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Limb bud cell cultures for estimating the teratogenic potential of compounds

Validation of the test system with retinoids

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Abstract. Mesenchyme cells, derived from embryonic limb buds, cultured at high cell density, multiply and differentiate into chondrocytes. Using alcian blue, a stain specific for cartilage proteoglycans, the degree of chondrogenesis can be visualized in the micromass cultures as well as quantified by extraction of the stain and spectrophotometric determination of its absorbance. In the presence of active retinoids chondrogenesis is concentration-dependently inhibited. For comparison of the activity of the various retinoids the concentration needed to reduce alcian blue staining by 50% was estimated. In order to validate whether the activity in limb bud cells can predict the teratogenic potential in vivo, the in vitro activity of 25 retinoids was compared with their in vivo teratogenicity observed mainly in rodents. For retinoids which were already in the biologically active form like those with a free carboxylic acid endgroup, there was a good quantitative correlation between the in vitro and in vivo activity. In contrast, the ethylester analog etretinate was slightly active and the ethylamine analog motretinide inactive in vitro but both were teratogenic in vivo. This finding may indicate that these retionoids were not metabolized to the active form in vitro. In conclusion, these results suggest that the limb bud cell culture system may be useful for a preliminary testing to select non-teratogenic retinoids. For the risk assessment in humans, however, the in vitro result should be verified in animal studies.

Key words: Retinoids – Teratogenicity in vivo – Teratogenicity in vitro – Limb bud cell cultures – Micromass culture

Introduction

During the last decade, various scientists developed in vitro methodology for the assessment of potential teratogenic risk. This task was also forced by the pressure imposed by society to reduce or replace experiments in the whole animal.

Although several in vitro tests are described (Legator 1982; Homburger and Goldberg 1985) only limited information is available on the validity of these tests. Presently it is imperative to validate and standardize each of the proposed tests before they can gain general acceptance. There are two ways for the validation of in vitro methods (Neubert 1985); (1) The use of a series of closely related chemicals of which the in vivo activity is known and which includes clearly positive and clearly negative compounds, (2) the use of a large series of unknown substances which are simultaneously tested in vivo and in vitro.

During the last years a large number of retinoids has been synthesized with marked quantitative differences in their biological activity including teratogenicity. Therefore, retinoids are an excellent class of compounds to validate an in vitro method developed for the determination of teratogenic potential. Primary cultures of limb bud cells have been proposed to be useful to detect the teratogenic potential of compounds (Wilk et al. 1980; Hassell and Horigan 1982; Guntakatta et al. 1984; Flint and Orton 1984; Kistler 1985). Mesenchymal cells of limb buds proliferate and differentiate into chondrocytes when cultured at high cell density (Umansky 1966; Caplan 1970). This differentiation process is inhibited by retinoids (Lewis et al. 1978; Gallandre et al. 1980; Kistler et al. 1985; Kistler 1985).

We here report on the activity of a selected number of retinoids in limb bud cells in vitro and compare this activity with their in vivo teratogenicity. For retinoids with a carboxylic acid endgroup there is a good quantitative correlation indicating that the limb bud cell test can be used for a preliminary assessment of the teratogenic potential of retinoids. The advantages but also the limitations of this in vitro test are then considered.

Materials and methods

Animals. Fü albino rats and mice, outbred stock and Swiss hare rabbits were obtained from the Institute of Biological and Medical Research (Füllinsdorf, Switzerland). Rats and mice were mated overnight and females which had a vaginal plug present the following morning were considered to be at day 1 post coitum (p.c.). Female rabbits were placed together with a male until copulation was observed. The day of copulation was designated as day 1 p.c.

Limb bud cell cultures. Details of the culture technique of limb bud cells were reported (Kistler et al. 1985; Kistler 1985). In brief, fore- and hindlimb buds of day 14 embryos were dissociated in calcium-magnesium-free Gey's balanced salt solution containing 0.1% trypsin, 0.1% EDTA and 50 mM tris, pH 7.3 at 37° C. The cell density was ad-

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justed to 2×10^7 viable cells/ml in CMRL medium containing 10% Nu-serum and antibiotics. High cell density cultures were set up by dispensing 20 µl of the cell suspension as a discrete drop in the centre of each well of a 24-well dish. The cells were allowed to settle and adhere to the dish for 1.5–2 h at 37° C before they were flooded with medium. Retinoids were dissolved in dimethylsulfoxide and added on day 1 of culture. An equivalent amount (0.25%) of the solvent was added to the control cultures.

The morphological differentiation was monitored by phase contrast microscopy. The accumulation of cartilage proteoglycans was determined by alcian blue staining. The bound dye was extracted from the cultures with 4 M guanidine hydrochloride and the absorbance at 600 nm was determined spectrophotometrically.

For the calculation of the inhibition concentration 50% (IC_{50}), the measured absorbance values m_i ($i = 1, \ldots, n$) of the concentration groups were divided by the mean value M of the corresponding control group: $q_i = m_i/M$. A mean value of the quotients q_i equal to 0.5 expresses therefore a 50% inhibition. The following logit model was fitted to the values q_i for each substance:

$$q_i = \frac{1}{1 + e^{-\beta(x_i - \mu)}} + \varepsilon_i$$

where x_i are the logarithms of the experimental concentrations, ε_i are the residues, and the estimated parameters μ and β are the logarithm of the inhibition concentration 50% and the slope, respectively. The calculations were performed with PROC NLIN from The Statistical Analysis System SAS (SAS-Institute, Cary, USA).

Embryotoxicity/teratogenicity studies in vivo. In our studies rape seed oil was the vehicle for all retinoids. The treatment was orally from days 7 to 16 p.c. in mice and rats and from days 7 to 19 p.c. in rabbits. Terminal necropsy was performed on days 19, 21 and 30 in mice, rats and rabbits, respectively. The number of fetuses, implantation and resorption sites was recorded. Fetuses were weighed and examined for external abnormalities. The head of each fetus was fixed and examined for cleft palate by dissection. In some pilot studies the beheaded body was prepared for skeletal examination using a NaOH-alizarin red S staining procedure.

Materials

Trypsin, CMRL-medium and antibiotics were obtained from GIBCO Europe (Glasgow, Scotland); the serum substitute Nu-serum from Collaborative Research Inc. (Lexington, MA); 24 well dishes from Costar (Cambridge, MA); the retinoids from F. Hoffmann-La Roche (Basle, Switzerland). All other materials were of reagent grade.

Results

Inhibition of chondrogenesis by retinoids in limb bud cell cultures

Mesenchyme cells, derived from embryonic limb buds, cultured at high density (micromass), multiply and differentiate into chondrocytes. Chondrogenesis is expressed morphologically by the formation of cartilage nodules and biochemically by the synthesis and accumulation of an extracellular matrix product, the cartilage proteoglycans. Using alcian blue, a stain specific for cartilage proteoglycans, the degree of chondrogenesis can be visualized in the micromass cultures (Fig. 1) as well as quantified by extraction of the stain and spectrophotometric determination of its absorbance (Kistler et al. 1985; Kistler 1985). In the presence of active retinoids chondrogenesis is inhibited in a concentration-dependent manner (Fig. 2). For comparison of the different activity of the various retinoids the concentration needed to reduce alcian blue staining by 50% is indicated. Computer-calculated IC_{50} values with the confidence limits for all the retinoids tested are shown in Table 1.

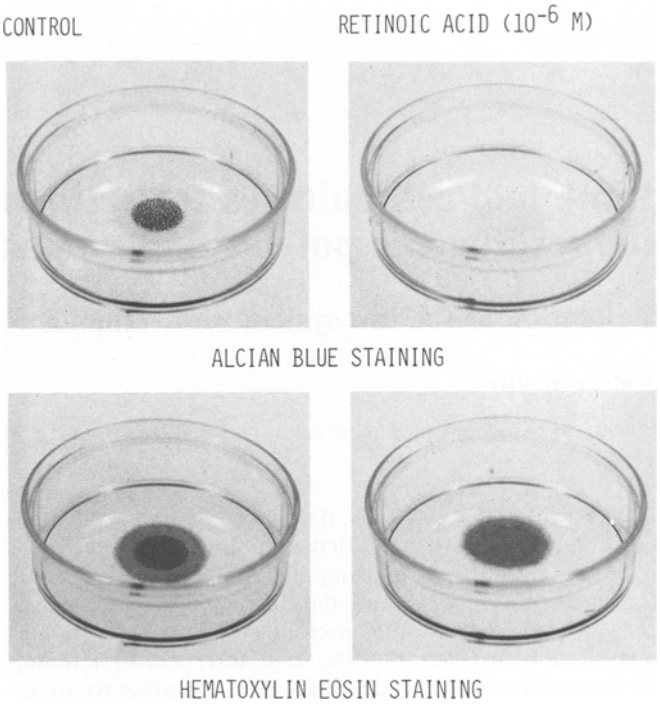


Fig. 1. Effect of retinoic acid on growth and differentiation of limb bud mesenchyme cells. Limb bud cells of rat embryos were cultured at high cell density (micromass cultures) for 7 days. In the presence of retinoic acid, chondrogenesis which was assessed by alcian blue staining, a stain specific for cartilage proteoglycan, was completely suppressed. However, hematoxylin-eosin staining demonstrated that the outgrowth of cells (fibroblast-like cells judged from phase contrast examination) from the spot of cells plated at day 0 was not affected. This finding indicated that retinoic acid was not cytotoxic but specifically inhibited chondrogenesis

Embryotoxicity/teratogenicity studies in vivo

Pilot studies were undertaken to investigate the possible embryotoxic/teratogenic effects of selected retinoids whose teratogenic potential was not yet known (Table 2). The most frequently observed malformations induced by retinoids in rodents are exencephaly, malformations of the head and eye, cleft palate and limb defects (Fig. 3). These malformations can be easily detected by external examination of fetuses at the time of cesarean section. Thus, in the pilot studies the fetuses were examined for external malformations and for cleft palate by dissection. In some studies the beheaded body was examined for severe skeletal malformations especially of the limbs. In general, the mal-

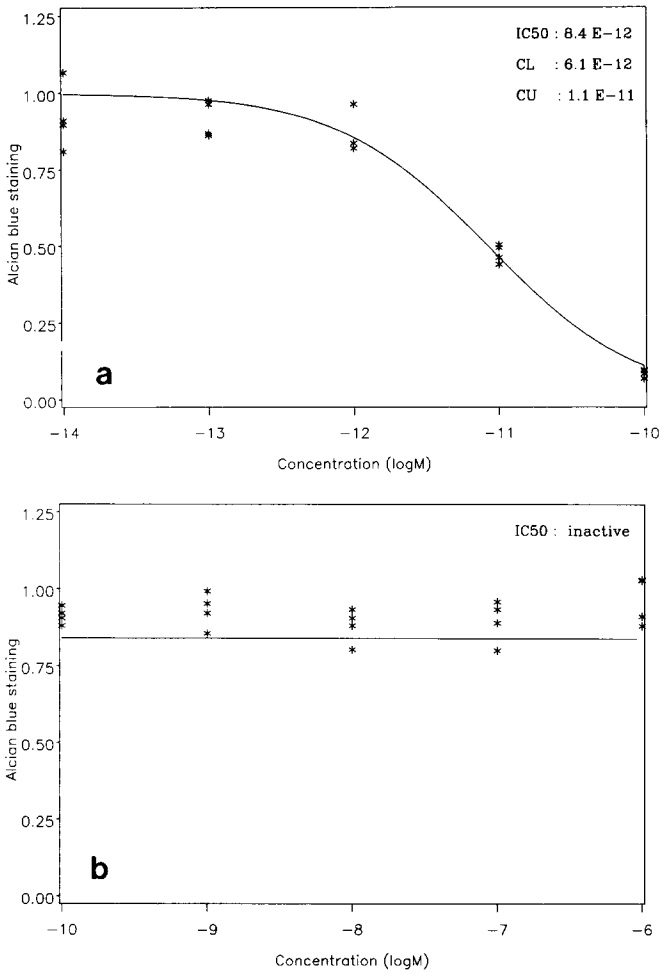


Fig. 2. Dose-response curves of two retinoids drawn from the computer-calculated IC_{50} estimation. **a** Retinoid No. 11, **b** retinoid No. 19. *CL* and *CU*, lower and upper confidence limits, respectively

formation pattern observed with the various retinoids was the same as caused by maternal hypervitaminosis A (Geelen 1979). Some representative, malformed fetuses are shown in Fig. 4. To enable a better comparison of the in vivo teratogenicity with the in vitro activity the lowest teratogenic dose in each species was determined and compiled in Table 1.

Comparison of the in vitro activity of retinoids with their teratogenicity in vivo

For retinoids containing a carboxylic acid endgroup there was a good quantitative correlation between the IC_{50} in limb bud cells and their in vivo teratogenicity (Fig. 5). A good correspondence was also found with the retinoid No. 19, lacking a terminal endgroup, which was inactive in the limb bud test and was not or only questionably teratogenic at the high dose of 300 mg/kg in rats. In addition, compounds 18, 21, 22, 23, 24 and 25 were active in vitro as well as in vivo. However, etretinate (No. 16) which was slightly active and motretinide (No. 17) which was inactive in vitro, were both markedly teratogenic in vivo. This lack of in vitro activity might reflect that these "prodrugs" were not metabolized to the active principle.

Besides the two "prodrugs" etretinate and motretinide, there was another retinoid whose activity in vitro did not correlate with the in vivo teratogenicity. Fenretinide (No. 20) was active in vitro but was not teratogenic in mice and rats up to the highest dose tested of 300 mg/kg.

Discussion

This study demonstrates that limb bud cell cultures can be used as a simple and rapid test for a preliminary estimation of the teratogenic potential of retinoids. Under the conditions used, the test is, however, limited to retinoids which are already in their biologically active form or can be metabolically activated within the cultured cells.

Table 1. Comparison of the IC_{50} values of retinoids in limb bud cell cultures with their in vivo teratogenicity

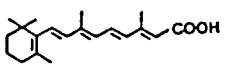
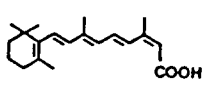
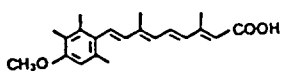
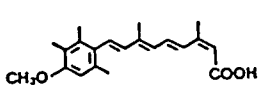
No.	Trivial name	Chemical structure	Limb bud cells (nM) IC_{50}	Confidence limits		In vivo teratogenicity, lowest teratogenic dose (mg/kg/day)		
				Lower	Upper	Mouse	Rat	Rabbit
Retinoids with a carboxylic acid end group								
1	Retinoic acid, tretinoin		80	50	100	4[a]	0.4–2[a]	2–10[a]
2	13-*cis*-Retinoic acid, isotretinoin		400	200	800	100	150[b]	10[b]
3	Etretin, Ro 10–1670		50	40	60	3[c]	15[c]	0.6[c]
4	13-*cis*-Etretin		200	200	400	100	≤3	≤3

Table 1. (continued)

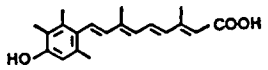
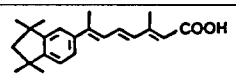
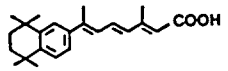
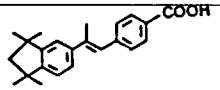
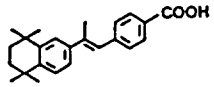
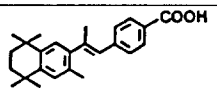
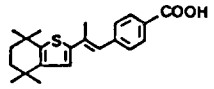
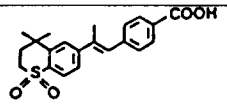
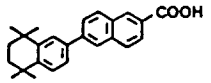
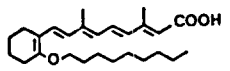
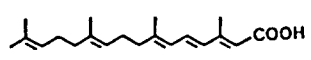
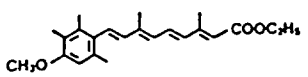
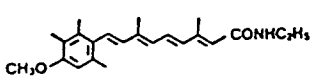
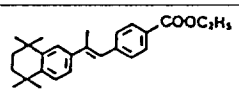
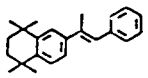
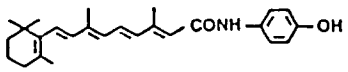
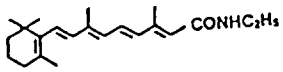
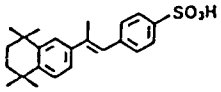
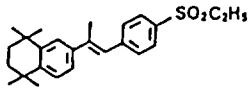
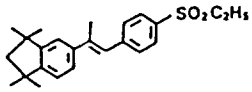
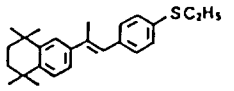
No.	Trivial name	Chemical structure	Limb bud cells (nM) IC_{50}	Confidence limits		In vivo teratogenicity, lowest teratogenic dose (mg/kg/day)		
				Lower	Upper	Mouse	Rat	Rabbit
Retinoids with a carboxylic acid end group								
5			2000	800	3000	100		
6			0.6	0.5	0.7	1	0.3	
7			0.3	0.2	0.4	0.1	0.3	
8			0.03	0.02	0.04	0.01	0.01	
9	Ro 13-7410, arotinoid, TTNPB		0.06	0.04	0.09	0.003	0.01	0.001
10	3-Methyl TTNPB		5	3	8	1	1	
11			0.008	0.006	0.01	0.003	0.001	
12			20	10	20	10	3	
13	TTNN		0.2	0.1	0.4	0.1	0.3	
14			800	90	6000	30		
15	Polyprenoic acid		800	600	1000	300		
Retinoids with a modified end group								
16	Etretinate, Ro 10-9359		3000	2000	8000	4[d]	8[d]	2[d]
17	Motretinide, Ro 11-1430		Inactive			5[e]	10[e]	5[f]
18	Ro 13-6298, TTNPB ethylester		0.1	0.06	0.2	0.005	0.015	0.01

Table 1. (continued)

No.	Trivial name	Chemical structure	Limd bud cells (nM) IC_{50}	Confidence limits Lower	Upper	In vivo teratogenicity, lowest teratogenic dose (mg/kg/day) Mouse	Rat	Rabbit
Retinoids with a modified end group								
19	Temarotene, Ro 15–0778		Inactive			>300	300 (?)	>300
20	Fenretinide, HPR		500	400	800	>300	>300	
21	N-Ethylretinamide		7000	1000	50000	300 (?)	300	
Retinoids with a sulfur-containing end group								
22	Arotinoid sulfonate		4	2	6	10		
23	Arotinoid ethylsulfone		8	4	20	1	3	>300
24			4000	2000	8000	30	30	>30
25	Arotinoid ethylsulfide		20	9	40	≤1	≤3	

[a] Kamm et al. 1984
[b] Kamm 1982
[c] Kistler and Hummler 1985
[d] Hummler and Schüpbach 1982
[e] Hummler H. Unpublished data
[f] Kistler A. Unpublished data

The potential of retinoids to induce teratogenic effects in animals (Geelen 1979) as well as in man (Rosa 1983; Happle et al. 1984; Stern et al. 1984; Lammer et al. 1985) is a marked handicap for the clinical use of this class of compounds, mainly in dermatology (Peck 1981, 1983; Bollag 1983). The importance of separating the undesirable side-effects of retinoids and especially the teratogenic potential, from their beneficial effects has been emphasized. To fulfil this goal, an early assessment of the teratogenic potential of retinoids is a prerequisite to enable the selection of compounds with low or no teratogenic activity for drug development. To fill this requirement a large number of analogs has to be tested, task which would be more suitable solved with a short-term in vitro test than with laborious teratological studies in vivo needing a large number of animals.

Many retinoids are available with marked differences in their biological activity over a dose range of more than 5 orders of magnitude (Loeliger et al. 1980; Kistler 1981, 1984, 1986). Because it was not yet possible to dissociate the desirable therapeutic effects from the side-effects of retinoids, teratogenicity is also induced over such a broad dose range. Thus, this class of compounds offers a powerful implement to accomplish whether the limb bud cell test can predict teratogenicity in vivo.

Characteristics of limb bud cell cultures

Cell and organ cultures provide effective tools to investigate the interaction and structure-activity relationship of retinoids and other compounds at a specific target site. In contrast to the complex responses in vivo which reflect absorption, distribution, metabolism and elimination of a compound as well as indirect effects due to action at other sites, responses in vitro are limited to the cells and tissues cultured. The comparison of effects in culture with responses in vivo may thus help to identify metabolic and

Table 2. Sumary of pilot embryotoxicity and teratogenicity studies with various retinoids

Retinoid No.	Species	Assessment	Dosage mg/kg	No. of dams	No. of resorpt./ No. of implant.	Perc. resorpt.	No. of fetuses/ dam	Fetal body weight (g)	No. of fetuses malformed/ examined	Observations fetuses	Observations dams
2	Mouse		10	7	12/109	11.0	13.9±1.5	1.4±0.2	0/ 97	NF	NF
			30	6	9/ 96	9.4	13.7±3.9	1.2±0.2	0/ 98	NF	NF
		T	100	8	20/120	16.7	12.5±0.8	1.3±0.2	15/100	3EX, 3CP, 1EO, 15LM	NF
		T+E (?)	300	8	26/133	19.5	13.4±4.9	1.2±0.2	34/107	1EX, 1HM, 14CP, 19LM	NF
4	Mouse		3	7	8/103	7.8	13.6±3.1	1.5±0.1	3/ 95	3HLC	NF
			10	7	4/108	3.7	14.9±2.3	1.6±0.2	1/104	1HLC	NF
			30	8	15/128	11.7	14.1±2.3	1.5±0.1	1/113	1HLC	NF
		T	100	8	16/119	13.4	12.9±3.2	1.5±0.1	1/103	1CP	NF
		T	200	8	9/128	7.0	14.6±2.3	1.5±0.1	21/119	20CP, 3EO, 1EC	NF
4	Rat	T	3	6	3/ 79	3.8	12.7±1.4	3.4±0.2	3/ 76	3CP	NF
		T	10	6	7/ 77	9.1	11.7±2.3	3.4±0.1	4/ 70	4CP	NF
		T	30	6	14/ 69	20.3	9.2±4.8	3.4±0.3	1/ 55	1CP	NF
		T	100	5	2/ 62	3.2	12.0±4.2	3.3±0.1	1/ 60	1CP	NF
		T	200	6	5/ 82	6.1	12.8±2.6	3.3±0.1	5/ 77	1HM, 4CP	NF
4	Rabbit	T+E	3	5	40/ 45	88.9	1.0±1.0	42.4±6.1	5/ 5	1EO, 1SB, 1LM, 2FLC, 4TM, 1EC	NF
		T+E	10	5	38/ 42	90.5	0.8±1.8	30.4±0.0	4/ 4	2HM, 4CP, 2EO, 3FLC, 3HLC, 1TM	slight HVA
		E	30	3	31/ 31	100.0	0.0±0.0	0.0±0.0	0/ 0		moderate HVA
5	Mouse		10	7	10/105	9.5	13.6±2.0	1.5±0.1	0/ 95	NF	NF
		E (?)	30	7	25/106	23.6	11.6±5.5	1.4±0.1	0/ 81	NF	NF
		T+E (?)	100	6	20/ 86	23.3	11.0±5.5	1.5±0.1	1/ 66	1EX, 1HM, 1EO	NF
		T+E	300	6	30/ 85	35.3	9.2±4.8	1.4±0.2	19/ 55	8EX, 10HM, 14CP, 2EO, 4HLC, 4TM	NF
6	Mouse		0.01	7	10/114	8.8	14.7±4.0	1.5±0.1	0/104	NF	NF
			0.03	6	11/ 97	11.3	14.2±1.9	1.4±0.1	1/ 86	1EX	NF
			0.1	7	10/103	9.7	13.0±5.7	1.6±0.2	1/ 93	1HLC	NF
			0.3	6	10/101	9.9	15.2±3.4	1.5±0.1	0/ 91	NF	NF
		T	1.0	6	15/100	15.0	13.8±5.9	1.6±0.1	10/ 85	5EX, 5HM, 1CL, 10CP, 1EO, 5LM	NF
6	Rat		0.01	8	4/ 94	4.3	11.3±3.4	3.3±0.3	2/ 90	2RM	NF
			0.03	8	12/100	12.0	11.0±4.6	3.4±0.4	2/ 88	1EC, 1RM	NF
			0.1	5	11/ 64	17.2	10.6±2.7	3.6±0.2	0/ 53	NF	NF
		T	0.3	8	4/106	3.8	12.8±0.7	3.5±0.2	21/102	1EX, 2HM, 1EO, 19LM	NF
		T	1.0	7	8/ 95	8.4	12.4±1.7	3.4±0.2	87/ 87	1EX, 4VB, 1HM, 19CP, 87LM	slight HVA
7	Mouse		0.003	7	19/ 91	20.9	10.3±4.9	1.5±0.2	1/ 72	1EX, 1HM, 1EO	NF
			0.01	8	21/123	17.1	12.8±5.0	1.4±0.1	1/102	1EX, 1HM, 1EO	NF
			0.03	8	24/120	20.0	12.0±4.5	1.5±0.2	1/ 96	1EX, 1HM	NF
		T	0.1	7	20/101	19.8	11.6±3.8	1.5±0.2	25/ 81	6EX, 6HM, 5EO, 1CP, 24LM	NF
		T+E	0.3	8	42/127	33.1	10.6±4.1	1.4±0.2	85/ 85	15EX, 15HM, 21EO, 65CP, 14RM, 85LM	NF
7	Rat		0.003	6	8/ 60	13.3	8.7±5.6	3.2±0.8	0/ 52	NF	NF
			0.01	8	8/ 92	8.7	10.5±5.4	3.1±0.6	0/ 84	NF	NF
			0.03	6	3/ 77	3.9	12.3±1.8	3.5±0.2	0/ 74	NF	NF
			0.1	8	10/103	9.7	11.6±3.3	3.4±0.3	0/ 93	NF	NF
		T	0.3	7	13/ 81	16.0	9.7±5.9	3.7±0.8	58/ 68	10EX, 14VB, 14HM, 13CP, 58LM	slight HVA
8	Mouse		0.003	7	8/117	6.8	15.3±2.8	1.5±0.0	0/109	NF	NF
		T	0.01	6	10/103	9.7	15.5±2.7	1.5±0.2	5/ 93	1HM, 4HLC	NF
		T	0.03	7	14/110	12.7	13.6±2.1	1.6±0.1	64/ 96	1EX, 2CP, 64HLC, 64LM	NF
		T+E	0.1	7	71/ 89	79.8	2.6±3.0	1.3±0.1	18/ 18	6EX, 17HM, 1EO, 1CL, 18CP, 18LM, 18TM	slight HVA
		E	0.3	7	97/ 97	100.0	0.0±0.0	0.0±0.0	0/ 0		moderate HVA

Table 2. (continued)

Retinoid No.	Species	Assessment	Dosage mg/kg	No. of dams	No. of resorpt./ No. of implant.	Perc. resorpt.	No. of fetuses/ dam	Fetal body weight (g)	No. of fetuses malformed/ examined	Observations fetuses	Observations dams
8	Rat		0.003	7	14/ 94	14.9	11.4±1.5	3.2±0.2	0/ 80	NF	NF
		T	0.01	6	3/ 76	3.9	12.2±1.2	3.7±0.3	9/ 73	2CP, 7LM	NF
		T+E (?)	0.03	8	17/ 97	17.5	10.0±4.6	3.0±0.2	80/ 80	22EX, 8VB, 80HM, 68CP, 7EO, 10ASM, 1RM, 8LM	NF
		T+E	0.1	8	92/ 94	97.9	0.3±0.7	2.5±0.0	2/ 2	2 with EX, HM, CP, EO, SB, ASM, TM, RM, LM, AGM	slight HVA
		E	0.3	8	103/103	100.0	0.0±0.0	0.0±0.0	0/ 0		moderate HVA
9	Mouse		0.0003	8	10/108	9.3	12.1±2.9	1.4±0.2	0/ 98	NF	NF
			0.001	6	7/ 91	7.7	14.0±4.6	1.4±0.3	0/ 84	NF	NF
		T	0.003	8	13/116	11.2	12.9±2.4	1.4±0.3	5/103	1EX, 4CP, 1EC	NF
		T	0.01	6	12/ 90	13.3	13.0±2.5	1.3±0.2	78/ 78	11EX, 1VB, 9HM, 74CP, 20EO, 78LM, 40TM	NF
9	Rat		0.0003	8	18/ 99	18.2	10.1±4.2	3.4±0.2	1/ 81	1EC	NF
			0.001	8	9/113	8.0	13.0±1.8	3.2±0.6	0/104	NF	NF
			0.003	8	8/110	7.3	12.8±1.5	3.3±0.2	0/102	NF	NF
		T	0.01	8	4/103	3.9	12.4±3.0	3.2±0.2	48/ 99	2EX, 1VB, 48CP	NF
9	Rabbit		0.0003	4	2/ 38	5.3	9.0±5.7	41.4±9.2	0/ 36	NF	NF
		T	0.001	5	7/ 32	21.9	5.0±2.0	43.2±7.1	7/ 25	4CP, 4TM	NF
		T+E	0.003	6	20/ 48	41.7	4.7±2.4	40.1±6.2	28/ 28	19HM, 27CP, 19EO, 1EC, 20LM, 13TM	slight HVA
10	Mouse		0.03	5	22/ 75	29.3	10.6±5.9	1.5±0.1	0/ 63	NF	NF
			0.1	5	8/ 69	11.6	12.0±1.9	1.6±0.1	0/ 74	NF	NF
		T (?)	0.3	4	1/ 56	1.8	13.8±1.7	1.6±0.1	1/ 66	1EC	NF
		T	1	7	9/105	8.6	13.7±1.4	1.7±0.1	40/ 96	3EX, 3HM, 3EO, 21CP, 40LM	NF
		T+E	3	7	47/103	45.6	8.0±5.2	1.4±0.1	56/ 56	20EX, 1VB, 56HM, 10EO, 56CP, 1EC, 4ACM, 1ASM, 17TM, 1RM, 56LM	NF
10	Rat		0.03	7	10/101	9.9	13.0±2.4	3.3±0.1	0/ 91	NF	NF
			0.1	8	10/113	8.8	12.9±2.7	3.3±0.2	0/103	NF	NF
			0.3	8	11/ 96	11.5	10.6±2.6	3.4±0.2	0/ 85	NF	NF
		T	1	8	21/112	18.8	11.4±2.8	3.0±0.2	89/ 91	17EX, 6VB, 89HM, 5EO, 84CP, 1CL, 3ASM, 2RM, 77LM	NF
		T+E	3	7	96/ 99	97.0	0.4±1.1	2.3±0.0	3/ 3	2EX, 3HM, 3CP, 3ASM, 1RM, 3TM, 3LM	slight HVA
11	Mouse		0.0001	6	13/ 89	14.6	12.3±1.9	1.4±0.2	1/ 76	1HLC	NF
			0.0003	7	13/110	11.8	13.9±2.8	1.5±0.2	3/ 97	1EO, 2HLC	NF
			0.001	5	5/ 79	6.3	14.8±3.1	1.4±0.1	0/ 74	NF	NF
		T+E	0.003	7	36/105	34.3	9.9±4.6	1.4±0.2	69/ 69	28EX, 61HM, 53CP, 16EO, 1SB, 69LM, 57TM	moderate HVA
11	Rat		0.0001	8	6/ 99	6.1	11.6±3.1	3.5±0.3	0/ 93	NF	NF
			0.0003	7	6/ 89	6.7	11.9±4.4	3.4±0.3	0/ 83	NF	NF
		T	0.001	8	9/ 95	9.5	10.8±2.1	3.6±0.3	68/ 86	30HM, 68LM	NF
		T	0.003	8	11/ 84	13.1	9.1±4.2	3.1±0.3	73/ 73	11EX, 7VB, 73HM, 59CP, 73LM	NF
		E	0.01	8	111/111	100.0	0.0±0.0	0.0±0.0	0/ 0		moderate HVA
12	Mouse		0.1	7	10/108	9.3	14.0±1.7	1.5±0.1	0/ 98	NF	NF
			0.3	5	4/ 65	6.2	12.2±2.6	1.4±0.2	0/ 61	NF	NF
			1	5	3/ 71	4.2	13.6±0.9	1.4±0.1	0/ 68	NF	NF
			3	6	10/ 58	17.2	8.0±5.0	1.6±0.1	0/ 48	NF	NF
		T	10	7	4/ 99	4.0	13.6±1.4	1.5±0.2	3/ 95	3EX, 3HM, 2EO	NF

Table 2. (continued)

Retinoid No.	Species	Assessment	Dosage mg/kg	No. of dams	No. of resorpt./ No. of implant.	Perc. resorpt.	No. of fetuses/ dam	Fetal body weight (g)	No. of fetuses malformed/ examined	Observations fetuses	Observations dams
12	Rat		0.03	7	4/ 95	4.2	13.0±3.2	3.5±0.1	0/ 91	NF	NF
			0.1	8	6/ 99	6.1	11.6±2.6	3.5±0.1	0/ 93	NF	NF
			0.3	7	5/ 98	5.1	13.3±1.1	3.5±0.1	1/ 93	1EC	NF
			1	8	5/106	4.7	12.6±1.9	3.2±0.3	0/101	NF	NF
		T	3	7	8/ 98	8.2	12.9±3.4	3.4±0.2	15/ 90	15CP	NF
13	Mouse		0.003	7	12/108	11.1	13.7±3.0	1.5±0.1	0/109	NF	NF
			0.01	7	5/106	4.7	14.3±1.9	1.5±0.1	1/101	1EC	NF
		T	0.03	8	17/106	16.0	11.1±3.5	1.5±0.2	2/ 89	1EX, 1EO, 1ASM	NF
		T	0.1	6	8/ 91	8.8	13.7±1.8	1.5±0.2	40/ 97	2EX, 2HM, 5CP, 40LM	NF
		T+E	0.3	5	23/ 65	35.4	8.2±6.0	1.3±0.2	42/ 42	13EX, 13HM, 1EO, 39CP, 10ASM, 8TM, 10RM, 42LM, 3AGM	NF
13	Rat		0.003	8	12/107	11.2	11.9±3.1	3.3±0.2	0/ 95	NF	NF
			0.01	8	6/ 97	6.2	11.4±2.5	3.4±0.2	0/ 91	NF	NF
			0.03	8	17/101	16.8	10.5±4.6	3.3±0.1	0/ 84	NF	NF
			0.1	8	8/101	7.9	11.6±2.3	3.4±0.2	0/ 95	NF	NF
		T	0.3	7	11/ 95	11.6	12.0±2.6	2.9±0.2	82/ 82	8EX, 6VB, 82HM, 71CP, 1SB, 73LM	NF
14	Mouse		10	8	15/117	12.8	12.8±3.7	1.6±0.1	0/102	NF	NF
		T+E	30	6	54/ 89	60.7	5.8±4.9	1.4±0.1	8/ 35	2EX, 3HM, 2CP, 3EO, 1AGM	NF
		E	100	7	89/ 89	100.0	0.0±0.0	0.0±0.0	0/ 0		NF
15	Mouse		3	3	4/ 48	8.3	14.7±2.5	1.5±0.1	0/ 58	NF	NF
			10	6	20/ 81	24.7	10.2±5.3	1.6±0.1	0/ 61	NF	NF
			30	6	6/ 86	7.0	13.2±2.3	1.6±0.1	0/ 92	NF	NF
			100	8	32/109	29.4	9.6±6.2	1.6±0.1	0/ 77	NF	NF
		T	300	4	4/ 57	7.0	13.3±1.0	1.5±0.3	2/ 53	1EO, 1CP	NF
		T+E	600	5	29/ 63	46.0	6.4±4.4	1.1±0.3	17/ 34	2EX, 4HM, 6EO, 16CP, 5LM	moderate HVA
18	Mouse*		0.001	7	13/ 89	14.6	10.9±3.3	1.4±0.1	0/ 76	NF	NF
		T	0.005	8	18/115	15.7	12.1±1.7	1.4±0.2	2/ 97	1EX, 1EO, 1LM	NF
		T+E	0.015	6	57/ 69	82.6	2.0±2.5	1.1±0.1	12/ 12	6EX, 11HM, 1EO, 12LM, 11TM	slight HVA
		E	0.03	8	92/ 92	100.0	0.0±0.0	0.0±0.0	0/ 0		moderate HVA
18	Rat*		0.001	5	4/ 24	16.7	4.0±4.3	4.4±0.4	0/ 20	NF	NF
			0.005	6	5/ 60	8.3	9.2±1.7	4.0±0.8	0/ 55	NF	NF
		T+E	0.015	6	15/ 51	29.4	6.0±2.5	2.9±0.2	36/ 36	20EX, 36HM, 7EO, 11SB, 36LM, 21TM, 1EC	slight HVA
		T+E	0.03	8	68/ 70	97.1	0.3±0.5	2.8±0.6	2/ 2	2EX, 2HM, 2SB, 2LM, 2TM	moderate HVA
		E	0.09	6	55/ 55	100.0	0.0±0.0	0.0±0.0	0/ 0		marked HVA
18	Rabbit		0.0001	4	10/ 27	37.0	4.3±1.3	43.6±3.3	0/ 17	NF	NF
			0.001	4	6/ 34	17.6	7.0±3.2	42.9±6.3	0/ 28	NF	slight HVA
		T	0.01	4	30/ 33	90.9	0.8±1.5	32.9±0.0	3/ 3	3HM, 3EO, 3LM, 2TM, 3EC	marked HVA
19	Mouse		10	5	7/ 72	9.7	13.0±3.8	1.6±0.1	0/ 65	NF	NF
			30	6	6/ 86	7.0	13.3±1.4	1.5±0.1	0/ 80	NF	NF
			100	7	20/107	18.7	12.3±5.6	1.5±0.1	0/ 87	NF	NF
			300	7	4/105	3.8	14.1±1.8	1.5±0.1	0/101	NF	NF
19	Rat		10	7	1/ 89	1.1	12.6±2.6	3.4±0.2	0/ 88	NF	NF
			30	6	4/ 79	5.1	12.5±2.0	3.3±0.2	0/ 75	NF	NF
			100	7	4/ 81	4.9	11.0±2.1	3.5±0.2	0/ 77	NF	NF
		T (?)+E	300	5	43/ 78	55.1	7.0±4.2	3.3±0.3	1/ 35	1CL, 1CP	mortality: 2/7
19	Rabbit		10	5	7/ 40	17.5	6.6±1.5	40.7±5.1	0/ 33	NF	NF
			30	4	4/ 29	13.8	6.2±2.4	37.1±4.5	1/ 25	1HM	NF
			100	6	12/ 53	22.6	6.8±0.7	31.8±8.9	0/ 41	NF	NF
		E	300	4	29/ 33	87.9	1.0±2.0	36.1±0.0	0/ 4	NF	mortality: 3/7

Table 2. (continued)

Retinoid No.	Species	Assessment	Dosage mg/kg	No. of dams	No. of resorpt./ No. of implant.	Perc. resorpt.	No. of fetuses/ dam	Fetal body weight (g)	No. of fetuses malformed/ examined	Observations fetuses	Observations dams
20	Mouse		30	8	12/119	10.1	13.4±2.3	1.6±0.2	0/107	NF	NF
			100	6	9/ 94	9.6	14.0±2.0	1.4±0.1	1/ 85	1CP	NF
			300	7	4/ 98	4.1	13.1±2.7	1.6±0.2	0/ 94	NF	NF
20	Rat		30	7	10/101	9.9	13.0±1.7	3.3±0.1	0/ 91	NF	NF
			100	7	7/ 93	7.5	12.3±2.1	3.2±0.2	0/ 86	NF	NF
			300	7	9/ 76	11.8	9.6±4.6	3.2±0.4	0/ 67	NF	NF
21	Mouse		10	7	9/107	8.4	14.0±1.4	1.5±0.1	0/ 98	NF	NF
			30	7	13/108	12.0	13.6±2.6	1.6±0.1	2/ 95	1HLC, 1EC	NF
			100	7	8/ 89	9.0	11.6±3.9	1.6±0.1	0/ 81	NF	NF
		T (?)	300	7	5/109	4.6	14.9±1.2	1.6±0.1	1/104	1EO	NF
21	Rat		3	7	3/ 89	3.4	12.3±2.6	3.5±0.1	0/ 86	NF	NF
			10	6	6/ 75	8.0	11.5±4.0	3.4±0.2	0/ 69	NF	NF
			30	8	4/ 89	4.5	10.6±3.3	3.6±0.2	0/ 85	NF	NF
			100	8	6/ 93	6.5	10.9±4.0	3.4±0.3	0/ 87	NF	NF
		T	300	6	6/ 82	7.3	12.7±1.0	3.2±0.3	6/ 76	1ASM, 1RM, 6LM	NF
22	Mouse		0.01	8	7/118	5.9	13.8±4.2	1.4±0.3	1/111	1EX, 1EO, 1CP	NF
			0.1	8	9/126	7.1	14.5±2.6	1.2±0.2	1/117	1SB	NF
			1	6	3/ 88	3.4	14.2±1.9	1.2±0.2	1/ 85	1CP	NF
		T	10	8	6/118	5.1	14.0±3.0	1.6±0.1	3/112	1EX, 3EO	NF
		T+E	30	8	29/115	25.2	10.6±3.6	1.3±0.1	85/ 86	26EX, 4VB, 69HM, 74EO, 70CP, 10LM	NF
		E	100	6	93/ 93	100.0	0.0±0.0	0.0±0.0	0/ 0		marked HVA
23	Mouse		0.3	7	11/ 89	12.4	11.1±2.4	1.6±0.2	0/ 78	NF	NF
			1	8	8/101	7.9	11.5±3.3	1.6±0.1	0/ 93	NF	NF
		T	3	8	20/110	18.2	11.1±2.9	1.5±0.2	89/ 90	1EX, 1HM, 89EO, 80CP	slight HVA
		T	10	7	11/ 89	12.4	11.0±4.6	1.2±0.1	78/ 78	2EX, 72HM, 78EO, 78CP, 62BM, 78LM	moderate HVA
		T+E	30	8	61/ 97	62.9	4.4±4.6	1.0±0.2	36/ 36	4EX, 36HM, 36EO, 36CP, 34BM, 36LM	marked HVA
23	Rat		0.3	8	3/107	2.8	13.0±1.1	3.4±0.2	0/104	NF	slight HVA
		T (?)	1	6	0/ 81	0.0	13.5±1.9	3.4±0.1	1/ 81	1CP	slight HVA
		T+E	3	8	70/ 98	71.4	3.5±3.9	2.9±0.3	12/ 28	4EX, 6VB, 10HM, 2EO, 5CP, 2SB	moderate HVA
		E	10	5	66/ 66	100.0	0.0±0.0	0.0±0.0	0/ 0		marked HVA
23	Rabbit		0.3	5	11/ 43	25.6	6.4±3.6	40.5±5.2	0/ 32	NF	NF
			1	5	12/ 40	30.0	5.6±3.2	38.8±6.6	0/ 28	NF	NF
			3	4	2/ 34	5.9	8.0±1.6	37.0±3.1	0/ 32	NF	NF
			10	4	7/ 26	26.9	4.8±3.3	40.5±1.6	0/ 19	NF	NF
		E	30	4	15/ 24	62.5	2.3±2.9	44.3±1.3	0/ 9	NF	NF
		E	100	4	23/ 27	85.2	1.0±1.4	46.4±0.1	1/ 4	1CP	mortality: 2/6
		E	300	4	26/ 28	92.9	0.5±1.0	45.1±0.0	0/ 2	NF	mortality: 2/6
24	Mouse		10	6	8/ 93	8.6	14.2±3.1	1.6±0.2	0/ 85	NF	NF
		T+E	30	7	53/108	49.1	7.9±7.7	1.7±0.1	3/ 55	2EO, 1CP	NF
		E	100	6	91/ 91	100.0	0.0±0.0	0.0±0.0	0/ 0		NF
24	Rat		1	7	15/ 94	16.0	11.3±1.3	3.3±0.2	0/ 79	NF	NF
			3	8	12/ 94	12.8	10.3±4.7	3.5±0.2	0/ 82	NF	slight HVA
			10	7	3/ 88	3.4	12.1±2.0	3.3±0.3	0/ 85	NF	moderate HVA
		T+E	30	5	49/ 57	86.0	1.6±1.1	2.9±0.7	5/ 8	2EX, 5HM	marked HVA
24	Rabbit		1	6	10/ 42	23.8	5.3±2.8	35.5±3.6	2/ 32	2TM	NF
			3	7	11/ 53	20.8	6.0±3.6	36.9±5.0	1/ 42	1EX	NF
			10	3	1/ 23	4.3	7.3±1.2	35.0±5.3	1/ 23	1VB	NF
			30	7	7/ 54	13.0	6.7±2.6	37.4±5.5	1/ 54	1TM	NF

Table 2. (continued)

Retinoid No.	Species	Assessment	Dosage mg/kg	No. of dams	No. of resorpt./ No. of implant.	Perc. resorpt.	No. of fetuses/ dam	Fetal body weight (g)	No. of fetuses malformed/ examined	Observations fetuses	Observations dams
25	Mouse	T	1	2	2/ 19	10.5	8.5±6.4	1.6±0.1	2/ 41	2CP	NF
		T	3	4	11/ 43	25.6	8.0±5.5	1.7±0.2	45/ 45	28HM, 28CP, 34EO, 35ASM, 45RM, 45LM	slight HVA
		T	10	3	6/ 33	18.2	9.0±7.8	1.5±0.2	30/ 30	1EX, 1VB, 29HM, 30CP, 30EO, 30ASM, 30RM, 30LM	slight HVA
		T	30	4	7/ 56	12.5	12.3±2.6	1.5±0.1	53/ 53	1EX, 2VB, 53HM, 53CP, 53EO, 53ASM, 53RM, 53LM	moderate HVA
		T+E	100	8	80/ 93	86.0	1.6±4.6	1.1±0.0	13/ 13	6EX, 1VB, 13HM, 13CP, 13EO, 13ASM, 13RM, 13LM	marked HVA
25	Rat	T+E	3	8	72/103	69.9	3.9±3.1	2.7±0.3	13/ 31	6EX, 1VB, 10HM, 6CP, 2ASM, 1RM	slight HVA
		T+E	10	8	98/101	97.0	0.4±0.7	2.5±1.0	2/ 3	1EX, 1VB, 1HM	moderate HVA

Abbreviations used in Table 2

T	teratogenic	*	CP not examined	SB	spina bifida
E	embryolethal	CP	cleft palate	LM	fore and/or hind limbs malformed
NF	no findings	CL	cleft lip	HLC	hind limbs contorted
EX	exencephaly	BM	back malformed	FLC	fore limbs contorted
VB	vertex bulge	TM	tail malformed	EC	ectopy of intestines
HM	head malformed	ASM	axial skeleton malformed	AGM	anal and genital malformations
EO	eyelids opened	RM	ribs malformed	HVA	hypervitaminosis A toxicity

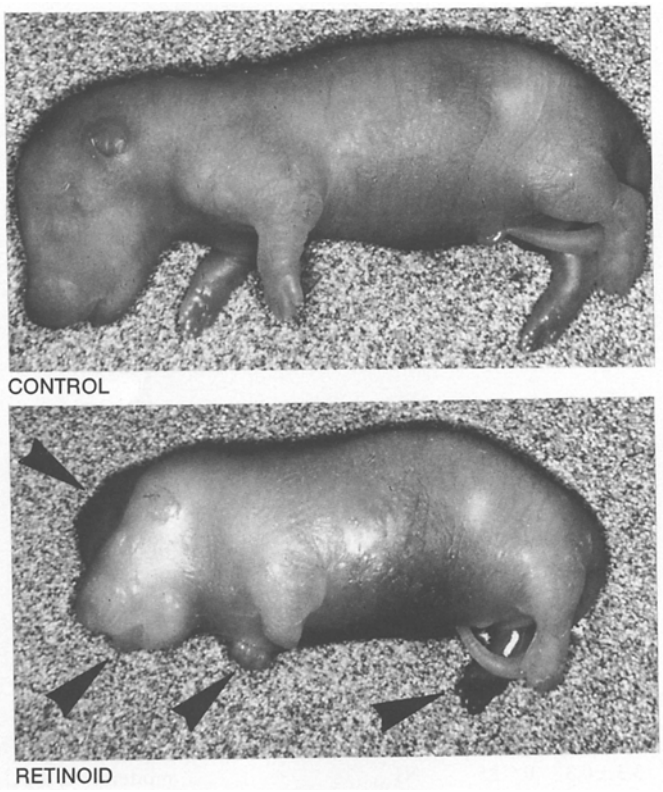


Fig. 3. Teratogenesis of retinoids. The most frequently observed malformations induced by retinoids in rodents are exencephaly, malformations of the head and eye, cleft palate and limb defects. These malformations can be easily detected by external examination of fetuses at the time of cesarean section. Here, the teratogenic effects were caused by treatment of rats from day 7 to 16 of gestation with retinoid No. 11 at the dose of 0.03 mg/kg

pharmacokinetic factors that influence the response in vivo.

The in vitro test applied here using limb bud cells to determine the teratogenic potential of retinoids has several advantages: (1) The test uses mammalian embryonic cells. (2) It relies on a well-established test system to study chondrogenesis. (3) The test is mechanistically related to the in vivo development. The skeletal system is a main target organ for teratogens. (4) There are quantifiable morphological and biochemical endpoints. The latter is specific for cartilage and can be easily quantified. (5) This quantification makes possible the estimation of IC_{50} values to compare compounds with different activity. (6) The culture system is simple and easy. (7) General cytotoxic effects can be distinguished from the specific inhibition of chondrogenesis. (8) The assay requires only small amounts of the chemical compounds. (9) The number of animals used is small. (10) The results can be obtained rapidly.

One major drawback of in vitro systems is that they usually lack the drug-metabolizing enzymes. This holds true also for the limb bud cells as shown with the ethylester retinoid etretinate (No. 16) which could be metabolically activated by the addition of esterase into the culture medium (Kistler 1985), indicating that etretinate was metabolized to etretin (No. 3), the free acid analog, and that etretin was the active metabolite. There are other possibilities for in vitro metabolic activation of prodrugs, like the use of liver microsomal preparations containing the drug-metabolizing enzymes (S9-fraction). Whether these preparations could metabolize all the prodrug retinoids to the active principle or could produce false positives as a result of unphysiological metabolites needs further investigation. Furthermore, such activation systems might influence chondrogenesis itself.

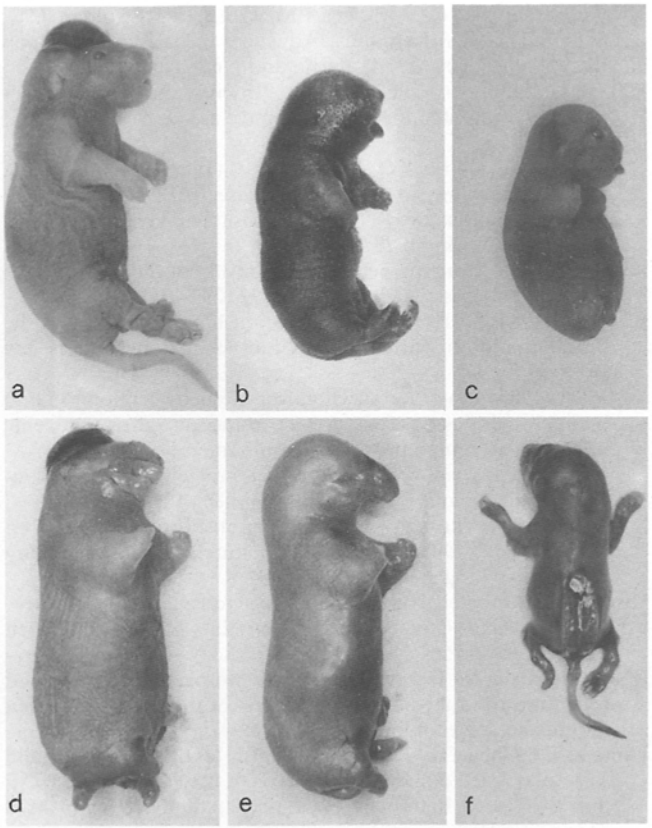


Fig. 4. Severe malformations induced by retinoids in rodents. The treatment of the dams was from day 7 through 16 of gestation. The species, retinoid No., dose and types of malformations according to the abbreviations used in Table 2 are given for each malformed fetus. **a** Mouse, No. 7, 0.3 mg/kg, EX, EO; **b** mouse, No. 8, 0.1 mg/kg, HM, LM; **c** mouse, No. 11, 0.01 mg/kg, HM, EO, LM, TM; **d** rat, No. 10, 3.0 mg/kg, EX, HM, LM, TM; **e** rat, No. 10, 3.0 mg/kg, HM, EO, LM, TM; **f** mouse, No. 22, 0.1 mg/kg, SB, HLC

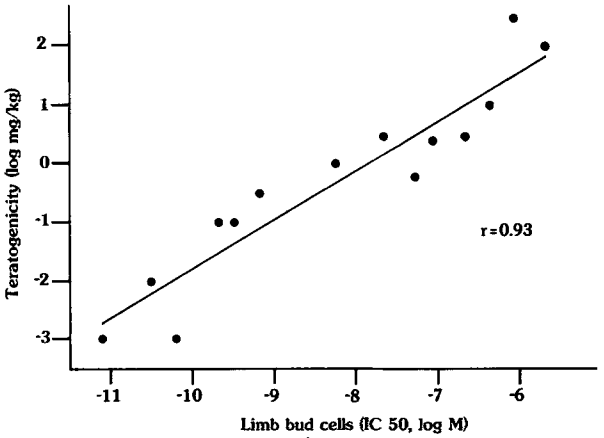


Fig. 5. Correlation between the in vitro activity in limb bud cells and in vivo teratogenicity of retinoids containing a carboxylic acid endgroup. For the in vivo teratogenicity the lowest teratogenic dose of the most sensitive species was used

In vivo teratogenicity of retinoids

In laboratory animals maternal hypervitaminosis A results in marked teratogenic effects affecting almost all body systems. Craniofacial malformations including defects of the eye, ear and brain (exencephaly) and cleft palate and severe skeletal malformations among others were reported (Geelen 1979). All these types of malformations were also found after treatment with retinoids. In general, the malformation pattern was similar with all retinoids tested.

The results of the pilot studies should be considered preliminary because only a small number of animals was treated and the teratological examination was limited usually to an external examination of the fetuses. When examining a larger number of animals in our routine teratological studies, we often noted that the no-effect level of teratogenicity was at a lower dose than that of the pilot studies. Thus, the lowest teratogenic dose of the pilot studies is an approximate estimation only.

For all the retinoids tested the lowest teratogenic dose was within 1 order of magnitude for mice and rats, with the exception of 13-*cis*-etretin (No. 4). With this retinoid a low incidence of cleft palates unrelated to dose was noted in rats over a wide dose range from 3–200 mg/kg. Cleft palates were also the main malformation found at 100 and 200 mg/kg in mice. In contrast, 13-*cis*-etretin was markedly embryolethal and teratogenic in rabbits, inducing several types of severe malformations at 3 mg/kg, the lowest dose tested.

The arotinoid ethylsulfone (No. 23) also caused a species-specific malformation pattern. In mice and rats the usual congenital malformations caused by retinoids were found but in rabbits this compound was embryolethal but did not induce teratogenic effects even at the high dose of 300 mg/kg.

Etretin (No. 3) was teratogenic in all three species tested but at rather different doses (Kistler and Hummler 1985). The lowest teratogenic dose in rabbits was 0.6 mg/kg but was 15 mg/kg in rats, a dose 25 × higher than that in rabbits. In mice the respective dose of 3 mg/kg was in between these two doses.

Isotretinoin (No. 2) is a similar example. The lowest teratogenic dose in mice, rats and rabbits was 100, 150 and 10 mg/kg, respectively but is below 1 mg/kg in man (Rosa et al. 1986). Therefore, with this retinoid the poor predictability of the teratogenic risk in humans from animal studies even if tested in three species is clearly demonstrated.

In summary, there is a large difference in species sensitivity for the retinoids and this varies from compound to compound.

Correlation between the activity of retinoids in vitro and in vivo

There was a good quantitative correlation between the results of the limb bud cell test and in vivo teratogenicity among the 15 retinoids tested containing a carboxylic acid endgroup. Of the other ten retinoids with a modified or sulfur-containing endgroup seven compounds also showed a good correlation. The remaining three were etretinate (No. 16), motretinide (No. 17) and fenretinide (No. 20). For the prodrug form etretinate, which was slightly active in vitro but markedly teratogenic in vivo, we demonstrated that it could be metabolically activated by esterase. Motretinide was inactive in vitro but teratogenic in vivo. This ethylamine retinoid probably represents another prodrug form which cannot be metabolically activated within the cells. Whether exogenous drug metabolizing systems could activate motretinide awaits further investigation.

Fenretinide represents an opposite example, which was not teratogenic in vivo in mice and rats but was active in limb bud cells as well as in whole embryo cultures (Steele et al. 1986). This could indicate that in vivo fenretinide was detoxified so that no active drug could reach the embryo.

In conclusion, the limb bud cell test appears to be useful for a preliminary estimation of the teratogenic potential of retinoids. It is obvious from this study that for retinoids with a carboxylic acid endgroup a reliable estimation may be obtained. However, the test system, under the conditions used, is limited to retinoids which are already in their biologically active form or can be metabolically activated within the cells. Thus, for the risk assessment in humans animal studies are still required to confirm the in vitro result because absorption, metabolism, distribution and elimination of new retinoids in the whole organism are unknown.

The limb bud cell test may be useful for the prediction of the teratogenic potential of other classes of compounds. However, as demonstrated with retinoids the limitations of the test systems should be borne in mind.

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