all-trans-RA to day-eight pregnant hamsters. Hamsters were dosed with 10.5 mg/kg all-trans-RA, and plasma was assayed by HPLC for concentrations of all-trans-RA (◆), 13-cis-RA (◆), and all-trans-4-oxo-RA (■). Concentrations at time points greater than 4 and 12 h for all-trans-RA and 13-cis-RA, respectively, were less than the analytical limit of detection (< 0.15 and 0.16 nmol/g, respectively). Each point is the mean value of separate determinations of plasma obtained from three hamsters. Disintegrations per minute were converted to nmol equivalents/ gram plasma for total radioactivity (□) following oral administration of [^{3}H]-all-trans-RA.

TABLE V.1

PHARMACOKINETIC PARAMETERS OF 13-CIS-RA

Parameter	Animal Number			Mean ± SD
	1	2	3	
$t_{1/2}$	3.48	4.35	5.41	4.41 ± 0.97
AUC (nmol.h/g)	20.37	21.86	24.91	22.38 ± 2.31

$t_{1/2}$ = Half-life for rapid elimination phase

AUC = Total area under plasma concentration vs. time curve

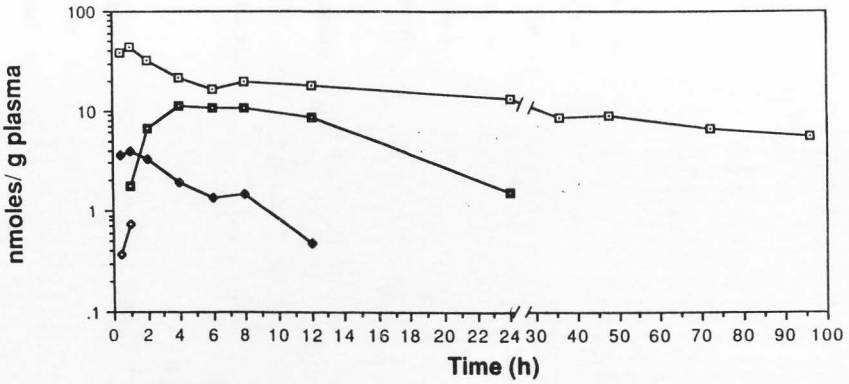


Figure V.5. Plasma concentrations of retinoids following administration of 13-cis-RA to day-eight pregnant hamsters. Hamsters were dosed with 10.5 mg/kg 13-cis-RA, and plasma was assayed by HPLC for concentrations of 13-cis-RA (◆), all-trans-RA (◆), and 13-cis-4-oxo-RA (■). Concentrations at time points greater than 1 and 12 h for all-trans-RA and 13-cis-RA, respectively, were less than the analytical limit of detection (< 0.15 and 0.16 nmol/g, respectively). Each point is the mean value of separate determinations of plasma obtained from three hamsters. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram plasma for total radioactivity (□) following oral administration of [^{3}H]-13-cis-RA.

The parent 13-cis-RA persisted for 12 h, after which it was undetectable (limit of detection = 0.15 nmol/g plasma. Two metabolites, all-trans-RA and 13-cis-4-oxo-RA, were present after a 10.5 mg/kg dose of 13-cis-RA (Fig. V.5). 13-cis-4-Oxo-RA was present in higher concentrations and persisted longer in the plasma than its parent, 13-cis-RA. Low concentrations of all-trans-RA were detected at 0.5 and 1 h (Fig. V.5).

The pharmacokinetic parameters of all-trans-4-oxo-RA are indicated in table V.2. all-trans-4-Oxo-RA reached peak plasma concentrations 0.5 h after dosing followed by exponential elimination with a $t_{1/2}$ of 5.67 h (Fig. V.6 and Table V.2). The AUC was 28.66 nmol.h/g.

The time-course of radioactivity for [^{3}H]-9-cis-retinal and [^{3}H]-retinyl acetate is shown in figures V.7 and V.8, respectively. Pharmacokinetic parameters for 9-cis-retinal and retinyl acetate were not calculated due to undetectable plasma concentrations. (The limit of detection was 0.56 and 0.18 nmol/g plasma, respectively, for 9-cis-retinal and retinyl acetate.) An unidentified, more polar metabolite of 9-cis-retinal appeared at 0.5 h, peaked at 1 h , and persisted for 4 h. The retention time of the peak did not correlate with that of all-trans-retinaldehyde, all-trans-RA, or 13-cis-RA.

DISCUSSION

Distribution

The radioactivity associated with retinoids studied here crossed the placental barrier and entered the conceptus. The placental transfer and concentration of vitamin A in placental and fetal tissues have been studied in a variety of species. Kochhar (1976) demonstrated transplacental passage of the label associated with [^{3}H]-all-trans-RA in mice. Creech-Kraft et al. (1987) determined the transplacental passage of all-trans-RA and 13-cis-RA and their

TABLE V.2

PHARMACOKINETIC PARAMETERS OF ALL-TRANS-4-OXO-RA

Parameter	Animal Number 1	2	3	Mean ± SD
$t_{1/2}$	4.32	3.54	9.15	5.67 ± 3.04
AUC	32.12	22.67	31.20	28.66 ± 5.21

$t_{1/2}$ = Half-life for rapid elimination phase

AUC = Total area under plasma concentration vs. time curve

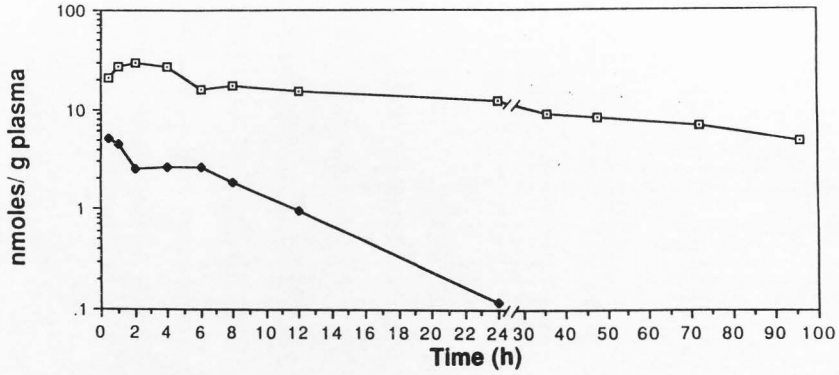


Figure V.6. Plasma concentration of retinoid following administration of all-trans-4-oxo-RA to day-eight pregnant hamsters. Hamsters were dosed with 11.0 mg/kg all-trans-4-oxo-RA, and plasma was assayed by HPLC for concentration of all-trans-4-oxo-RA (◆). Each point is the mean value of separate determinations of plasma obtained from three hamsters. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram plasma for total radioactivity (□) following oral administration of [^{3}H]-all-trans-4-oxo-RA.

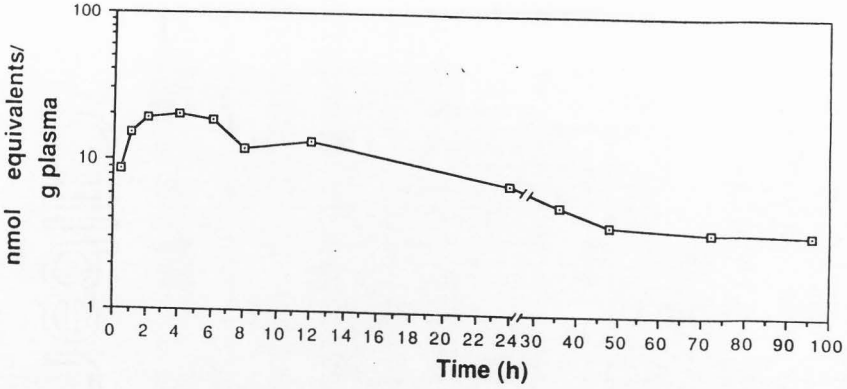


Figure V.7. Plasma concentration of radioactivity following oral administration of 10.5 mg/kg [^{3}H]-9-cis-retinal to day-eight pregnant hamsters. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram plasma for total radioactivity.

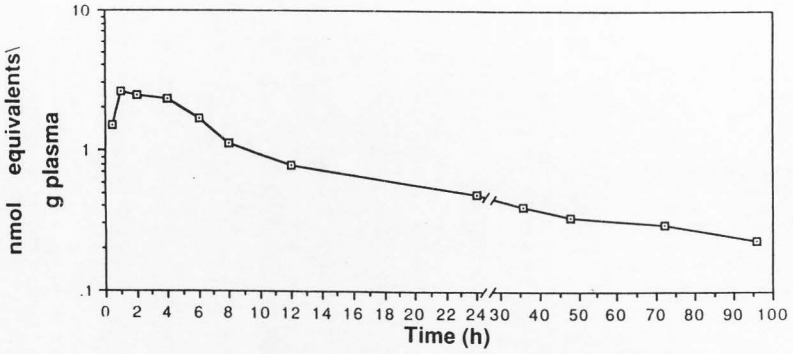


Figure V.8. Plasma concentration of radioactivity following oral administration of 11.2 mg/kg [^{3}H]-retinyl acetate to day-eight pregnant hamsters. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram plasma for total radioactivity.

corresponding metabolites, all-trans-4-oxo-RA and 13-cis-4-oxo-RA, in NMRI mice. Each compound was transferred to the embryo, but transfer of the trans isomers was accomplished to a much greater extent than the 13-cis isomers; the trans isomers' concentration was 20-fold more than the 13-cis isomers' (Creech-Kraft et al., 1987). Based on radioactivity, the results here indicate neither isomer had appreciable accumulation in the fetus 96 h following dosing. However, this does not rule out the possibility of this occurring at earlier stages of embryogenesis. It must be recognized that much of the radioactivity in circulation was in forms other than the parent compound and recognizable metabolites at the time of tissue sampling. It may be assumed that only a small fraction of radioactivity in tissues represents active chemicals.

In a human abortus obtained 72 h after the last oral dose of 13-cis-RA (isotretinoin; Accutane®), circulating maternal concentrations were 15 and 10 ng/ml, respectively, for 13-cis-RA and all-trans-RA. The embryo (cephalic region) contained 1,200 and 2,800 ng/g of 13-cis-RA and all-trans-RA, respectively, and the placental concentrations of 13-cis-RA were 2,800 - 3,000 ng/g. Circulating concentrations of the primary metabolite of 13-cis-RA, 13-cis-4-oxo-RA, were also detected in the maternal blood and embryo (cephalic region) amounting to 32 and 74 ng/g, respectively (E. Lammer, personal communication). Retinol concentrations were 600, 400, and 300-400 ng/g corresponding to maternal blood, embryo, and placenta. It is unknown to what extent the 4-oxo metabolites of the trans and 13-cis isomers contribute to the teratogenicity of the parent compound. In hamsters a single oral dose of all-trans-4-oxo-RA administered on day eight of gestation resulted in teratogenicity equal to that of all-trans-RA (ED_{50} = 10.5 mg/kg for all-trans-RA; ED_{50} = 9.6 mg/kg for all-trans-4-oxo-RA) (chapter IV).

Pharmacokinetics

Several investigators (Wang et al., 1980; Kalin et al., 1981) have reported nonexponential elimination of all-trans-RA from mouse serum. Other investigators have indicated that all-trans-RA was eliminated from rat serum following first-order kinetics as the dose was decreased (Swanson et al., 1980; Swanson et al., 1981). The saturable elimination of all-trans-RA differs from the exponential elimination of 13-cis-RA observed in mice (Kalin et al., 1982) and hamsters (Table V.1).

Isotretinoin (13-cis-RA) is rapidly absorbed from the G.I. tract and undergoes enterohepatic circulation in humans (Colburn et al., 1983a; Khoo et al., 1982; Goodman et al., 1982). Peak plasma concentrations occurred between 1 and 4 h after administration (Khoo et al., 1982; Goodman et al., 1982; Lucek and Colburn, 1985; Colburn and Gibson, 1985). Human pharmacokinetic data could be described by bi- or triexponential equations (Khoo et al., 1982), but a recycling model fit the plasma data best. The presence of food in the gut and the type of pharmaceutical preparation (Colburn et al., 1983b; Shelley et al., 1982) can affect the absorption of the drug. Peak circulating plasma concentrations and bioavailablility were greater when the drug was administered following a meal than when the gut was empty (Colburn et al., 1983b). The elimination half-lives in man ranges from 4 (Kerr et al., 1982) to 13 to 14 (Colburn et al., 1983a) to 25 h (Goodman et al., 1982; 1983) Neither the parent nor its metabolite, all-trans-RA, is eliminated in the urine; they are primarily eliminated in the feces (Khoo et al., 1982).

The primary metabolites of all-trans-RA and 13-cis-RA in hamsters are the corresponding 4-oxo compounds (Frolik et al., 1979; 1980; Frolik, 1981; Vane et al., 1982; Colburn and Gibson, 1985; Brazzell et al., 1983). In humans receiving multiple doses of 13-cis-RA, the circulating concentration of 13-cis-

4-oxo-RA exceeded that of the parent compound (Brazzell et al., 1983). This was also observed with single oral doses administered to hamsters in this study. The production of the 4-oxo metabolites is catalyzed by hamster liver and tracheal tissues (Frolik et al., 1979; Roberts, 1981). In vivo 13-cis-RA is metabolized to all-trans-4-oxo-RA (Lucek and Colburn, 1985; McCormick et al., 1983). In hamsters, all-trans-RA and 13-cis-RA can be metabolized to 13-cis-4-oxo-RA in vitro (Roberts and Frolik, 1979; Frolik, 1981). The degradation of all-trans-RA can proceed by three differing pathways: epoxidation to all-trans-5,6-epoxyretinoic acid, reduction in the length of the polyene side-chain, and loss of the terminal carboxyl carbon. In hamsters all-trans-RA is metabolized to both 13-cis-RA and all-trans-4-oxo-RA (Fig. V.4) and that 13-cis-4-oxo-RA and all-trans-RA are metabolic products of 13-cis-RA (Fig. V.5). This was also the case for patients with advanced cancer who metabolized 13-cis-RA to the trans isomer (Goodman et al., 1982). Again, the extent to which the trans isomers contribute to the teratogenicity of the 13-cis isomeric forms is unknown. The amount of all-trans-RA found the human conceptus described above points toward the conclusion that all-trans-RA may play a major role in the teratogenicity of 13-cis-RA in humans.

Considerably less is known regarding the metabolism of 9-cis-retinal and retinyl acetate. Retinol and its esters are hydrolyzed in the gut and are subsequently re-esterified and transported to the liver via chylomicrons (Goodman, 1984). Retinaldehyde can also be reduced to retinol (the free acid forms cannot be reduced), esterified, and transported in the same way that retinol can (Goodman, 1984). In light of the fact that neither 9-cis-retinal nor retinyl acetate was present in maternal plasma in appreciable amounts, it is reasonable to assume that these two retinoids are metabolized to retinol and subsequently to retinyl esters, thus accounting for their high concentration in

the liver. Retinoic acid is a metabolic product of retinyl acetate in mice (Ito et al., 1974). This is in contrast to the present results, where no retinoic acid was detected. It is unknown why the [^{3}H]-label associated with treatment of [^{3}H]-9-cis-retinal and [^{3}H]-retinyl acetate accumulated to a large extent in the lungs (Figs. V.2 and V.3).

CHAPTER VI

PHARMACOKINETICS, TISSUE DISTRIBUTION, AND PLACENTAL PERMEABILITY OF ALL-TRANS- AND 13-CIS-N-ETHYL RETINIAMIDES IN PREGNANT HAMSTERS

INTRODUCTION

The retinamides (amide retinoid derivatives) are, in general, less toxic on an acute and chronic basis than free acid or esterified retinoids (Howard and Willhite, 1986). N-Ethyl-13-cis- and N-ethyl-all-trans-retinamide and motretinid (the ethyl amide derivative of the human and animal teratogen, etretinate) were not teratogenic in Syrian hamsters after administration of oral doses up to 15 times an equimolar teratogenic dose of all-trans-retinoic acid (all-trans-RA) (Willhite and Shealy, 1984; Howard et al., 1986). Presented here are the results of studies on the distribution (Phase I) and pharmacokinetic parameters (Phase II) of N-ethyl-13-cis-retinamide (CNERA) and its stereoisomer, N-ethyl-all-trans-retinamide (NERA) in pregnant hamsters. The present results are compared and contrasted to the results of previous investigators who studied the metabolic fate and tissue profiles of retinamides in rodents.

MATERIALS AND METHODS

Chemicals

Radioactive N-ethyl-13-cis-[11-^{3}H]-retinamide ([^{3}H]-CNERA; 3.21 Ci/mmole in toluene) and N-ethyl-all-trans-[11-^{3}H]-retinamide ([^{3}H]-NERA; 3.21 Ci/mmole in toluene) were obtained from SRI International, Menlo Park, CA, under the courtesy of the Biological and Chemical Prevention Program, Chemical and Physical Carcinogenesis Branch, Division of Cancer Cause and Prevention,

National Cancer Institute, NIH, Bethesda, MD. The radiolabeled retinoids were chromatographed by isocratic high performance liquid chromatography (HPLC) on a Radial Pak 8 C_{18} column with a mobile phase (2 ml/min) of acetonitrile:water (8:2, v/v) and monitored at 325 nm. After integration of the area under the curve, the 13-cis and all-trans isomeric preparations were 68.3 and 82.5 percent pure, respectively. Subsequent attempts to purify the retinamides to greater chemical purity were unsuccessful due to the inherent radiolability of these retinoids, yet a final radiochemical purity of 95% was achieved.

An aliquot of the radioactive retinamide in toluene was pipetted into a volumetric flask, and the cold retinamide was added and dissolved. The solution was then diluted in Tween 20 (polyoxyethylesorbitan monolaurate, Sigma Chemical Co., St. Louis, MO), such that the final toluene concentration was 20%.

Nonlabeled retinamides (CNERA, SoRI Lot No. C273-79-1JLF and NERA, SoRI Lot No. 9785-141-1JLF) were synthesized, characterized, and obtained as previously described (Willhite and Shealy, 1984). These retinoids were assayed by HPLC and found to be 99.8 percent pure. All retinoids were stored under argon at -80^{o}C in amber glassware.

Animals

Animals were purchased, bred, and housed as described in chapter III.

Treatment

Two separate studies were performed: 1) Comparative distribution and placental permeabilities were considered, and 2) the comparative pharmacokinetic parameters of the parent retinamides were evaluated. In the first study (Phase I), six pregnant hamsters were given a single oral intubation

of either CNERA or NERA at 11.4 mg/kg (35 μmol/kg; 20 μCi/animal) in Tween 20 at 10:00 A.M. on day eight of pregnancy. The quantity of retinamide administered was a dose equivalent to 10.5 mg/kg all-trans-RA, a dose equimolar to the teratogenic ED_{50} of all-trans-RA in hamsters (Willhite and Shealy, 1984). Blood was collected from the orbital sinus and counted for total radioactivity as described below. At 96 h after dosing, the dams were killed, and selected tissues, including the conceptus and the placenta, were collected, digested, and the radioactivity counted as described below. Six additional animals were dosed with an identical amount of tritiated compound; Three were killed at 4 h and three at 24 h after dosing. Tissues from these animals were likewise collected and the residual radioactivity determined.

Blood and Tissue Collection Schedule

Blood was collected from the post-orbital plexus in heparinized glass hematocrit tubes at 0.5, 1, 2, 4, 6, 8, 12, 24, 36, 48, 72, and 96 h after intubation of the radiolabeled retinoid. Animals were killed in a saturated CO_2 atmosphere at 4, 24, or 96 h after intubation. The heparinized blood was centrifuged immediately after collection in a microhematocrit centrifuge for 10 min to obtain plasma. The plasma was weighed, placed into scintillation vials, and mixed with 200 μl of 70% aqueous perchloric acid (Fisher Scientific, Pittsburgh, PA). The plasma solution was incubated at 60°C for 4 h. The incubated solution was cooled to room temperature and mixed with 500 μl of 30% hydrogen peroxide (Fisher Scientific). The samples were subsequently incubated at 60°C for 4 h to decolorize. Samples were cooled to room temperature, and 15 ml of scintillation fluid were added (see chapter V). Tissues were homogenized with two volumes of distilled water, and a 150 μl aliquot was taken to be processed as described above. The residual

radioactivity was counted in a Beckman LS 3801 scintillation counter. Tritium activity was quantified by the use of an external standard quench correction mode.

High Performance Liquid Chromatography

In the second phase of the study, whole blood was collected, centrifuged, and weighed as described above. Three hundred ul of HPLC grade methanol (Fisher Scientific) were added to each plasma sample (90-150 μg), the samples purged with argon and stored at -80°C, until chromatographic separations could be performed. Samples were thawed in a dark room at room temperature and then centrifuged at 10,000 x g for 30 min. The supernatant was decanted and filtered through 0.2 μm microfilter (Bioanalytical Systems, West Lafayette, IN) at 1,000 x g for 5 min. The methanol extracted samples were analyzed by HPLC using a Varian Model 5000 chromatograph equipped with a Spherisorb ODS 5-μm column (Universal Scientific, Atlanta, GA) and a Spherex C18 5-cm X 4.6 cm pre-column (Phenomonex, Rancho Palos Verdes, CA). The HPLC grade acetonitrile and water (J. T. Baker, Jackson, TN) were filtered (0.2 um) and vacuum-degassed prior to use. Two hundred μl of extracted plasma were injected onto the column. Both retinoids were assayed at 340 nm (Varian Varichorm UV/VIS spectrophotometer) with acetonitrile-1% aqueous ammonium acetate (85:15, v/v) as the eluent with a flow rate of 1.2 ml/min. Under these conditions, retinamide retention times were 8.05 and 9.66 min for CNERA and NERA, respectively. All procedures were performed under yellow light with as little exposure of the samples to air as possible. The retinoids were quantified by peak height (Hewlett-Packard model 3390 A integrator) with an external standard and identified by their retention times. Plasma concentration values

were fitted to a curve, and the area under the curve (AUC) was calculated, as described in chapter V.

RESULTS

Distribution

Radioactivity was distributed to all tissues sampled 4 h after a single dose of 11.4 mg/kg of [^{3}H]-CNERA (Fig. VI.1). The urinary bladder contained the highest concentrations of residual radioactivity/g tissue at 4 h followed by the concentrations in the liver>conceptus>adrenal>perirenal fat. Radioactivity was rather evenly distributed among the remaining tissues at 4 h. The concentrations of residual radioactivity in peripheral tissues declined in a time-dependent fashion (Fig. VI.1); at 96 h following administration of [^{3}H]-CNERA hepatic tissues contained the highest concentrations of radioactivity. The ratio of radioactivity in the conceptus versus that in the maternal plasma was 1.5:1 after 4 h and was less than one after 24 or 96 h. In the placenta, substantially lower concentrations of radioactivity were detected; the ratio of activity in the conceptus versus that in the placenta was 10.5:1 at 4 h, and this ratio declined to 1:1 at 24 and 96 h.

Distribution of total radioactivity after administration of [^{3}H]-NERA essentially mirrored that observed after [^{3}H]-CNERA administration (Fig. VI.2). Radioactivity was concentrated primarily to the urinary bladder and the maternal liver at 4 h after a single oral dose of NERA and was distributed essentially equally to all other tissues sampled. Again, as was the case with CNERA, the radioactivity decreased in a time-dependent fashion. After 96 h, the detectable radioactivity was always greater in tissues from animals treated with [^{3}H]-CNERA as compared to the activity recovered after an identical dose of its trans isomer. The ratio of total radioactivity in the conceptus compared

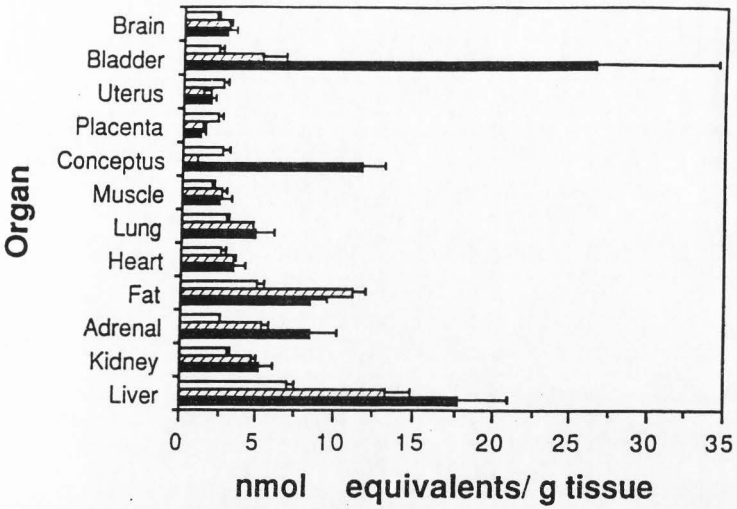


Figure VI.1. Distribution of radioactivity in pregnant hamsters following oral administration of 11.4 mg/kg [^{3}H]-CNERA. Tissues were sampled 4 (▬), 24 (▨), and 96 h (□) after dosing. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue. Bars represent S.E.M.

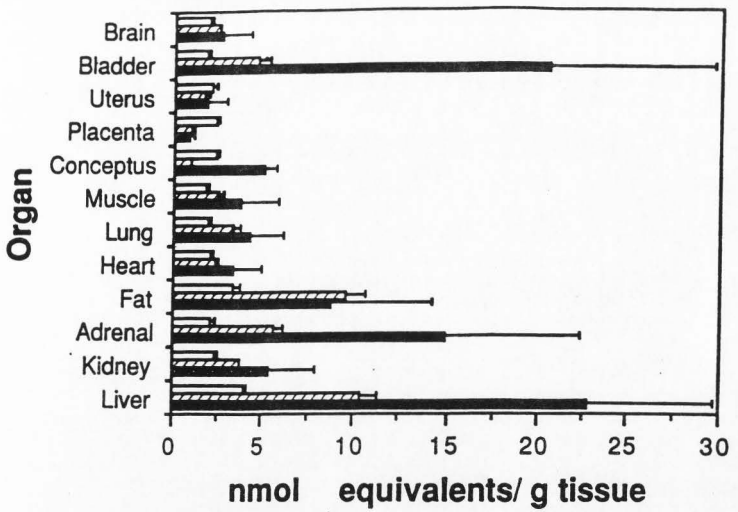


Figure VI.2. Distribution of radioactivity in pregnant hamsters following oral administration of 11.4 mg/kg [^{3}H]-NERA. Tissues were sampled 4 (▨), 24 (▩), and 96 h (□) after dosing. Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue. Bars represent S.E.M.

to the maternal plasma was 1:1 at 4 h after administration; the ratio of radioactivity in the conceptus compared to that in the placenta was 6.5:1. Within 96 h of an oral dose of [^{3}H]-NERA, the ratio of total radioactivity in the conceptus to that in the placenta had declined to 1:1. It is important to emphasize here that these values represent those for total radioactivity.

Pharmacokinetics

The pharmacokinetic parameters for CNERA and NERA (Tables VI.1 and VI.2) were obtained from nonlinear regression fittings of animal plasma concentration-time values (Figs. VI.3 and VI.4). The AUC for CNERA was 22.5 percent greater than for NERA (Tables VI.1 and VI.2). NERA was absorbed essentially instantaneously; whereas, CNERA reached a peak circulating concentration 1.84 h after dosing. Two elimination half-time values were observed for CNERA, yet only one was noted for NERA (Tables VI.1 and VI.2). The elimination half-time values for CNERA were 1.01 and 1.99 h for the rapid and slow elimination phases, respectively. The elimination $t_{1/2}$ for NERA was 3.14 h.

The plasma concentration-time curves for CNERA and NERA are shown in figures VI.3 and VI.4, respectively. Both retinamides achieved mean peak circulating concentrations of approximately 1 nmol/g plasma, followed by exponential elimination. Detectable concentrations of either parent retinamide were absent from the plasma after 12 h (limit of detection = 0.6 nmol/g plasma for both retinoids), yet from the measurements of total radioactivity (Figs. VI.3 and VI.4), unidentified metabolites of these retinoids persisted in the circulation for extended periods of time. The CNERA was metabolized in situ to NERA (Fig. VI.3), and the parent NERA was metabolized in the hamster to

TABLE VI.1

PHARMACOKINETIC PARAMETERS OF CNERA

Parameter	Animal Number 1	2	3	Mean ± SD
$t_{1/2}^{\pi}$	0.30	0.63	0.35	0.43 ± 0.18
$t_{1/2}^{\alpha}$	1.42	0.61	0.99	1.01 ± 0.41
$t_{1/2}^{\beta}$	1.42	3.05	1.50	1.99 ± 0.92
Time to peak concentration (h)	2.00	1.77	1.74	1.84 ± 0.14
AUC (nmol.h/g)	3.57	3.96	4.04	3.86 ± 0.25

$t_{1/2}^{\pi}$ = Half-life for absorption

$t_{1/2}^{\alpha}$ = Half-life for rapid elimination phase

$t_{1/2}^{\beta}$ = Half-life for slow elimination phase

AUC = Total area under plasma concentration vs. time curve

TABLE VI.2

PHARMACOKINETIC PARAMETERS OF NERA

Parameter	Animal Number			Mean ± SD
	1	2	3	
$t_{1/2}$	2.40	3.98	3.04	3.14 ± 0.79
AUC (nmol.h/g)	2.42	2.98	3.58	2.99 ± 0.58

$t_{1/2}$ = Half-life for rapid elimination phase

AUC = Total area under plasma concentration vs. time curve

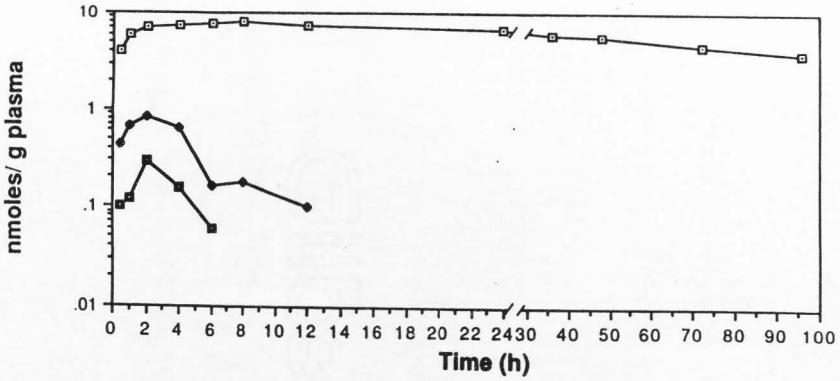


Figure VI.3. Plasma concentrations of retinamides following oral treatment with 11.4 mg/kg CNERA to day-eight pregnant hamsters. Concentrations at time points greater than 12 and 6 h for CNERA (◆) and NERA (■), respectively, were less than the analytical limit of detection (< 0.6 mnol/g). Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue for total radioactivity (⊡) following oral administration of [^{3}H]-CNERA. Each point is the mean value of separate determinations of plasma obtained from three hamsters.

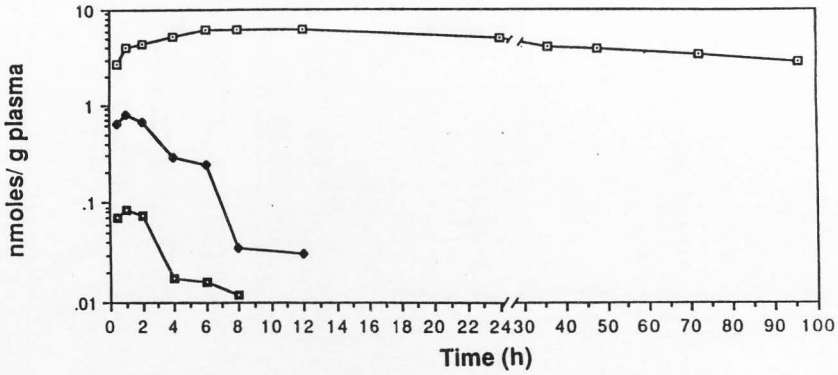


Figure VI.4. Plasma concentrations of retinamides following oral treatment with 11.4 mg/kg NERA to day-eight pregnant hamsters. Concentrations at time points greater than 12 and 8 h for NERA (◆) and CNERA (■), respectively, were less than the analytical limit of detection (< 0.6 mnol/g). Disintegrations per minute (DPM) were converted to nmol equivalents/ gram tissue for total radioactivity (□) following oral administration of [^{3}H]-NERA. Each point is the mean value of separate determinations of plasma obtained from three hamsters.

its cis isomer (Fig. VI.4). Detectable amounts of all-trans- or 13-cis-retinoic acid were not generated in vivo.

DISCUSSION

Distribution

The data presented in this chapter were consistent with those of Hultin et al. (1985) who demonstrated that N-(4-hydroxyphenyl) retinamide (4-HPR) concentrated in rat liver 8 h after cessation of oral dosing at 5 mg/kg/d for 5 days. In mice dosed orally with 10 mg/kg N-(2-hydroxyethyl) retinamide (N-HOERA), the parent retinoid was found in the liver, lung, fat, kidney, brain, heart, spleen, muscle, testes, and bladder (Kalin et al., 1982). In the case of the N-HOERA, the bladder had the longest elimination $t_{1/2}$ of all tissues sampled; this was consistent with the observation made here that the radioactivity associated with an oral dose of CNERA or NERA was accounted for in large measure in the bladder. From this observation one may conclude that bladder tissue may reflect urinary elimination of the parent retinamide and/or its metabolic products. The amount of radioactivity remaining in the adipose tissue 96 h after dosing with [^{3}H]-CNERA and [^{3}H]-NERA was 5-fold greater for these lipophilic retinamides, as contrasted to the concentrations of residual radioactivity associated with administration of the more polar all-trans-RA, 13-cis-retinoic acid, or their metabolite, all-trans-4-oxoretinoic acid (see Chapter V). Again, as stated in chapter V, it must be recognized that much of the radioactivity in circulation was in forms other than the parent compound and recognizable metabolites at the time of tissue sampling. It may be assumed that only a small fraction of radioactivity in tissues represents active chemicals.

Pharmacokinetics

Kalin et al. (1982) investigated the disposition of N-HOERA in orally dosed mice. This retinoid exhibited a single plasma $t_{1/2}$ of 2.9 h. This value is similar to the elimination $t_{1/2}$ for NERA of 3.14 h (Table V.2). When N-HOERA was administered intravenously its elimination was described best by a 2-compartment system (Wang et al.,1980). The two elimination half-lives were 0.3 and 1.5 h, corresponding to the rapid and slower elimination phases (Wang et al., 1980). Pretreatment of mice with phenobarbital, 3-methylcholanthrene, or N-HOERA decreased the AUC values of N-HOERA by 39, 30, and 13%, respectively, indicating that retinoid metabolism may be inducible (Kalin et al., 1984).

Detectable amounts of all-trans-RA in plasma were not observed after oral treatment with either CNERA or NERA. This agrees with the data of Swanson et al. (1981; 1980), who observed no production of all-trans-RA in rats that received 4-hydroxyphenyl retinamide. The three retinamides, CNERA, NERA, and motretinid, are teratogenically inactive in hamsters (Willhite and Shealy, 1984; Howard et al., 1986). Kochhar et al. (1987) suggested that after very large doses of motretinid to mice, limited biotransformation of the amide to the acid confers teratogenic activity. It is the free acid form of retinoids which binds to cellular retinoic acid-binding protein (cRABP) in hamster tissues (Brandes et al., 1983; personal observation, see chapter VIII). Thus, it can be assumed that the retinamides are teratogenically inactive, because they are not biotransformed to a free acid in appreciable amounts at position C15, thereby not binding with the putative receptor cRABP.

CHAPTER VII

PHARMACOKINETICS, TISSUE DISTRIBUTION, AND PLACENTAL PERMEABILITY OF A RETINOIDAL BENZOIC ACID DERIVATIVE (AROTINOID Ro 13-7410) IN HAMSTERS

INTRODUCTION

The arotinoids, the third generation of retinoids, contain one or two aromatic rings in their cyclic end group, side-chain, or polar terminus. The arotinoid Ro 13-6298 (arotinoid ethyl ester) has been effective in patients who showed resistance to etretinate for treatment of severe generalized psoriasis and psoriatic arthritis (Tsambaos and Orfanos, 1983). The pharmacology of arotinoids has been reviewed (Bollag, 1985).

The arotinoids are also the most potent hamster (Flanagan et al., 1987) and mouse (Zimmermann et al., 1985) retinoid teratogens reported to date. In hamsters the free acid arotinoid Ro 13-7410 (TTNPB) is at least 750 times as teratogenic as all-trans-retinoic acid (all-trans-RA). The pharmacokinetics, tissue distribution, and placental permeability of Ro 13-7410 were determined in hamsters to assess the possibility whether the in vivo disposition properties of this chemical may account for its high toxicity.

MATERIALS AND METHODS

Chemicals

The free acid arotinoid p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl-6,7-$^{3}H_{2}$)-propenyl]-benzoic acid ([^{3}H]-Ro 13-7410, 22.8 Ci/mmole in ethanol) was obtained from SRI International, Menlo Park, CA, under the courtesy of the Biological and Chemical Prevention Program, Chemical and

Physical Carcinogenesis Branch, Division of Cancer Cause and Prevention, National Cancer Institute, NIH, Bethesda, MD. When the radiolabeled retinoid was chromatographed by isocratic high performance liquid chromatography (HPLC) on a Radial Pak 8 C_{18} column, using ethanol:1% acetic acid (8:2, v/v) as the mobile phase, and monitored at 304 nm, it was found to be 88.9 percent pure. Nonlabeled Ro 13-7410 was received from Hoffmann-La Roche, Inc., Nutley, NJ. Cold Ro 13-7410 was 99% pure when assayed by HPLC at 304 nm using a previously described HPLC system (Willhite and Shealy, 1984).

Animals

Animals were purchased, bred, and treated as described in chapter III.

Treatments

The nonlabeled retinoid was dissolved in a small volume of reagent grade acetone, and the radiolabeled retinoid was pipetted into the acetone-retinoid solution. The acetone-ethanol-retinoid solution was then solubilized in Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma, St. Louis, MO), such that the final acetone or ethanol concentration did not exceed 5 percent. The retinoid solution was administered at a rate of 0.5 ml/100 g body weight.

Two separate studies were performed: 1) The comparative distribution and placental permeabilities were considered, and 2) the comparative pharmacokinetic parameters of the parent arotinoid were evaluated. In the first study (Phase I), six pregnant hamsters were given a single oral intubation of 100 μg/kg [^{3}H]-Ro 13-7410 (35 μCi/animal) in Tween 20 at 10:00 A.M. on day eight of pregnancy. Blood was collected from the orbital sinus and counted for total radioactivity as in chapter V. At 96 h after dosing, the dams were killed, and selected tissues, including the conceptus and the placenta, were collected, digested, and the radioactivity counted as described

below. Six additional animals were dosed with an identical amount of tritiated compound; three were killed at 4 h, and three were killed at 24 h after dosing. Tissues from these animals were likewise collected and the residual radioactivity determined. In the second study (Phase II), three animals were dosed with 1000 μg/kg [^{3}H]-Ro 13-7410; plasma was collected, chromatographed, and fractions collected for the amount of radioactivity present.

Blood and Tissue Collecting Schedule

Blood and tissues were collected and processed as described in chapter V.

High Performance Liquid Chromatography

Plasma was extracted and chromatographed as described in chapter V. The retinoids were eluted with acetonitrile-1% aqueous ammonium acetate (85:15) at 1.0 ml/min. One-half ml fractions were collected, processed, and counted in a Beckman LS 3801 liquid scintillation counter. Nonradioactive Ro 13-7410 was used to determine the retention position of the parent molecule. Tritium activity was quantified in DPMs via the use of an external standard quench correction mode.

RESULTS

Distribution

Radioactivity was evenly distributed to all sampled tissues, except the liver, by 4 h after a 100 μg/kg dose of [^{3}H]-Ro13-7410. The liver contained the highest amount of radioactivity (2800 pmol eq./g tissue) followed by the conceptus (580 pmol eq./g tissue) (Fig. VII.1). The ratio of radioactivity in the conceptus versus the maternal plasma was 5.4:1 at 4 h. At 24 and 96 h the ratio of radioactivity in the conceptus versus the maternal plasma was less than one. The amount of radioactivity in tissues declined in a time-dependent

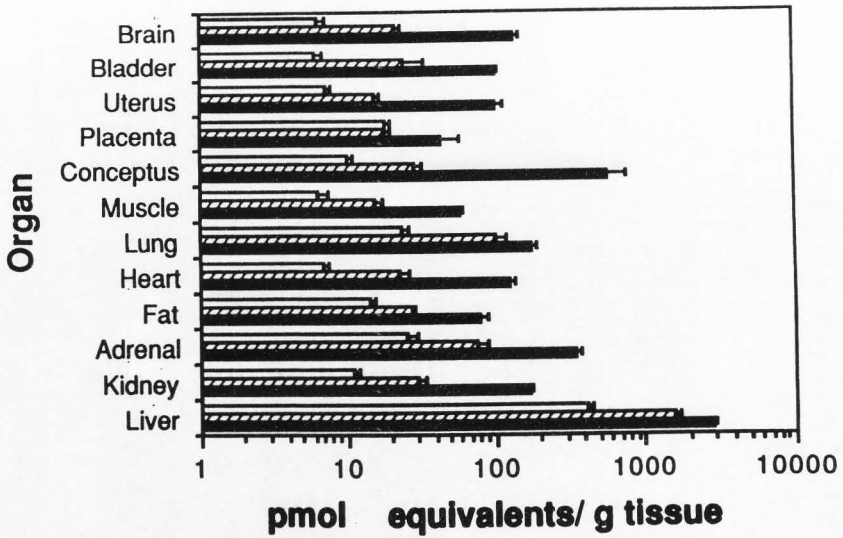


Figure VII.1. Distribution of radioactivity in pregnant hamsters following oral administration of 100 µg/kg [^{3}H]-Ro 13-7410. Tissues were sampled 4 (▪), 24 (▨), and 96 h (□) after dosing. Disintegrations per minute (DPM) were converted to pmol equivalents/ gram tissue. Bars represent S.E.M.

fashion with the most radioactivity remaining in the liver at 96 h (400 pmol eq./g tissue) (Fig. VII.1). Ninety-six h after dosing with 100 μg/kg [^{3}H]-Ro 13-7410, the liver contained at least 15-fold more radioactivity than any other sampled tissue.

The distribution of radioactivity in liver, conceptus, placenta, and uterus, following administration of either 100 or 1000 μg/kg [^{3}H]-Ro 13-7410, is shown in figure VII.2. As was the case of a 100 μg/kg dose, the liver contained the most radioactivity 24 h after a 1000 μg/kg dose, having at least 36 times as much radioactivity as the conceptus, placenta, or uterus (Fig. VII.2).

Pharmacokinetics

Pharmacokinetic parameters of Ro 13-7410 (Table VII.1), following a 1000 μg/kg oral dose, were generated as described in chapter V. The retinoid accumulated in the plasma, reaching peak circulating concentrations at 3.37 h (Fig. VII.3). Elimination proceeded biexponentially with elimination half-lives of 1.52 and 2.45 h, corresponding to the rapid and slow elimination phases. The AUC was 1634.4 pmol.h/g. An unknown more polar metabolite (based on its retentivity) was present at 0.5 h. This metabolite reached its peak at 12 h, comprising 92% of the total radioactivity present in the plasma.

DISCUSSION

Distribution

Arotinoids are at least 750-fold more potent teratogens than all-trans-RA in hamsters (Flanagan et al., 1987). If pharmacokinetics alone are responsible for the increased teratogenic potency, at least a 750-fold increase in the concentration of arotinoids in the embryo would be required; however, this was not observed based on radioactivity (Figure VII.1). The increased teratogenic

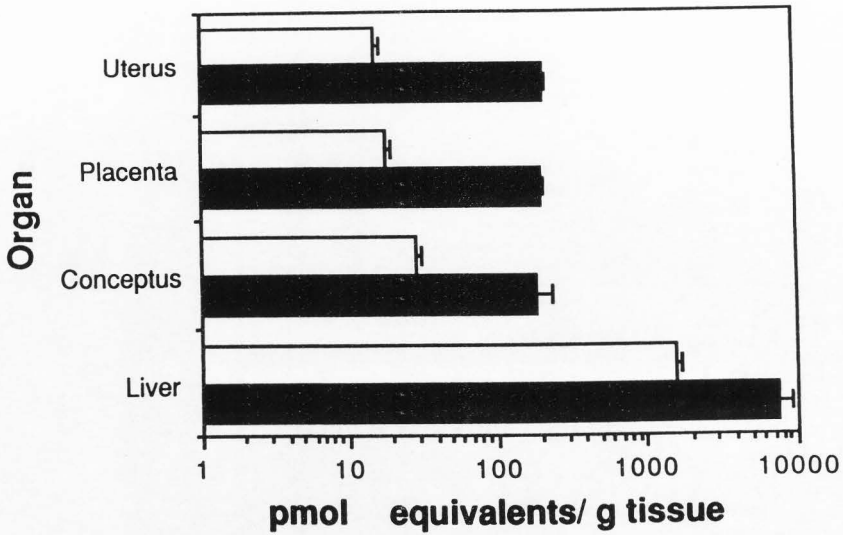


Figure VII.2. Distribution of radioactivity 24 h following oral administration of 100 (□) or 1000 µg/kg (■) [^{3}H]-Ro 13-7410 to pregnant hamsters. Disintegrations per minute (DPM) were converted to pmol equivalents/ gram tissue. Bars represent S.E.M.

TABLE VII.1

PHARMACOKINETIC PARAMETERS OF RO13-7410
FOLLOWING A 1000 μg/kg DOSE

Parameter	Animal Number 1	2	3	Mean ± SD
$t_{1/2}^{\alpha}$	2.11	1.79	0.65	1.52 ± 0.77
$t_{1/2}^{\beta}$	2.11	1.79	3.45	2.45 ± 0.88
Time to peak concentration (h)	3.18	5.00	1.93	3.37 ± 1.54
AUC (pmol.h/g)	1698.34	1712.14	1492.84	1634.44 ± 122.8

$t_{1/2}^{\alpha}$ = Half-life for rapid elimination phase

$t_{1/2}^{\beta}$ = Half-life for slow elimination phase

AUC = Total area under plasma concentration vs. time curve

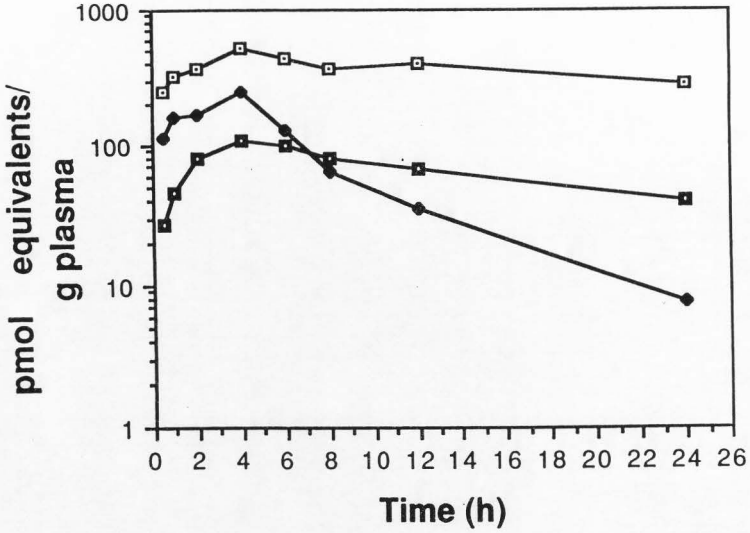


Figure VII.3. Plasma concentration of Ro 13-7410 following oral administration of 1000 µg/kg [^{3}H]-Ro 13-7410 to day-eight pregnant hamsters. Plasma was assayed by HPLC and 0.5 ml fractions were collected and counted for radioactivity corresponding to the retention time of Ro 13-7410 (◆). Total radioactivity in plasma following a single oral dose of 100 (◈) or 1000 (⊡) µg/kg [^{3}H]-Ro 13-7410. Disintegrations per minute (DPM) were converted to pmol or pmol equivalents/ gram plasma.

potency of arotinoids must be, in part, dependent on the retinoid's affinity for its receptor. Creech-Kraft et al. (1987) have demonstrated that all-trans-RA accumulates more than 13-cis-RA versus the maternal plasma, thus providing an explanation of the more potent teratogenic activity of all-trans-RA. Nau and Scott (1986) hypothesized that this concentration phenomenon is due to pH differences in embryo and maternal plasma. The pH of the embryo was found to be 0.4 pH units higher in the embryo thereby trapping weak acids. In the hamster, all-trans-RA is twice as potent a teratogen as 13-cis-RA (Willhite and Shealy, 1984). Pharmacokinetic differences may play a role in their differing potencies. However, in the mouse limb bud (Kwarta et al., 1985) and in chick wing buds (Maden and Summerbell, 1986) all-trans-RA serves as a better ligand for cellular retinoic acid-binding protein (cRABP) than 13-cis-RA. In the mouse limb bud, all-trans-RA had a 315-fold greater affinity for cRABP than 13-cis-RA (Kwarta et al., 1985). The in vivo accumulation of all-trans-RA in mouse neural tissues is saturable and competitive, indicating the accumulation is dependent upon a receptor (Dencker et al., 1987). It must be recognized that much of the radioactivity in circulation was in forms other than the parent compound and recognizable metabolites at the time of tissue sampling. It may be assumed that only a small fraction of radioactivity in tissues represents active chemicals.

Pharmacokinetics

Pharmacokinetic reports of arotinoids are scarce, because very low doses (0.05-0.1 mg) are required for biologic activity and because the techniques for identifying low (pg) concentrations of arotinoids have not been developed. One man dosed with 0.1 mg Ro 13-6298 had circulating levels of 807 and 630 pg/ml after 3 and 4 h, respectively (Lambert et al., 1985). Even though

comparisons based on one observation may mean little, this compares to a peak circulating level of 69.7 ng/g plasma in the hamster following a 1 mg/kg dose.

The elimination half-times for Ro 13-7410 were 1.52 and 2.45 h for the rapid and slower phases, respectively. These $t_{1/2}$s are shorter than those observed for either 13-cis-RA or all-trans-4-oxo-RA (chapter V) or for either retinamide (chapter VI). The appearance and persistence of a more polar metabolite in the plasma is consistent with the metabolic patterns observed for all-trans-RA and 13-cis-RA (chapter V). The extent to which this unidentified metabolite may contribute to the teratogenic potency of Ro 13-7410 is unknown. The pharmacokinetic disposition and distribution patterns of Ro 13-7410 may not account for its high teratogenicity, as compared with all-trans-RA.

CHAPTER VIII

CORRELATION OF BINDING AFFINITIES OF RETINOIDS TO cRABP AND THEIR TERATOGENIC POTENCY IN HAMSTERS

INTRODUCTION

Cellular retinoic acid-binding protein (cRABP) has 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 limb buds of the mouse, and in embryonic chick skin (Chytil and Ong, 1983; Rainier et al., 1983; Kwarta et al., 1985; Sani and Hill, 1974). In the mouse limb bud, all-trans-RA has a 315-fold greater affinity with cRABP than 13-cis-retinoic acid (13-cis-RA) (Kwarta et al., 1985). However, it is unknown if binding with cRABP is obligatory for teratogenic activity.

Retinoids vary over several orders of magnitude in their abilities to induce teratogenesis in hamsters (Howard and Willhite, 1986; Howard et al., 1987; chapter IV.) To assess the role of cellular retinoic acid-binding protein (cRABP) in retinoid-induced teratogenesis, the ability of these retinoids to displace high-specific activity [^{3}H]-all-trans-retinoic acid from cRABP was investigated.

MATERIALS AND METHODS

Chemicals

The retinoid all-trans-retinoic acid (all-trans-RA) was purchased from Sigma (St. Louis, MO). Isotretinoin (13-cis-retinoic acid, 13-cis-RA) was purchased from Kodak (Rochester, NY). The retinoids Ro 12-4824, Ro 10-1670, Ro 13-7410, Ro 15-0778, Ro 8-8717, Ro 10-9359, and Ro 11-1430 were gifts of

Hoffmann-LaRoche (Nutley, NJ). The retinoids SRI 5631-96, SRI 4657-47, and SRI 7167-67 were obtained from SRI International (Menlo Park, CA). The retinoids N-ethyl-all-trans-retinamide (NERA) and N-ethyl-13-cis-retinamide (CNERA) were obtained from SoRI (Birmingham, AL). All retinoids were greater than 95% pure by reversed phase HPLC.

Bovine serum albumin (BSA) and myoglobin were purchased from Sigma. 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/mmole; 98.8%) was purchased from NEN Research Products (Boston, MA). The 3-(N-morpholino)-propanesulfonic acid (Mops) was purchased from Sigma.

Preparation of Cytosol

Timed-pregnant hamsters were purchased from the Charles River Breeding Laboratories (Wilmington, MA). Animals were killed in excess CO_2. Whole day-12 hamster fetuses or day-eight hamster embryos were collected and placed in a centrifuge tube containing an equal volume 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 x g (4^{o}C) for 0.5 h, and the supernatant was subsequently centrifuged for one h at 105,000 x g in an ultracentrifuge (Beckman SW 41 Ti rotor). The protein concentration of the supernatant was determined using the Pierce protein assay reagent (Pierce, Rockford, IL) using BSA as the standard. Cytosol was frozen at -80^o C until use.

Labeling of Cellular Retinoic Acid-Binding Protein

Frozen cytosol was thawed at 4° C overnight. The cytosol was diluted with fresh tissue homogenization buffer to a final protein concentration of 1.04 mg/ml. An aliquot of 480 µl of diluted cytosol was pipetted into incubation tubes. Ten µl of [^{3}H]-all-trans-RA (100 nM, 2.63 µCi) were added to each tube. An additional 10 µl of ethanol were added with or without a 200-fold molar excess of all-trans-RA to individual tubes. The retinoid-protein mixture was incubated for 12 h overnight at 4° C in the dark. The incubation was stopped 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 x g for 10 min and the supernatant subsequently filtered through a 0.45-µm filter to remove all traces of charcoal prior to chromatography. Handling of all retinoid solutions was done under yellow light.

High Performance Size Exclusion Chromatography

Methods for high performance size exclusion chromatography (HPSEC) are described by Rainier et al. (1983). A Varian Model 5000 liquid chromatograph equipped with a SOTAPhase GF-200, 20 nm, 10 µm, 7.1 X 300 mm column and a SOTAPhase GF-200, 7.1 X 75 mm guard column (Rainin, Emeryville, CA) was used. The elution buffer contained 10 mM Mops, 2 mM 2-mercaptoethanol, 20 mM potassium acetate, 1 mM EDTA, pH 7.5. The buffer was filtered through a 0.2-µm filter prior to use. Buffer was prepared fresh daily. The eluent was pumped at a flow rate of 1.0 ml/min and monitored at 280 nm using a Varian Varichrom UV/Visible spectrophotometer. Two hundred µl of filtered supernatant were injected onto the column. Myoglobin and BSA were used to as standards. The elution position of [^{3}H]-RA-cRABP was determined by

collecting 0.5-ml fractions of the eluent. Fractions were counted in 4 ml of scintillation cocktail in a Beckman LS 3801 liquid scintillation counter. DPMs were quantified via an external standard mode.

Saturation Analysis

Day-12 hamster fetus cytosol (500 ug protein in 480 ul) was incubated in the dark at 4^{o} C for 12 h with 10 ul [^{3}H]-all-trans-RA (1-465 nM in 95% ethanol) and 10 ul of ethanol. The incubation was terminated with the addition of 100 ul ice-cold dextran-coated charcoal (2.5% charcoal, 0.25% dextran). Samples were centrifuged and filtered as described above. One-half ml fractions were collected and counted as described above. Specific binding was calculated by the amount of radioactivity in the 10-11 ml fraction (Fig. VIII.1), and a Scatchard analysis (Scatchard, 1949) was performed.

Competitive Binding Assay

Competition studies were performed by incubating protein samples (500 ug protein in 480 ul fresh homogenization buffer) at 4^{o} C for 12 h with 100 nM [^{3}H]-all-trans-RA (2.63 uCi) in 10 ul ethanol and 10 ul ethanol containing from 0.1 to 200-fold molar excess of unlabeled retinoid analogs. The incubation mixtures were treated with dextran-coated charcoal, centrifuged, filtered, chromatographed, and counted as described above.

RESULTS

Presence of cRABP

The presence of cRABP was identified first in day-eight hamster embryos. The amount of cRABP present was 2.2 $\pm$ 0.05 pmoles/mg protein. Day-12 hamster fetuses contained 8.9 pmoles/mg protein. When incubated with 100 nM [^{3}H]-all-trans-RA and separated by HPSEC, peaks of radioactivity were

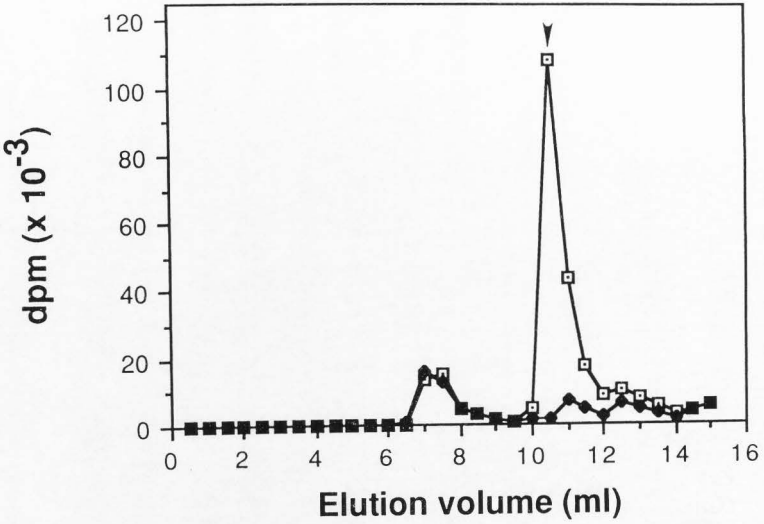


Figure VIII.1. High performance size exclusion chromatographic analysis of day-12 hamster fetus cRABP. Day-12 hamster fetus cytosol was incubated with 100 nM [^{3}H]-all-trans-RA in the absence (□) or presence (◆) of a 200-fold molar excess of unlabeled all-trans-RA. The arrow indicates the elution volume of myoglobin. Aliquots (200 ul) of dextran-treated charcoal assay mixtures were analyzed.

observed (Fig. VIII.1). The first peak of radioactivity was not displaced by incubation with 200-fold molar excess unlabeled all-trans-RA. The peak that eluted at the same elution volume as myoglobin (molecular mass=17,000) was abolished by incubating with 200-fold molar excess cRABP (Fig. VIII.1). This is evidence for the presence of a specific binding protein for retinoic acid.

Saturation Analysis

Scatchard plots of one set of data from day-12 hamster fetus cRABP yielded an apparent dissociation constant (K_d) of 12.7 nM, and the total specific binding capacity was estimated to be 11 pmoles cRABP/ mg protein (Fig. VIII.2).

Competitive Binding Assay

The relative binding affinities of various retinoids to day-12 hamster fetus cRABP were determined in competition experiments. First, the cytosol was incubated with 100 nM [^{3}H]-all-trans-RA and 200-fold excess of each of the 14 retinoids (Table VIII.1). Etretinate (Ro 10-9359), NERA, CNERA, motretinid (Ro 11-1430), SRI 7167-67, Ro 8-8717, and Ro 15-0778 did not displace any [^{3}H]-all-trans-RA from cRABP. Slight competition was noted for SRI 4657-47 at 200-fold molar excess. The retinoids Ro 13-7410, Ro 10-1670 (the free acid of etretinate), Ro 12-4824 (all-trans-4-oxo-retinoic acid), 13-cis-RA, and SRI 5631-96 displaced [^{3}H]-all-trans-RA. Varying the amount of free ligand (competitor) to the assay displaced different amounts of [^{3}H]-all-trans-RA, and concentration to displace 50% of the label from the protein (DC_{50}) was calculated (Fig. VIII.3 and VIII.4). The calculated DC_{50} for each retinoid is shown in table VIII.1.

The retinoids may be ranked according to their ability to displace [^{3}H]-all-trans-RA from cRABP: all-trans-RA = Ro 12-4824 > Ro 13-7410 > SRI 5631-

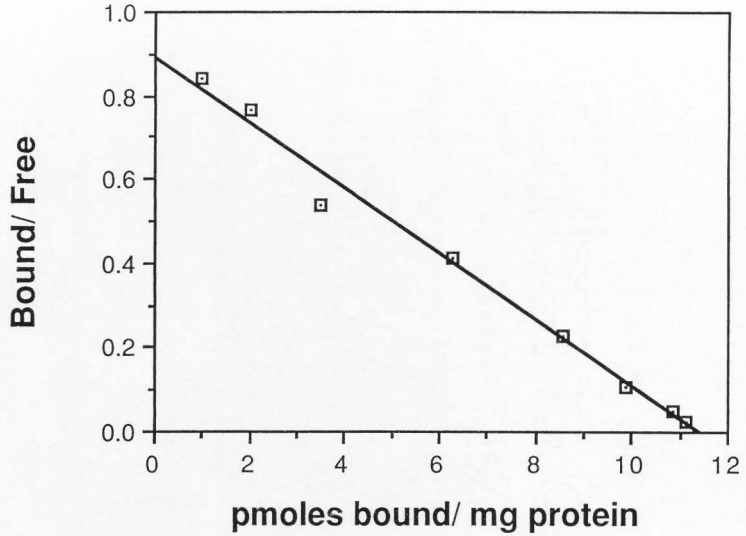
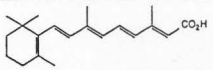
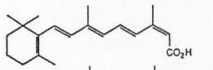
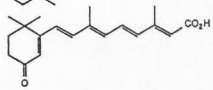
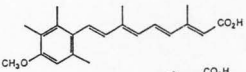
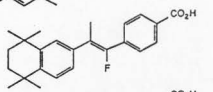
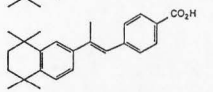
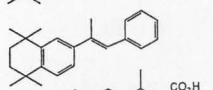
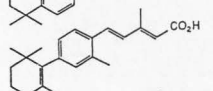
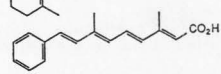
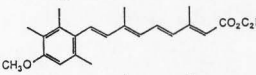
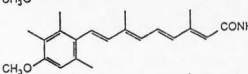
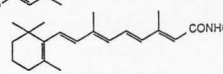
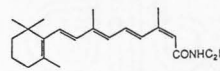
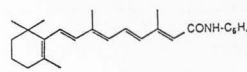


Figure VIII.2. Scatchard analysis for all-trans-RA with day-12 hamster fetus cRABP. (See text for details.)

TABLE VIII.1

MOLECULAR STRUCTURES AND BINDING AFFINITIES OF RETINOIDS

STRUCTURE	CODE	DC_{50} (M)
	all-trans-RA	4.7×10^{-7}
	13-cis-RA	2.6×10^{-6}
	Ro12-4824	3.4×10^{-7}
	Ro10-1670	6.4×10^{-6}
	SRI 5631-96	2.2×10^{-6}
	Ro13-7410	6.2×10^{-7}
	Ro15-0778	NC
	SRI 4657-47	NC
	Ro8-8717	NC
	Ro10-9359	NC
	Ro11-1430	NC
	NERA	NC
	CNERA	NC
	SRI 7167-67	NC

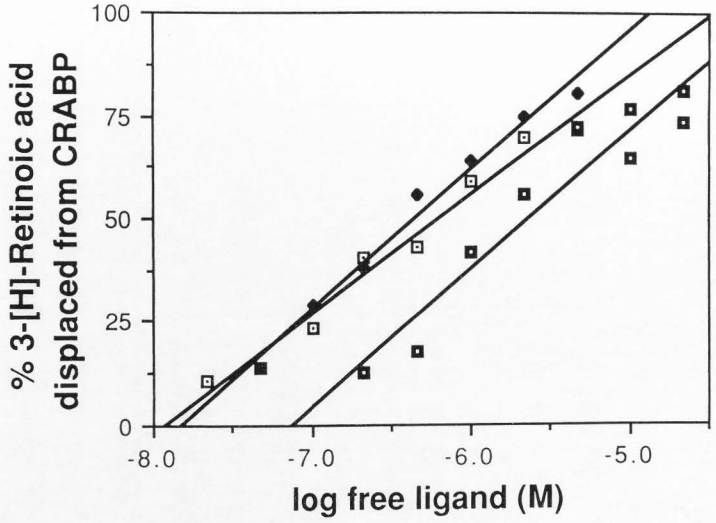


Figure VIII.3. Competition of retinoid analogs with day-12 hamster fetus cRABP. The displacement of [^{3}H]-all-trans-RA occurred with increasing concentrations of all-trans-RA (◆), Ro 13-7410 (⊡), or 13-cis-RA (◘).

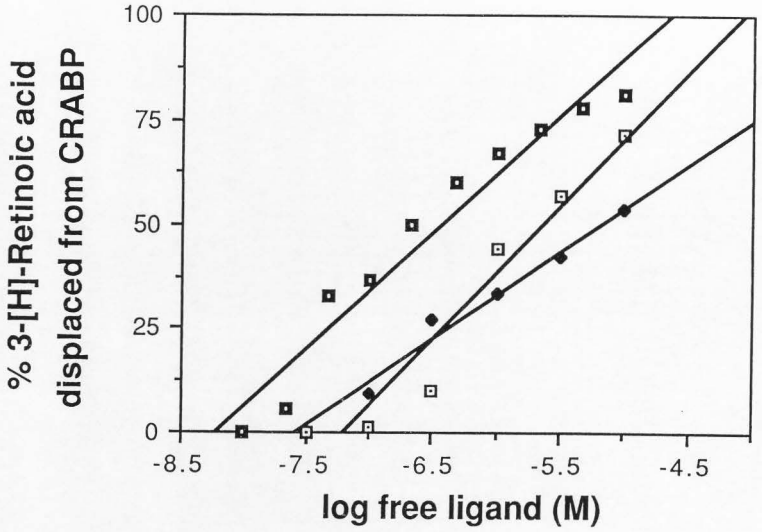


Figure VIII.4. Competition of retinoid analogs with day-12 hamster fetus cRABP. The displacement of [^{3}H]-all-trans-RA occurred with increasing concentrations of SRI 5631-96 (⊡), Ro 12-4824 (◘), or Ro 10-1670 (◆).

96 > 13-cis-RA > Ro 10-1670. Those with little or no affinity include SRI 4657-47, SRI 7167-67, NERA, CNERA, Ro 15-0778, Ro 8-8717, Ro 11-1430, and Ro 10-9359.

DISCUSSION

Cellular retinoic acid-binding protein (cRABP) exists in the embryonic day-eight hamster, as well as in the day-12 hamster fetus. The protein has a high degree of specificity for all-trans-RA. The saturation analysis plot was linear which is indicative of the exsistence of a single receptor site. This was also the conclusion reached by Kwarta et al. (1985) and Maden and Summerbell (1986).

From competition study data, binding with cRABP was dependent on a free acid at position C15. Retinoids without a polar terminus (Ro 15-0778) or those retinoids that contain ester or amide substitutions at C15 failed to displace [^{3}H]-all-trans-RA. This is consistent with reports of cRABP isolated from chick wing buds (Maden and Summerbell, 1986) and from hamster tissues (Brandes et al., 1983). A 15-fold difference in the binding affinities of various retinoids occurred. Ro 13-7410 had approximately 2-fold lower affinity with cRABP than all-trans-RA. However, Ro 13-7410 is at least 750 times more teratogenic in the hamster than all-trans-RA. The ethyl ester congener of etretin is twice as potent a teratogen in the hamster as all-trans-RA (Williams et al., 1984). Etretin served as the poorest ligand with cRABP. The primary metabolite of all-trans-RA in hamsters is all-trans-4-oxoretinoic acid (Ro 12-4824) (Frolik et al., 1979; 1980; Frolik, 1981). It bound with cRABP just as well as all-trans-RA, and it is as teratogenic as all-trans-RA (chapter IV). Isotretinoin (13-cis-RA) bound to cRABP with approximately 10-fold lower affinity than all-trans-RA, and it is one-half as teratogenic in hamsters as all-

trans-RA (Willhite and Shealy, 1984). This compares with the data of Maden and Summerbell (1986), who found that in the chick wing bud, Ro 13-7410 has 2-fold greater affinity with cRABP than all-trans-RA, and 13-cis-RA had a 6-fold greater affinity with cRABP. Etretin (Ro 10-1670) was the poorest ligand for cRABP, having an 18-fold lower affinity with cRABP. The present results contrast with those of Kwarta et al. (1985), who demonstrated that, in the mouse limb bud cRABP, all-trans-RA had a 315-fold greater affinity than 13-cis-RA.

A structure-activity relationship resulted, such that retinoids which bound with cRABP were teratogenic, and retinoids which failed to bind with cRABP were teratogenically inactive (unless they are esters). However, no apparent correlation emerged between in vivo teratogenic potency and the observed DC_{50}.

Petkovich et al. (1987) have recently demonstrated that a nuclear receptor for retinoic acid exists and that it belongs to a family of nuclear receptors similar to steroid receptors. It has been shown that [^{3}H]-all-trans-RA is translocated into the nucleus (Chytil and Ong, 1979) prior to differentiation. Retinoids may be modifying gene expression with a mechanism analogous to steroid receptor interactions (Pratt, 1984), whereby retinoic acid binds to cRABP, and the cRABP-retinoic acid complex subsequently is transported into the nucleus, where it can modify gene expression (Sporn and Roberts, 1983).

CHAPTER IX

SUMMARY AND CONCLUSIONS

Retinoids possess a high degree of biologic activity both in vivo and in vitro. Orally administered retinoids induce toxic side-effects, the worst of which is the severe terata that can occur if retinoid treatments occur during the first trimester of gestation. In an attempt to broaden the therapeutic index of retinoids, many investigators have synthesized several thousand retinoid analogs. From this research, I have demonstrated that consistent patterns of retinoid biologic activity emerge between cultured cell, cell-free assay systems, the hamster tracheal organ culture, and teratogenic potency. Thus, it appears that retinoids that possess therapeutic value also possess teratogenic activity.

The teratogenicity of retinoids containing either tetramethylated tetralin (Ro 13-6307 or Ro 13-2389) or tetramethylated indane (Ro 13-4306) ring systems substitutions was compared with the teratogenic potency of all-trans-retinoic acid. Single oral doses, administered to Syrian Golden hamsters at 10:00 a.m. on day 8 of gestation, induced a syndrome of malformations identical to that induced by treatment with all-trans-retinoic acid. These retinoids failed to induce signs of maternal hypervitaminosis A at doses associated with a significant teratogenic response. The tetramethylated tetralin retinoids and indane retinoid were 18 and 2.4 times as embryotoxic on a molar basis, respectively, as all-trans-retinoic acid. Introduction of a supplementary ring in the side-chain restricted polyene chain flexibility and maintained the hydrophobic plane of the chain. The present results are consistent with previous studies showing that the presence of or biotransformation to a free acid congener was necessary for retinoid teratogenic activity in hamsters and

that increasing conformational restriction of acidic retinoids increased teratogenic activity.

The teratogenic potency of congeners of all-trans-retinoic acid (all-trans-RA) containing modifications or substitution of the naturally occurring β-cyclogeranylidene ring was determined in Golden hamsters and compared to that of all-trans-RA. The following ring-modified retinoids were evaluated: phenyl (Ro 8-8717), furyl (Ro 8-9750), 4-methoxy-2,3,6-trimethylphenyl (Ro 21-6667), which also has a thiomethylene group in place of the trans-8,9 double bond of the etretin side-chain, 4-hydroxy-2,3,6-trimethylphenyl (Ro 11-4768), 2-chloro-3,6-dimethyl-4-methoxyphenyl (Ro 12-0995), 2-(1-methoxyethyl)-5,5-dimethyl-1-cyclopentenyl (Ro 10-1770), 2-acetyl-5,5-dimethyl-1-cyclopentenyl (Ro 8-7699), and 10,11-epoxy-11,11-dimethyl (juvenile hormone III), which also has the bonds saturated, corresponding to the 7,8- and 11,12- double bonds of the retinoid skeleton. The retinoids Ro 12-4824, Ro 12-4825, SRI 2712-24 had C4-keto, C18-hydroxyl, and C18-methyl substituents, respectively. Motretinid (Ro 11-1430) had both 4-methoxy-2,3,6-trimethylphenyl ring and ethyl amide polar group modifications. Single oral retinoid doses administered to pregnant dams at 10:00 A.M. on day eight neither induced signs of hypervitaminosis A nor induced weight loss in any of the treated groups. Teratogenically active retinoids induced a malformation syndrome identical to that induced by all-trans-RA. At retinoid doses that were associated with malformations in all of the fetuses, embryolethality remained near that of vehicle-treated controls. The phenyl retinoid Ro 8-8717 was embryolethal but was not teratogenic. The ethyl amide derivative of the human and animal teratogen etretinate, motretinid, was teratogenic only at the highest dose administered, 350 mg/kg. The retinoids Ro 12-4824, Ro

at the highest dose administered, 350 mg/kg. The retinoids Ro 12-4824, Ro 12-4825, Ro 8-7699, and SRI 2712-24 were as potent as all-trans-RA. The chlorine-substituted retinoid, Ro 12-0995, was 6-fold more teratogenic than all-trans-RA, and the cyclopentene retinoid, Ro 10-1770, was 19 times more potent than all-trans-RA. The retinoids with furyl or epoxy group substitution for the cyclohexenyl ring were devoid of teratogenic activity up to equimolar doses of 75 mg/kg of all-trans-RA, and Ro 21-6667 was teratogenically inactive at a dose equivalent to 150 mg/kg of all-trans-RA. Major modifications of the β-cyclogeranylidene ring can be made without altering teratogenic activity. The ring need not be six-membered and can have decreased lipophilicity through the incorporation of polar groups compared to retinoic acid but must have sufficient lipophilic substituents to provide the necessary mass for interaction with the retinoid receptor. The present results indicate that those ring-modified retinoids having cell differentiating activity also show teratogenic activity.

Additionally, I have investigated several aspects (pharmacokinetics and retinoid affinity with cellular retinoic acid-binding protein, cRABP) in an attempt to explain the large variation of retinoid-induced teratogenic potency. In distribution studies with all-trans-RA, 13-cis-RA, all-trans-4-oxo-RA, 9-cis-retinal, retinyl acetate, CNERA, NERA, and Ro 13-7410, the radiolabel associated with each retinoid crossed the placenta and entered the conceptus. These retinoids, or a metabolite of these retinoids, is present in the conceptus. Thus, the teratogenically inactive CNERA and NERA (and/or their metabolites) reach the target tissue, but fail to induce a teratogenic response.

The pharmacokinetic disposition and distribution properties of all-trans-RA, 13-cis-retinoic acid, all-trans-4-oxoretinoic acid, 9-cis-retinal, and retinyl

acetate were determined in pregnant hamsters. The retinoid all-trans-RA was eliminated rapidly from the maternal blood (elimination $t_{1/2}$ = 0.5 h) and was metabolized to its isomer, 13-cis-retinoic acid, and to all-trans-4-oxoretinoic acid, both of which persisted in higher amounts and for longer periods of time. Isotretinoin (13-cis-retinoic acid) exhibited a longer elimination half-life of 4.41 h; it was metabolized to the trans isomer and also to 13-cis-4-oxoretinoic acid. The all-trans-4-oxoretinoic acid persisted in the maternal plasma for 24 h and had an elimination half-life of 5.57 h. No amount of parent compound of either 9-cis-retinal or retinyl acetate was detected in the maternal plasma. The free acid retinoids were distributed to all sampled tissues; the lowest amount of radioactivity was found in the perirenal fat. The aldehyde and acetate ester congeners were distributed primarily to the lung and liver with lesser amounts present in remaining tissues sampled.

The pharmacokinetic parameters of N-ethyl-13-cis-retinamide (CNERA) and N-ethyl-all-trans-retinamide (NERA) were determined in pregnant hamsters. CNERA was eliminated from the maternal plasma following two separate constants; whereas, NERA was eliminated exponentially. Both CNERA and NERA were biotransformed to NERA and CNERA, respectively, but neither was metabolized to a free acid in appreciable amounts. The bioavailability of the retinamides was 10-fold lower than for free-acid retinoids. The retinamides are teratogenically inactive, apparently because they are not biotransformed to a free acid at C15.

Arotinoid Ro 13-7410 was investigated to determine its in vivo pharmacokinetic profile in pregnant hamsters. The arotinoid exhibited two elimination half-times of 1.52 and 2.45 h, corresponding to the rapid and slower elimination phases, respectively. Ro 13-7410 was distributed to all

sampled tissues by 4 h with the highest concentrations occurring in hepatic tissues. Peak concentrations of 200 pmol/g plasma were observed 3.5 h following a dose of 1000 ug/kg. A polar metabolite appeared at 0.5 h and reached peak circulating concentrations at 12 h after dosing. The pharmacokinetic disposition of Ro 13-7410 may not account for its high teratogenic potency.

All-trans-RA is twice as potent a teratogen as 13-cis-RA; however, all-trans-RA's elimination half-life, area under the curve, and peak plasma concentration were less than or equal to the same parameters observed with 13-cis-RA. No amount of parent 9-cis-retinal was detected in the maternal plasma, yet it is one-half as teratogenic as all-trans-RA. The retinoid Ro 13-7410 had elimination half-lives similar to those observed for the retinoids 13-cis-RA and all-trans-4-oxo-RA, yet it is 1000-fold more potent teratogen than all-trans-RA. Therefore, an obvious correlation between teratogenic potency, peak plasma concentration, area under the curve, or elimination half-life was not observed.

The binding affinities of 14 retinoid analogs were determined with day-12 hamster fetus cellular retinoic acid-binding protein (cRABP) by utilization of high-specific activity [^{3}H]-all-trans-RA. High performance size exclusion chromatography (HPSEC) was employed for isolation of cRABP. Retinoids that failed to compete with [^{3}H]-all-trans-RA were also teratogenically inactive, with the exception of the ethyl ester of etretin, etretinate, which does compete. Of retinoids that did compete, all-trans-RA = Ro 12-4824 < Ro 13-7410 < SRI 5631-96 < 13-cis-RA < etretin. Binding with cRABP may be essential for teratogenic activity, but binding affinities of retinoids with cRABP do not correlate with their in vivo teratogenic potency.

A putative intracellular receptor for retinoids is cellular retinoic acid-binding protein (cRABP). If cRABP is the ultimate receptor that mediates retinoid-induced teratogenicity, a structure-activity relationship should exist between retinoid binding affinities with cRABP and the retinoids' in vivo teratogenic potency. I determined the binding affinity of a series of retinoids with cRABP. Retinoids that were teratogenically inactive (i.e., amides, retinoids lacking a polar terminus, or retinoids without sufficient ring substituents) failed to bind with cRABP. Retinoids that bound with cRABP were also found to exhibit teratogenic activity. However, the binding affinities of retinoids that bound with cRABP varied by 15-fold, compared with a 2,000-fold variation for teratogenic activity. Subsequently, retinoid binding with cRABP may be an obligatory step in retinoid-induced teratogenesis.

In view of the fact that retinoids are metabolized (i.e., isomerized and/or oxidized) to other teratogenic forms, the total amount of active retinoid present in the target tissue needs to be assessed. Further studies evaluating this aspect of retinoid-induced teratogenicity should be initiated. Additionally, in vivo competition studies utilizing displacement of radiolabelled all-trans-RA from target tissues with other teratogenically active retinoids may provide this important structure-activity relationship. This information would enable researchers to determine if retinoid-induced teratogenicity is receptor-mediated.

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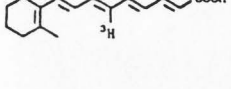
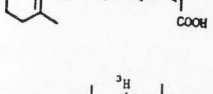
APPENDIX

MOLECULAR STRUCTURES OF RETINOIDS USED IN CHAPTERS V, VI, AND VII

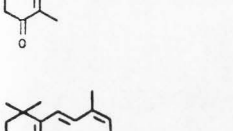
[10,11-$^{3}H_2$]-all-trans-retinoic acid



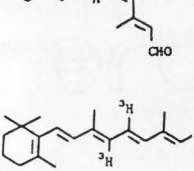
[11-^{3}H]-13-cis-retinoic acid



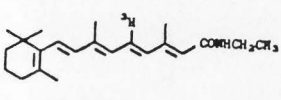
[11-^{3}H]-all-trans-4-oxo-retinoic acid



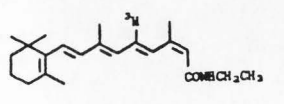
[11-^{3}H]-9-cis-retinal



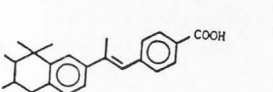
[10,11-$^{3}H_2$]-retinyl acetate



[11-^{3}H]-N-ethyl-all-trans-retinamide



[11-^{3}H]-N-ethyl-13-cis-retinamide



[5,6-$^{3}H_2$]-Ro 13-7410

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PROFESSIONAL ORGANIZATIONS

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PUBLICATIONS

Howard, W.B. and C.C. Willhite. 1986. Toxicity of retinoids in humans and animals. J. Toxicol., Toxin Reviews. 5(1):55-94.

Howard, W.B., C.C. Willhite, and R.P. Sharma. 1987. Structure-toxicity relationships of tetramethylated tetralin and indane analogs of retinoic acid. Teratology, 36(3):303-311.

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ABSTRACTS

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Howard, W.B. and F. deNoyelles. 1984. Phytoplankton develop resistance to atrazine herbicide. Mountain-West Chapter of the Society of Toxicology, Logan, Utah.

GRANTS

Sharma, R.P., C.C. Willhite, and W.B. Howard. 1988. Mechanisms of retinoid-induced malformations. March of Dimes Birth Defects Foundation, White Plains, NY. $50,000.

AWARDS

FASEB Summer Research Conference on MICRONUTRIENTS: RETINOIDS, 1986.

Teratology Young Investigator Award, 1987.

PERSONAL

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