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Comparative teratogenicity of nine retinoids in the rat

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Summary. The comparative teratogenicity of nine retinoids in Wistar rats was investigated. The compounds studied and dose levels tested (mg/kg) were: all-*trans*-retinoic acid (TRA), 6.25, 12.5, 25, 50, 100; etretinate (ETR), 25, 50; acitretin (ACIT), 25, 50; 13-*cis*-retinoic acid (13CRA), 100, 200; and five retinamides, each at 300 and 600 mg/kg, *N*-(4-hydroxyphenyl)-retinamide (4HPR); *N*-tetrazol-5-ylretinamide (TZR); *N*-butylretinamide (NBR); *N*-ethylretinamide (NER); 13-*cis*-*N*-ethylretinamide (13CNER). Retinoids were administered by oral intubation on days 10 and 11 *post coitum* (p.c.). Dams were killed on day 22 p.c. and examinations carried out to assess teratogenic potential. TRA, ETR, ACIT, 13CRA and 4HPR increased the incidence of resorptions. The incidence of abnormal fetuses, irrespective of the specific abnormalities induced, was markedly increased (50–100%) by TRA, ETR, ACIT, 13CRA and 4HPR, whereas TZR and NBR caused moderate increases (20–50%), and NER and 13CNER induced mild increases (10–20%). The incidences of CNS, craniofacial and urinogenital defects were generally high with TRA, ETR, ACIT and 13CRA. Cardiac vessel defects were markedly increased by 4HPR. Using a number of criteria, a generalized ranking order of the toxicity of the compounds was drawn up: TRA > ETR > ACIT > 13CRA > 4HPR > TZR≡NBR > NER≡13CNER. The ranked order of relative in-vivo teratogenicity for the nine retinoids is compared with a previously reported in-vitro assessment of the compounds using a rat whole embryo culture technique.

Keywords: teratology, rat, retinoids

Retinoids are the natural and synthetic analogues of vitamin A. Although several retinoids have been tested in man (Saurat 1989), at present only 13-*cis*-retinoic acid (isotretinoin, 13CRA) and etretinate (ETR) are in widespread clinical use, mainly for the

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treatment of dermatological disorders (Peck 1984; Gollnick 1987; Orfanos *et al.* 1987). However, the systemic administration of retinoids in man is frequently associated with a spectrum of adverse side-effects (Turton *et al.* 1985; Silverman *et al.* 1987; David *et al.* 1988; Teelman 1989) and the more widespread therapeutic use of these compounds is limited by their toxicity (Bollag 1983, 1985; Yob & Pochi 1987). In addition, 13CRA and ETR are teratogenic in humans at therapeutic dose levels (Benke 1984; Chen 1985; Lammer *et al.* 1985; Rosa *et al.* 1986). The predominant malformation pattern in man includes defects of the heart, thymus, craniofacial and central nervous systems. Experimental studies with retinoids in laboratory animals (Hummler & Schüpbach 1981; Kamm *et al.* 1984; Kochhar *et al.* 1984; Kistler & Hummler 1985; Kochhar & Penner 1987) have demonstrated similar patterns of developmental abnormalities to those reported in human exposure.

In recent years there have been numerous reports describing the development of in-vitro methods in different fields of toxicity testing. This trend has been evident in the evaluation of compounds for teratogenic potential (Brown & Fabro 1982; Neubert 1985). In general, three types of model systems have been developed for use in in-vitro teratology testing: embryo culture, organ culture and cell culture (Kochhar 1981; Beck 1982). However, it is widely considered that the purpose of such in-vitro screens will be for the preliminary estimation of potential teratogenicity, and the identification of compounds which require further evaluation *in vivo*, rather than to establish definitely the teratogenic potential of a compound (Brown & Fabro 1982; Manson *et al.* 1982; Neubert 1985; Kistler 1987). Furthermore, at present there is still widespread debate about the advantages and disadvantages of the various in-vitro techniques (Fantel 1982; Sadler *et al.* 1982; Kistler 1985; Neubert 1985) and the validation and predictability of the culture systems (Kimmel *et al.* 1982; Kochhar 1982; Flint & Orton 1984; Schmid 1985; Kistler 1987).

Two in-vitro methods which have been widely used in the study of the teratogenic potential of retinoids are whole embryo culture (WEC) (Kochhar 1975; Klug *et al.* 1985; Sadler *et al.* 1985; Cockroft & Steele 1987; Cicurel & Schmid 1988) and primary embryonic limb bud cell culture (LBCC) (Hassell & Horigan 1982; Kistler 1985; Renault *et al.* 1989). Recent reports on retinoid effects on embryonic development have demonstrated the usefulness of both techniques, for example WEC in the rat and mouse (Goulding & Pratt 1986; Bechter & Hall 1987; Goulding *et al.* 1988; Klug *et al.* 1989; Watanabe & Pratt 1991) and the rat LBCC assay (Gallandre *et al.* 1980; Wilk *et al.* 1980; Kistler 1985, 1987).

Kistler (1985, 1987) investigated the activity of a series of retinoids in inhibiting chondrogenesis in rat LBCC and compared the results with the in-vivo teratogenicity of the compounds. Discussing the validity of the culture technique, it was concluded that, in general, there was good correlation between in-vitro and in-vivo activity and that the LBCC assay could be used for the preliminary assessment of the teratogenic potential of retinoids (Kistler 1985, 1987).

Following the studies on retinoids by Kistler using both rat LBCC and in-vivo experiments, we reported on the in-vitro activity of 10 retinoids in rat WEC (Steele *et al.* 1987). We now present results on the comparative teratogenicity of nine retinoids *in vivo* in the rat. The findings *in vitro* and *in vivo* are compared by a relative ranking of teratogenic potential to assess the validity of the WEC technique in the study of retinoid teratogenicity. A preliminary report on aspects of the findings has been published (Turton *et al.* 1989).

Materials and methods

Animal maintenance and mating procedure

Ten-week-old outbred female Wistar rats

(Harlan Olac Ltd, Blackthorn, Bicester, Oxon OX6 0TP) were maintained under Specific Pathogen Free conditions at 19–23°C, with 40–60% relative humidity and a 12-hour light-dark cycle. Animals were fed a maintenance diet (PRD, SDS Ltd, Witham, Essex CM8 3AD). Drinking water from the mains supply, and diet, were available *ad libitum*. Female rats were mated overnight with 10-week-old males of the same stock. The morning on which a vaginal plug was found was designated as day 1 *post coitum* (p.c.).

Test compounds and dosing

The structures of the retinoids tested are shown in Fig. 1. ETR, acitretin (etretin, ACIT) and 13CRA were supplied by Roche Products Ltd (PO Box 8, Welwyn Garden City, Herts AL7 3AY), and all-*trans*-retinoic acid (TRA) was purchased from Sigma Chemical Co (Poole, Dorset BH17 7TG). The retinamides *N*-(4-hydroxyphenyl) retinamide (4HPR), *N*-butylretinamide (NBR), *N*-ethylretinamide (NER), and 13-*cis*-*N*-ethylretinamide (13CNER) were provided by the National Cancer Institute (courtesy of Dr C. Smith and Dr M.B. Sporn) and the Southern Research Institute (Dr Y.F. Shealy). *N*-tetrazol-5-ylretinamide (TZR) was supplied by courtesy of Dr F. Frickel (BASF, Aktiengesellschaft, Ludwigshafen, FRG). Retinoids were suspended in maize oil, the suspensions being prepared daily, immediately prior to dosing. The suspensions were administered by oral intubation in an administration volume of 5 ml/kg body weight on day 10 and 11 p.c. Retinoid dose levels, and the numbers of animals in each treatment group, are set out in Table 1. Control rats received maize oil only.

Teratology study

Dams were killed on day 22 p.c. by CO_2 overdose for caesarean section. The positions of live fetuses *in utero* and of early and late embryonic deaths was noted. Live young were weighed, sexed, and examined externally under a stereomicroscope for gross developmental malformations before killing by a dorsal subcutaneous injection of pentobarbitone sodium (Euthatal, May & Baker Ltd, Eccles, Manchester M30 7RT). Alternate fetuses were preserved in alcoholic Bouin's fixative for subsequent sectioning and examination for internal abnormalities. The remaining fetuses were examined internally, and skeletal defects assessed using Alizarin Red S staining. The uterus was removed from each dam and stained with ammonium sulphide for the demonstration of implantation sites. The ovaries were removed and fixed in formalin for subsequent counting of corpora lutea.

Results

Raw data from the experiments are presented in Table 1. Twenty-seven specific abnormalities have been classified under one of seven headings ('Groups of defects') based, in general, upon anatomical site. Also, each specific abnormality has been defined as being either an externally or internally observed defect.

There were no striking differences between each of the four control groups in either the number of implantations per dam or the type and incidence of the specific abnormalities (Tables 1 and 2). For ease of reference and presentation in subsequent Tables, the data from the four control groups have been combined and treated as a single group (see Table 2). However, the range of the data in the four control groups was taken into account in assessing subsequently the comparative effects of the nine retinoids.

Based upon the average number of implantations per dam demonstrated by the combined control group (Table 3), and the individual control groups (Table 2), none of the retinoids, at the doses tested, significantly affected the number of implantations per dam (Table 3). At all of the doses studied however, TRA, ETR, ACIT, 13CRA and 4HPR produced clear increases in the percentage of resorptions compared to controls.

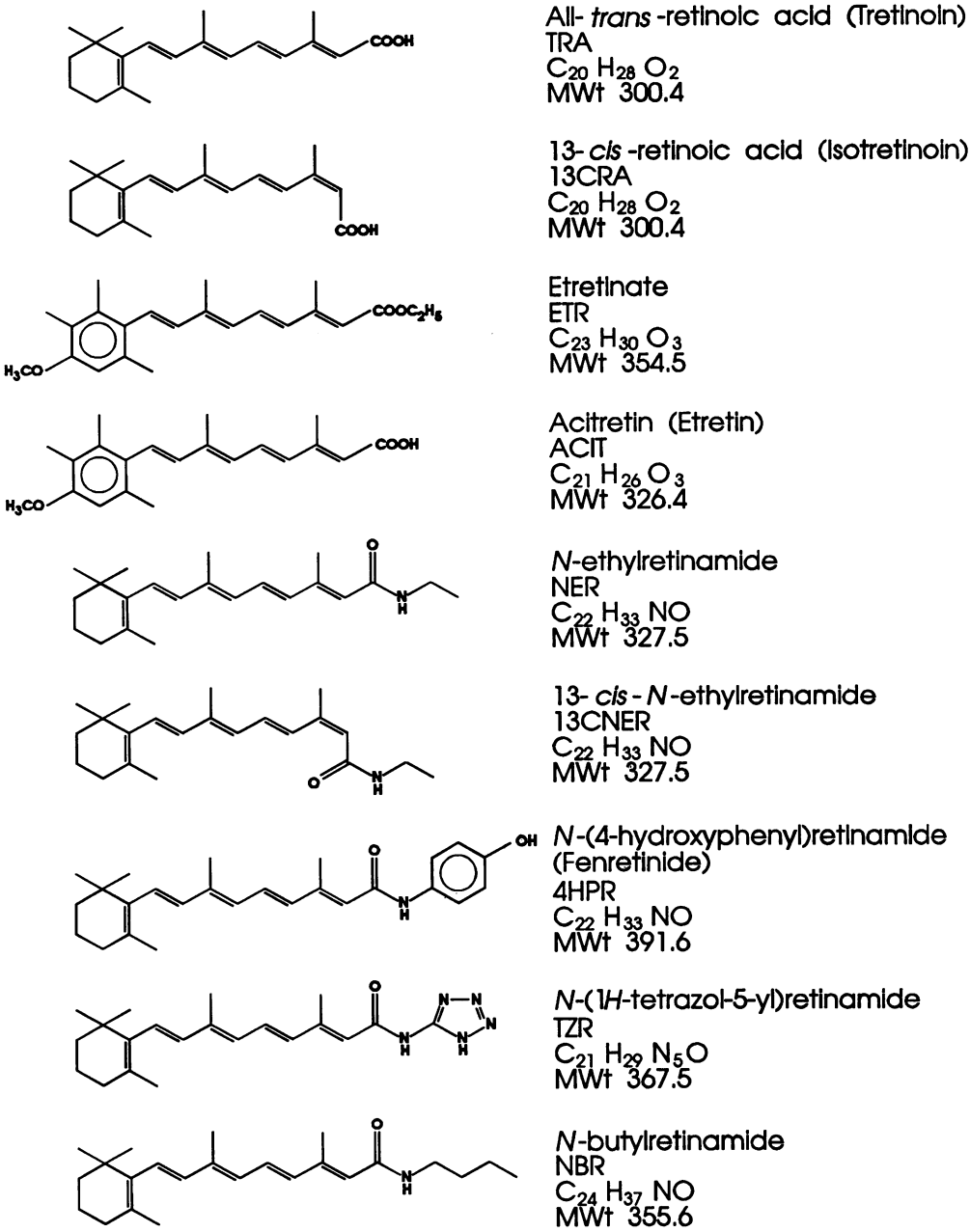

Fig. 1. Chemical structures of the retinoids tested.

Furthermore, for each of these five retinoids, percentage resorptions were greatest at the higher doses, suggesting a dose–response relationship. This was particularly evident over the five concentrations of TRA tested. However, TZR, NBR, NER, and 13CNER had little or no effect on the percentage of fetuses resorbed compared to controls (Table 3).

Table 1. Raw data from the study; information on litters and abnormalities recorded in four control groups and retinoid treatment groups[a]

	Externally (E) or internally (I) observed abnormality	Retinoid treatment group; dose levels of retinoids (mg/kg)																								
		C1[b]	TRA[c]					13CRA		C2	ETR		ACIT		C3	NER		13CNER		4HPR		C4	TZR		NBR	
		—	6.25	12.5	25	50	100	100	200	—	25	50	25	50	—	300	600	300	600	300	600	—	300	600	300	600
No. of dams		4	4	6	4	4	4	4	4	3	4	4	4	4	7	7	4	5	5	5	8	4	5	4	4	4
No. of implantations		55	53	76	53	47	53	51	41	36	53	52	53	53	87	89	52	64	65	66	90	48	68	54	48	50
No. of resorptions		4	11	48	48	47	53	13	32	2	16	32	6	28	5	7	3	4	5	21	83	2	3	5	1	5
No. of fetuses examined		51	42	28	4	0	0	38	9	34	37	20	47	25	82	82	49	61	60	45	7	46	65	49	47	45
Groups of defects[d]																										
(1) *CNS defects*																										
Exencephaly[e]	E+I	0	10	19	1	—[f]	—[f]	1	5	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Microcephaly	I	0	0	12	1	—	—	0	1	0	1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0
Hydrocephaly	I	0	2	0	0	—	—	0	0	0	6	4	3	3	2	0	1	0	1	1	0	0	0	0	0	0
Anencephaly	I	0	0	2	0	—	—	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Spina bifida	E+I	0	0	0	0	—	—	0	8	0	8	16	2	12	0	0	0	0	0	0	0	0	0	0	0	0
(2) *Craniofacial defects*																										
Mandibulofacial abnormalities[g]	E	0	3	12	1	—	—	7	7	0	37	20	1	8	0	0	0	0	0	1	4	0	0	0	0	0
Otic abnormalities[h]	E	0	2	13	4	—	—	30	9	0	24	20	1	17	0	0	0	0	0	0	0	0	0	1	2	0
Protruding tongue[i]	E	0	10	27	3	—	—	34	4	0	11	7	1	11	0	0	0	0	0	0	0	0	0	0	0	1
Secondary cleft palate	I	0	2	6	0	—	—	3	0	0	24	17	0	17	0	0	0	0	0	0	1	0	0	0	0	0
Optic abnormalities[j]	E+I	0	11	24	3	—	—	2	2	0	29	18	0	1	0	3	2	1	0	7	3	0	0	0	0	0
(3) *Urinogenital defects*																										
Hydronephrosis	I	0	23	22	1	—	—	22	7	0	28	9	21	17	2	0	0	0	2	3	1	0	2	2	1	1
Ureteric abnormalities[k]	I	1	28	22	1	—	—	28	8	1	18	9	27	17	1	9	3	7	8	7	0	1	17	9	9	6
Renal/bladder/genital agenesis	I	0	3	7	3	—	—	8	2	0	8	11	5	10	0	0	0	0	0	0	0	0	0	0	0	0
Undescended testes	I	0	0	2	0	—	—	1	2	0	5	2	3	9	1	2	0	0	1	7	1	0	0	0	0	0
Ectopic kidney	I	0	0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
(4) *Cardiac vessel defects*																										
Abnormal vessel origin from aortic arch	I	0	0	0	0	—	—	0	1	0	2	6	0	0	0	0	0	0	0	33	7	0	0	0	0	0
Other vessel abnormalities[l]	I	0	0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	12	4	0	0	0	0	0
(5) *Haemorrhage*																										
Subcutaneous haemorrhage	E	0	4	0	0	—	—	3	0	0	0	0	0	0	2	3	2	0	0	14	6	0	3	5	8	1
Cranial haemorrhage	I	1	0	1	0	—	—	1	0	4	15	1	2	3	6	0	0	1	0	20	2	0	0	0	0	0
(6) *Caudo-rectal defects*																										
Imperforate anus	E	0	0	2	4	—	—	1	8	0	27	20	2	16	0	0	0	0	0	0	0	0	0	0	0	0
Caudal agenesis	E	0	0	1	4	—	—	4	8	0	32	20	2	19	0	0	0	0	0	0	0	0	0	0	0	0
(7) *Other defects*																										
Hypoplastic/hyperplastic thymus	I	0	0	0	1	—	—	0	2	0	22	14	0	2	1	1	1	4	0	2	3	0	0	0	0	0
Pulmonary abnormalities[m]	I	0	0	0	0	—	—	0	0	0	0	0	0	0	0	0	1	0	0	0	3	0	0	0	0	0
Herniated diaphragm	I	0	0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Lateral displacement of oesophagus	I	0	0	0	0	—	—	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
Left-sided umbilical artery	I	4	11	9	0	—	—	8	3	3	2	4	13	1	2	6	7	5	6	14	2	2	8	5	12	6
Hind limb abnormalities	E	0	0	0	1	—	—	0	1	0	3	11	0	6	0	0	0	0	0	0	0	0	0	0	0	0

[a] In the main body of the Table, figures show the number of fetuses in which each particular abnormality was observed. [b] Four control groups, C1 to C4. [c] Retinoid abbreviations as in Materials and methods. [d] In general, abnormalities have been grouped according to anatomical site. [e] Including cranioschisis. [f] Dashes under 50 and 100 mg/kg TRA indicate that no fetuses were available for examination due to 100% resorption. [g] Mandibulofacial abnormalities include at least one of: microstomia, micrognathia, agnathia, maxillary retrocession, cleft lip, and unfused jaw. [h] Otic abnormalities include at least one of: anotia, microtia, and low-set ears. [i] Associated with other craniofacial abnormalities, e.g. shortened snout. [j] Optic abnormalites include at least one of: anophthalmia, exophthalmia, microphthalmia, folded retina, no Harderian gland, and ablepharia. [k] Convoluted, or slightly, moderately or severely dilated ureter. [l] Other vessel abnormalities include at least one of: separate innominate origin, retro-oesophageal aorta, no right subclavian artery, interrupted aorta, and common aortic/ductus trunk. [m] Pulmonary abnormalities include at least one of: alveolar haemorrhage, immature lungs, and nodulation of lung lobe.

Table 2. Summarized data for embryonic development in each of the four control groups[a] and the mean values

	Control groups				
	1	2	3	4	Mean
No. of implantations/dam	13.8	12.0	12.4	12.0	12.6
No. of resorptions (%)	7	6	6	4	5.8
No. of abnormal fetuses (%)	4	14	12	2	8.0
No. of externally abnormal fetuses[b] (%)	2	0	2	0	1.0
No. of internally abnormal fetuses[b] (%)	2	14	11	2	7.3
Mean fetal body weight (g±s.d.)	5.2±0.3	5.3±0.6	5.1±0.4	5.1±0.4	5.2

[a] See Table 1.
[b] Abnormalities defined as externally or internally observed are given in Table 1.

The incidence of abnormal fetuses, irrespective of the nature of the specific abnormality, was increased by all of the compounds tested compared to controls (Table 3). The incidence was markedly increased (defined as a 50–100% incidence) by TRA, ETR, ACIT, 13CRA, and 4HPR, and moderately increased (defined as a 20–50% incidence) by TZR and NBR. There was a mild increase (defined as a 10–20% incidence) in the occurrence of abnormal fetuses in the NER- and 13CNER-treated groups compared to the combined control group (8.0% incidence) (Table 3). However, particularly for the 600 mg/kg doses of both NER and 13CNER, the incidence of abnormal fetuses was within, or extremely close to, the upper range of the incidences seen in the four individual control groups (Table 2). To be classified as abnormal, a fetus must have shown either an externally or an internally observed abnormality as set out in Table 1. Although neither externally nor internally observed abnormalities predominate in the resultant classification of an abnormal fetus (Table 3), there is a tendency for the incidence of internally observed abnormalities to exceed that of externally observed abnormalities.

The *maximum possible* number of specific abnormalities, for any one of the seven groups of defects, for any one experimental group (i.e. retinoid dose level), is given by the multiplication of the number of fetuses examined (Table 1) by the number of specific abnormalities listed in each of the seven groups of defects (Table 1). The *actual* number of specific abnormalities, for any one of the seven groups of defects, for any one experimental group (i.e. retinoid dose level), is derived from the summation of the number of specific abnormalities in each of the seven groups of defects, as listed in Table 1. By dividing the *actual* occurrence by the *maximum possible* occurrence and multiplying by 100, the percentage incidence for any group of defects at any retinoid dose level may be calculated. These data are presented in Table 4 with, in addition, the 'Average incidence' calculated for all of the seven groups of defects. Presentation of data in this fashion allows the rapid assessment of the most severe groups of defects for each of the retinoid dose levels. The exceptions to this are the two highest doses of TRA, which produced 100% resorption of implants and thereby provided no fetuses for examination.

It is seen (Table 4) that the incidence of CNS defects was mild to moderate at the higher doses of TRA, ETR and 13CRA while the lower doses of these compounds produced only mild incidences. There was no

Table 3. Effect of retinoids on embryonic development: summarized data for the combined control group and the retinoid treatment groups[a]

	Retinoid treatment group; dose levels of retinoids (mg/kg)																					
	Control[b]	TRA					ETR		ACIT		13CRA		4HPR		TZR		NBR		NER		13CNER	
	0	6.25	12.5	25	50	100	25	50	25	50	100	200	300	600	300	600	300	600	300	600	300	600
No. of implantations/dam	12.6	13.3	12.7	13.3	11.8	13.3	13.3	13.8	13.3	11.8	12.8	12.8	13.2	11.3	13.6	13.5	12.0	12.5	12.7	13.8	12.8	13.0
No. of resorptions (%)	5.8	20.8	63.2	90.6	100	100	30.2	61.5	11.3	52.8	25.5	78.1	31.8	92.2	4.4	9.3	2.1	10.0	7.9	5.8	6.3	7.7
No. of abnormal fetuses (%)	8.0	85.7	100	100	—[c]	—[c]	100	100	61.7	100	100	100	97.8	100	30.8	28.6	31.9	20.0	19.5	12.3	19.7	15.0
No. of externally abnormal fetuses (%)[d]	1.0	35.7	100	100	—	—	100	100	6.4	88.0	100	100	31.1	100	4.6	16.3	21.3	8.9	4.9	4.1	0	0
No. of internally abnormal fetuses (%)[d]	7.3	85.7	96.4	100	—	—	100	100	61.7	100	100	100	97.8	100	26.2	18.4	19.2	13.3	14.6	10.2	19.7	15.0
Mean fetal body weight (g)	5.2	4.6	4.4	3.9	—	—	3.4	4.4	4.9	4.4	4.5	4.0	4.7	4.9	5.2	5.3	5.0	5.1	5.2	4.9	5.2	4.9

[a] Data for number of dams, number of implantations, number of resorptions and number of fetuses examined, for each retinoid treatment group, are presented in Table 1.
[b] Combined control group; mean values from four control groups (see Table 2).
[c] Dashes under 50 and 100 mg/kg TRA indicate that no fetuses were available for examination due to 100% resorption.
[d] Abnormalities defined as externally or internally observed are given in Table 1.

Table 4. Percentage incidences of groups of defects in the combined control group and the retinoid treatment groups[a,b]

	Retinoid treatment group; dose levels of retinoids (mg/kg)																					
	Control[c]	TRA					ETR		ACIT		13CRA		4HPR		TZR		NBR		NER		13CNER	
Group of defects	0	6.25	12.5	25	50	100	25	50	25	50	100	200	300	600	300	600	300	600	300	600	300	600
CNS	0.2	5.7	23.6	10.0	—[d]	—	8.1	21.0	2.6	13.6	0.5	31.1	0.5	0	0	0	0	0	0	0.4	0	0.3
Craniofacial	0	13.3	58.6	55.0	—	—	67.6	82.0	1.3	43.2	40.0	48.9	3.6	22.9	0	0.4	0.9	0.5	0.7	0.8	0.3	0
Urinogenital	0.7	25.7	37.9	25.0	—	—	31.9	31.0	23.8	42.4	31.1	42.2	8.0	5.7	5.9	4.5	4.3	3.1	2.7	1.2	2.3	3.7
Cardiac vessel	0	0	0	0	—	—	2.7	15.0	0	0	0	5.6	50.0	78.6	0	0	0	0	0	0	0	0
Haemorrhagic	3.1	4.8	1.8	0	—	—	20.3	2.5	2.1	6.0	5.3	0	37.8	57.1	2.3	5.1	8.5	1.1	1.8	2.0	0.8	0
Caudo-rectal	0	0	3·6	66.7	—	—	53.2	66.7	2.8	46.7	4.4	59.3	0	0	0	0	0	0	0	0	0	0
Other defects	0.9	4.4	5.4	8.3	—	—	12.2	24.2	4.6	6.0	3.5	11.1	6.3	26.2	2.1	1.7	4.3	2.2	1.4	3.1	2.5	1.7
Average incidence	0.7	7.7	18.7	23.6	—	—	28.0	34.6	5.3	22.6	12.1	28.3	15.2	27.2	1.5	1.7	2.6	1.0	0.9	1.1	0.8	0.8

[a] Abnormalities making up each group of defects are given in Table 1.
[b] The method of calculating the percentage incidence data, for any group of defects, is given in Results.
[c] Combined control group; mean values from four control groups (see Table 2).
[d] Dashes under 50 and 100 mg/kg TRA indicate that no fetuses were available for examination due to 100% resorption.

evidence of an effect with 4HPR, TZR, NBR, NER, and 13CNER, at the doses tested, on the appearance of CNS defects. The incidence of craniofacial defects was marked, with the higher doses of TRA and both doses of ETR, and moderate with both doses of 13CRA and the higher doses of ACIT and 4HPR. Urinogenital defects showed a moderate incidence with TRA, ETR, ACIT and 13CRA, while the occurrence particularly of ureteric abnormalities (Table 1) accounted for low incidences (defined as less than 10%) of urinogenital defects with 4HPR, TZR, NBR, NER and 13CNER. The very marked increase in the occurrence of cardiac vessel defects at both doses of 4HPR was striking. With the exception of the low to mild incidences of these lesions at the higher dose of 13CRA and both doses of ETR, cardiac vessel defects were totally absent from all other groups. 4HPR also produced moderate and marked increases in the incidence of haemorrhage at the low and high doses respectively. With the exception of 4HPR and possibly the lower dose of ETR, the incidence of haemorrhage with other retinoids was generally difficult to distinguish from its occurrence in controls. The highest dose of TRA which provided fetuses for examination (25 mg/kg), the higher dose of 13CRA, and both doses of ETR, produced marked incidences in the appearance of caudo-rectal defects while a moderate increase was produced by the higher dose of ACIT. Aside from the possible small increases in the appearance of caudo-rectal defects caused by the low dose of 13CRA and ACIT, the other compounds had no effect on the incidence of these changes. With regard to 'other' defects (see Table 1), ETR produced mild to moderate increases in the incidence of these effects, due to an increased level of thymic disorders. With the exception of 4HPR, all the other retinoids tested raised the incidence of 'other' defects to a low or mild level almost exclusively through an increased incidence of left-sided umbilical arteries (Table 1).

The relative adverse effects on embryonic development of each of the nine retinoids tested may be judged by a number of criteria including: the percentage of resorptions, the percentage of abnormal fetuses, the percentage of externally or internally observed abnormal fetuses (Table 3) and by the average incidence of abnormalities (Table 4). By assessing the relative toxicity of the com-

Table 5. Ranked orders for the teratogenicity of the nine retinoids tested based on several criteria for assessing adverse effects on embryonic development

Criteria for assessing toxicity	Ranked order[a]
1. No. of resorptions (%)	TRA > ETR > ACIT > 13CRA > 4HPR > TZR≡NBR≡NER≡13CNER
2. No. of abnormal fetuses (%)	TRA > ETR≡ACIT > 13CRA > 4HPR > TZR≡NBR > NER≡13CNER
3. No. of externally observed abnormal fetuses (%)	TRA > ETR≡13CRA > ACIT > 4HPR > TZR≡NBR > NER > 13CNER
4. No. of internally observed abnormal fetuses (%)	TRA > ETR≡13CRA > ACIT > 4HPR > TZR≡NBR≡NER≡13CNER
5. Cumulative criteria[b]	TRA > ETR > ACIT > 4HPR > TZR≡NBR > NER > 13CNER ↑13CRA↑
6. Compiled from 'Average incidence'[c]	TRA > ETR > ACIT > 13CRA > 4HPR > TZR≡NBR > NER≡13CNER

[a] The two highest doses of TRA caused 100% resorptions and therefore there were no fetuses for examination. However, TRA has to be considered the most toxic retinoid tested.

[b] Compiled from criteria 1 to 4.

[c] Compiled from the 'Average incidence' of defects (Table 4).

Table 6. Ranked orders for the teratogenic potential of selected retinoids *in vivo*[a] and *in vitro*

In vivo/In vitro	Origin of data	Ranked order
In vivo	Kistler[b]	TRA > ETR > ACIT > 13CRA > NER > 4HPR
In vivo	Present study[c]	TRA > ETR > ACIT > 13CRA > 4HPR > TZR≡NBR > NER≡13CNER
In vitro[d]	Kistler[b]	ACIT > TRA > 13CRA > 4HPR > ETR > NER
In vitro[e]	Steele *et al.*[f]	TRA≡ACIT≡13CRA > 4HPR≡TZR≡NER≡13CNER > NBR≡ETR

[a] In the rat.
[b] Data from Kistler (1987), Table 1.
[c] Data from Table 5, Criterion No. 6.
[d] LBCC technique.
[e] WEC technique.
[f] Data from Steele *et al.* (1987) (Tables 1–4).

pounds using these criteria, a series of ranking orders can be produced. These are presented in Table 5. Although the ranking has been achieved by a number of criteria, the objective was to attain an overall generalized ranked order of relative toxicity. On that basis, TRA was judged the most toxic compound assessed and ranked first under all criteria as fetal development was completely prevented at the higher doses of TRA used (Table 1). The ranking orders of the other eight retinoids were similar, regardless of the criteria employed (Table 5). In general, the compounds TRA, ETR, ACIT and 13CRA displayed a greater adverse effect on embryonic development than did 4HPR, which in turn was more toxic than the other retinamides TZR, NBR, NER and 13CNER.

Discussion

An important goal in reproductive toxicology at the present time is the development of in-vitro screening techniques which are capable of accurately predicting the toxic effects of compounds on mammalian embryonic development *in vivo*. The purpose of establishing such procedures is to provide simple, fast, accurate, reliable and inexpensive methods for the initial evaluation of compounds for adverse effects on embryonic development, without the delay and complexities which are associated with conventional in-vivo teratology safety evaluation studies (Kochhar 1980; Brown & Fabro 1982). However, for such in-vitro systems to be useful, they must accurately predict the in-vivo teratogenicity of pharmaceutical and environmental compounds. With this in mind, considerable effort is being expended to 'validate' a variety of in-vitro techniques by comparing in-vitro results with the responses shown by mammalian embryos *in utero* (Kochhar 1981, 1982; Saddler *et al.* 1982; Neubert 1985). The purpose of the present investigation was to evaluate the teratogenic potential of nine retinoids in the rat, and compare these findings with an earlier assessment of the compounds made *in vitro* using a rat WEC technique (Steele *et al.* 1987). The in-vivo and in-vitro results were also to be compared with the findings of Kistler (1987) who correlated the in-vivo teratogenicity of a series of retinoids in the rat with their in-vitro toxicity using a LBCC technique.

The malformations induced by the nine retinoids in the present study (Table 1) were characteristic of those previously reported to be induced by retinoids in rodents (Shenefelt 1972; Geelen 1979; Willhite *et al.* 1984; Kistler & Hummler 1985; Zimmermann *et al.* 1985). However, the types of malformations induced were dependent to a degree on the

chemical structures of particular retinoids. For example, retinoids with a free acid terminal (TRA, ACIT, 13CRA) induced caudo-rectal and CNS defects whereas, generally, these effects were not induced by the retinamides. Also, apart from very low incidences with TRA and 13CRA (1 of 74 and 1 of 47 fetuses, respectively), ETR and its major metabolite ACIT were the only compounds to induce hind limb abnormalities.

The incidence of adverse effects on embryonic development caused by the *cis*-isomer, 13CRA, were considerably lower than those of the *trans*-isomer, TRA (Table 1). This is in agreement with the findings of Willhite and Shealy (1984), Creech-Kraft *et al.* (1987) and Klug *et al.* (1989). These authors suggested that the teratogenicity of the *cis*-form depends on its metabolic isomerization to the *trans*-configuration. The incidence of malformations induced by the *cis*-isomer 13CNER, and the *trans*-isomer NER, however, were too low to allow a meaningful comparison of relative teratogenicity to be made between these two isomers. A very high incidence of cardioaortic defects was observed in 4HPR-treated animals (Table 1). Such effects were not looked for in a previous rodent study with 4HPR, which involved an external examination of the fetuses only (Kistler 1987), but have been described in humans following retinoid exposure (Chen 1985; Lammer *et al.* 1985; Rosa *et al.* 1986) and in response to retinoid administration in laboratory animals (Shenefelt 1972; Geelen 1979). It should be noted that variation in the incidence and distribution of malformations in experimental studies on retinoid teratogenicity will result from the use of different experimental protocols and dosing regimens (Shenefelt 1972). The experimental protocol in the present study, with retinoid administration on days 10 and 11 p.c., was designed to expose embryos to retinoids *in vivo* over approximately a 48-hour period at a particular stage of pregnancy. This was to parallel the 48-hour exposure period in the earlier in-vitro WEC experiments (Steele *et al.* 1987).

The results of the present study allow the nine retinoids under investigation to be placed in a series of ranking orders of in-vivo teratogenic potential, using a variety of criteria for assessing toxicity (Table 5). It is seen that the ranked orders of the compounds are similar. TRA possessed the highest teratogenic potential, followed by ETR and the retinoids with a free acid terminal (ACIT and 13CRA). It has been shown, in relation to the toxicity of ETR and ACIT, that ACIT is the free acid analogue and the main metabolite of ETR (Kistler & Hummler 1985; Bechter & Hall 1987; Reiners *et al.* 1988). Table 5 shows that the five retinamides in this study were consistently less potent than TRA, ETR, ACIT and 13CRA, but their ranked orders of teratogenicity were always comparable.

The ranked orders of in-vivo toxicity may be used to compare the present results with the in-vivo findings reported by Kistler (1987) in the rat for six of the nine retinoids: TRA, ETR, ACIT, 13CRA, 4HPR, NER (Table 6). The in-vivo data of Kistler are directly comparable with the present findings, with TRA being more toxic than its isomer 13CRA, and ETR and ACIT being more potent than 13CRA but less potent than TRA. However, with 4HPR and NER, the results of Kistler contrast with the present data, his findings showing that NER was teratogenic at 300 mg/kg whereas 4HPR was not toxic at 300 mg/kg, the highest dose level tested. Tables 1 and 5 demonstrate that, in our hands, 4HPR was more potent than NER, although both retinoids produced abnormalities at both 300 and 600 mg/kg. Taken together, the results suggest that structural modification of the terminal group at C15 can result in reduced teratogenic potential, as indicated by Willhite and Shealy (1984), but such modification did not prevent teratogenesis.

When the retinoids from the present study, and from the in-vivo investigation of Kistler (1987), are placed in their ranked orders of teratogenic potential, the findings may also be compared with the in-vitro

results from the LBCC system (Kistler 1987) and WEC assays (Steele *et al.* 1987), respectively (Table 6). The in-vitro LBCC information, and the in-vivo data of Kistler, both show the grouping together of the more potent retinoids TRA, ACIT and 13CRA, and the pairing of the less active retinamides 4HPR and NER. *In vitro*, however, ETR shows a low toxicity, which compares with the retinamides, whereas *in vivo* the toxicity of ETR compares with TRA, ACIT and 13CRA. Similarly, the in-vitro figures of Steele *et al.* (1987) with WEC, and the present in-vivo information, also show a directly comparable pattern, each demonstrating a grouping of the more potent retinoids TRA, ACIT and 13CRA, and a group of less active retinamides. Furthermore, here also, ETR *in vitro* demonstrated a low toxicity comparable to the retinamides, whereas *in vivo* the toxicity found a parallel with that of TRA, ACIT and 13CRA. Analysed in this way there is, in general, a good correlation between the LBCC assay and the in-vivo information of Kistler (1987), and the WEC system of Steele *et al.* (1987) and the current data. However, ETR gives an anomolous result with both in-vitro techniques and it is of interest that Bechter and Hall (1987) evaluated a rat WEC technique in an examination of the teratogenic potential of ETR, with an enzymatic drug activation system included in the culture medium. Using a rat liver homogenate (S-9 mix) or esterase (carboxylic-ester hydrolase) as the metabolizing system, the toxicity of ETR was greatly increased, clearly demonstrating the need for biological activation to the free acid for the expression of teratogenic potential.

We therefore conclude, using the ranked order of teratogenic potential for the nine retinoids, that there is broad agreement between the current in-vivo findings in the rat, and the previous results with the in-vitro WEC technique (Steele *et al.* 1987). ETR however gave an anomalous result *in vitro*, confirming that this compound requires metabolic activation for adverse effects on embryonic development to become evident. It therefore appears that the WEC technique can be used as a rapid and simple preliminary screen for the assessment of the teratogenic potential of retinoids which are in the biologically active form. However, for the assessment of teratogenic risks in humans, in-vitro findings must be substantiated by in-vivo studies in experimental animals.

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