Bull. Environ. Contam. Toxicol. (1995) 54:363–369

Environmental Contamination and Toxicology

Developmental Toxicity of Cyproconazole, an Inhibitor of Fungal Ergosterol Biosynthesis, in the Rat

K. Machera

Laboratory of Pesticide Toxicology, Benaki Phytopathological Institute, 8 Stephanou Delta Street, GR-145 61 Kifissia, Athens, Greece

Received: 24 January 1994/Accepted: 15 August 1994

Cyproconazole is a tertiary triazole-alcohol with antifungal activity. Triazoles are very widely used in agriculture and their antifungal activity is related to the inhibition of fungal steroid demethylation, with primary site of action the cytochrome P-450 (Buchenauer 1987).

Various effects of triazoles, such as reduction in fertility of the experimental animals through delay of ovulation, suppression of estradiol levels and inhibition of granulosa cell steroidogenesis have been observed (Milne et al. 1987; Wickings et al. 1987).
The developmental toxicity of several triazoles has been reported, and significant differences in the teratogenic potential between the various molecules are evident. The compound triadimefon when administered to pregnant rats caused maternal toxicity from the dose of 30 mg/kg and embryotoxicity at 100 mg/kg. At the doses of 75 and 100 mg/kg occasional cleft palates were observed and the no-observed effect level (NOEL) for the fetuses was 30 mg/kg (FAO 1981; 1983 b). Similar effects concerning maternal toxicity and embryolethality were observed on rats after the administration of triadimenol. The main effects observed on the embryonic development were skeletal abnormalities. The NOEL for the developmental toxicity of triadimenol is approximately 30 mg/kg (FAO 1989 b). When bitertanol was administered to rats at doses 10, 30 and 100 mg/kg a decrease in maternal body weight throughout the study and fetal body weight was observed from the dose of 30 mg/kg. Teratogenicity was observed at 100 mg/kg, cleft palate being the main effect (FAO 1983 a). When higher doses of bitertanol were tested on rats, caused acaudia, exophthalmus, hypognathia and cleft palate (Vergieva 1990). Embryolethality and teratogenicity were also observed when flusilazole was tested at doses up to 250 mg/kg. The main fetal abnormalities detected were cleft palate, absence of innominate artery, skeletal variations and retarded development. The NOEL determined was 2 mg/kg (FAO 1989 a). When higher doses of flusilazole were tested on rats, caused exophthalmus, hypognathia, macroglossia and cleft palate (Vergieva 1990). The administration of 1,2,4- triazole to rats at doses up to 200 mg/kg caused maternal toxicity, embryotoxicity and teratogenicity. The principal fetal abnormalities were cleft palate and malformations of the hind legs at the

dose of 200 mg/kg. The NOEL is 100 mg/kg for the dams and less than this for the fetuses (Renhof 1988 a and b).
When propiconazole was tested on rats at doses up to 300 mg/kg exhibited maternal toxicity and fetotoxicity at the dose of 300 mg/kg. Also at the same dose delay of ossification was observed (Ciba-Geigy 1992).

This work is part of a project aimed to study the developmental toxicity of triazole fungicides. In this report, the developmental toxicity of cyproconazole on Wistar rats is presented.

MATERIALS AND METHODS

The test compound, cyproconazole was of technical grade purity and kindly provided by Sandoz Ltd (Switzerland) through Geopharm (local representative). Initially, a range-finding test was performed for the detection of the maximal tolerated dose on the basis of maternal and fetal lethality. In this preliminary test a dose of 100 mg/kg was administered to 10 pregnant experimental animals. The doses administered during the main experiment were 20, 50, and 75 mg/kg body weight, in a volume of 10 mL/kg. The test compound was dissolved in carboxymethyl cellulose for both the preliminary and the definitive test. The same volume of the carrier was administered to the controls.

Female Wistar rats weighing about 200 g, fed with a standard diet, were allowed to mate with males of the same stock overnight, and vaginal smears were examined in the following morning for spermatozoa. The day on which spermatozoa were found was taken as the first day of gestation. The females were randomly distributed in four groups, one control and three experimental with 20 animals per group. The test compound was daily administered by gavage to pregnant rats from the 6th to the 16th day of gestation. The animals were weighed daily and examined for general condition and behaviour. They were sacrificed on the 21st day of gestation and the number of implantations, resorption sites and live and dead fetuses were recorded.

The fetuses were weighed, examined for external abnormalities, and crown-rump dimensions were taken. Approximately half of them were fixed in Bouin's fluid for the visceral examination, and the remaining fetuses were fixed in 10% ethanol, for the examination of skeletal abnormalities. The bodies were dissected according to the technique of Barrow and Taylor (1969). Heads were sectioned and skeletons were prepared and examined according to the technique of Wilson (1965). The data were analyzed by ANOVA followed by Duncan's multiple range comparison procedure.

RESULTS AND DISCUSSION

Signs of maternal toxicity, such as increased mortality, symptoms of intoxication, or reduction of body weight throughout the study were not observed in any of the treated animals of the definitive experiment. However, the general condition of the dams from the preliminary study at the dose of 100 mg/kg was not very good. A statistically significant decrease on the body weight gain at the early period of administration, 6th to 11th day, was observed in all the treated groups when compared to controls. This difference was more pronounced in animals treated

Table 1. Effects of cyproconazole on the parameters of pregnancy in the rat (means ± SD).

Effects	Cyproconazole(mg/kg)				
	0	20	50	75	100
No. of litters	17	20	20	20	10[2]
No. total implants per litter	10.06±2.28	9.00±2.79	8.50±2.01	9.25±2.15	9.00±2.49
No. fetuses per litter	9.64±2.40	7.60±3.30	5.20±2.90[1]	4.90±3.60[1]	1.60±3.24[1]
No. live fetuses per litter	9.53±2.37	7.55±3.25	4.95±3.05[1]	4.70±3.67[1]	0.40±0.84[1]
No. dead fetuses per litter	0.06±0.24	0.05±0.22	0.20±0.41	0.20±0.52	1.2±3.16[1]
No. resorptions per litter	0.41±0.51	1.40±2.43	3.35±3.15[1]	4.25±4.19[1]	7.40±3.37[1]
Fetal length (cm)	3.45±0.13	3.34±0.31	3.16±0.26[1]	3.16±0.21[1]	2.68±0.57[1]
Fetal body weight (g)	4.34±0.65	3.87±0.74[1]	3.43±0.79[1]	3.40±0.74[1]	2.70±0.60[1]

1 : Statistically significant different from the control group.
2 : Seven of ten litters were totally resorbed.

Table 2. External, visceral and skeletal observations on fetuses from dams treated orally with cyproconazole from day 6 to 16 of gestation.

Effects	Cyproconazole (mg/kg)				
	0	20	50	75	100
Externalobservations No. of fetuses observed/No. of litters	162/17	151/20	99/20	94/17	12[2]/3
Anophthalmia unilateral	0	0	1(1)[1]	0	0
Microphthalmia unilateral	0	0	0	1(1)	0
Micrognathia of mandibula	0	0	0	2(1)	0
Kinky tail	0	1(1)	0	1(1)	1(1)
Cachectic	0	4(2)	0	0	3(1)
Visceral observations No. of fetuses observed/No. of litters	100/17	86/20	54/20	50/17	12/3
Cleft palate	0	2(2)	11(5)	9(4)	11(2)
Internal hydrocephaly of moderate degree	0	5(3)	4(3)	10(9)	10(3)
Full blown internal hydrocephaly	0	0	6(3)	6(6)	2(2)
Ureterohydronephrosis	1(1)	5(4)	4(4)	6(6)	2(2)
Skeletonobservations No. of fetuses observed\No. of litters	62/17	65/20	45/20	44/17	-
Large fontanelles	1(1)	2(2)	7(3)	2(2)	-
Very large fontanelles and cranial sutures, delay of ossification	0	0	30(5)	17(4)	-

1 :In parentheses, number of affected litters.
2 :Eight of the twelve fetuses examined were found dead.

with 100 mg/kg.

The effects of the test compound cyproconazole from both the preliminary and the main study, on the parameters of pregnancy, i.e. number of implantation sites, number of live fetuses, number of dead fetuses, number of resorption sites per litter, mean fetal length and mean fetal body weight are presented in Table 1. A dramatic increase in the incidence of resorption sites and dead fetuses per litter were observed at the dose of 100 mg/kg. The total number of fetuses and the number of live fetuses per litter were decreased. A statistically significant decrease in the fetal body weight and length was also observed. At the doses of 75 and 50 mg/kg, statistically significant increases in the number of resorption sites were observed. In addition, statistically significant reductions in the total number of fetuses, number of live fetuses and decreases in the fetal length and body weight were observed. At the dose of 20 mg/kg a decreased fetal body weight was the only effect observed.

The effects of the test compound on fetal development, as they can be evaluated by the external, visceral and skeletal examination of the fetuses, are summarized in Table 2. The external abnormalities observed, were of low incidence and cannot be associated with the administration of the test compound. The visceral examination revealed a series of important abnormalities such as cleft palate, hydrocephaly and uretero-hydronephrosis. Cleft palate was observed at a frequency of 2.3, 20.4, 18.0 and 85.0 % at the doses of 20, 50, 75 and 100 mg/kg, respectively. No cases of cleft palate were observed in the animals of the control group. Hydrocephaly with lateral ventricles affected was evaluated as hydrocephaly of moderate degree, and hydrocephaly with both lateral and third ventricles affected was evaluated as full blown internal hydrocephaly. Full blown internal hydrocephaly was observed on the fetuses from the dose of 50 mg/kg and the incidence was 11, 12 and 17% at the doses of 50, 75 and 100 mg/kg respectively. Hydrocephaly of moderate degree affected the fetuses even at the dose of 20 mg/kg, and the incidence was 6, 7, 20 and 83% at the doses of 20, 50, 75 and 100 mg/kg, respectively. No cases of hydrocephaly were observed in the control animals. Ureterohydronephrosis was another abnormality developed at frequency of 1.0, 5.8, 7.4, 12.0 and 16.6 % at the doses of 0, 20, 50, 75 and 100 mg/kg, respectively. The above three abnormalities appeared in a dose-correlated response.
All the fetuses from the range-finding study (100 mg/kg) were examined only viscerally due to extensive fetotoxicity.

The skeleton examination of the fetuses from the definitive study revealed a dose-dependent retardation of ossification. The main effects caused by the higher doses administered (50 and 75 mg/kg) were the following: absence of one or several ossification centres of sternum, absence of the 13th rib, less than three metatarsal ossification centres, incomplete development of skull bones, distinctly larger fontanelles and wider cranial sutures. The effect on the size of the cranial fontanelles was evaluated as severe, which was usually followed by a general delay of the ossification and of moderate degree which was observed even at the lower doses. The total frequency of the effect on the size of the cranial fontanelles was developed at frequencies of 1.6, 3.1, 60.0 and 43.0 % at the doses of 0, 20, 50 and 75 mg/kg, respectively.

The observed effects of cyproconazole are in agreement with the effects reported elsewhere, related to other triazoles. The majority of triazoles examined were found to cause maternal toxicity and embryotoxicity. Teratogenicity appeared in most of the studied cases at maternally toxic levels. Cleft palate was the most characteristic teratogenic effect caused by the studied triazoles (FAO 1981; FAO 1983; FAO 1989; Vergieva 1990; Renhof 1988 a & b). Skeletal variations and retardation of ossification have also been associated with the administration of flusilazole and propiconazole (FAO 1989 a; Ciba-Geigy 1992). Hydrocephaly has also been mentioned as an abnormality after the administration of other triazoles, but at non-statistically significant incidence. Hydrocephaly was one of the main abnormalities, after the administration of cyproconazole, observed in our experiment.

However, the teratogenic potential greatly varies among the different triazole molecules. The results from a pilot study in our laboratory with the fungicides penconazole and fenbuconazole, are indicative of a variation in the developmental toxicity among triazoles.
No maternal toxicity was observed when penconazole, a triazole without other functional groups, was tested at a single dose of 300 mg/kg. Slight increase in the resorptions was observed but at no statistically significant levels when compared to the control group. Delay of ossification was observed in 65% in the fetuses of the test group, with the respective incidence in the control group being 10%. In addition, one fetus out of 76 exhibited unilateral anophthalmia and two fetuses omphalocele. On the other hand, when fenbuconazole, a triazole with a nitrilo group, was tested at doses 0, 20, 50 and 200 mg/kg embryolethality was detected from the dose of 50 mg/kg. A statistically significant decrease in the total number of fetuses was observed at the dose of 200 mg/kg. In all the experimental litters, fetuses with haematoma on various sites of the body were observed at a very high incidence. In addition, fetuses with abnormally large heart were detected at a dose correlated incidence. The main effect was observed on the auricles which were very hyperplastic and in many cases expanding over the whole area of ventricles. In these cases pericardium was also severely affected, showing an extreme enlargement and very loose appearance. Remarkable distention of the first part of the aortic arch was also observed. In addition, hypoplasia of the lungs was observed at significant incidence, at the highest dose of 200 mg/kg.

The above data indicate that cyproconazole has embryo- / fetal toxicity and teratogenic potential. Maternal toxicity was clearly observed at the dose of 100 mg/kg. At other doses a delay in the maternal body weight gain was the only effect observed on the treated dams. This effect was not persistent throughout the study. Fetotoxicity was observed from the dose of 50 mg/kg and teratogenicity even at the no-maternal-toxicity dose or the non fetotoxic dose of 20 mg/kg. The main teratogenic effects were cleft palate, hydrocephaly and severe retardation of ossification. From these results it can be concluded that the NOEL for maternal toxicity and fetotoxicity is 20 mg/kg and for teratogenicity is definitely lower.

For the clarification of differences regarding the developmental toxicity among the group of triazoles and in many cases for the determination of NOEL for teratogenicity, further studies need to be carried out.

The teratogenic hazard of triazoles, is probably caused by a combination of

bioavailability and cytochrome P-450 mediated metabolism by the embryo cells to form a putative toxic metabolite (Flint et al. 1986). On the other hand the antifungal activity of triazoles is related to the inhibition of fungal steroid demethylation, through binding to cytochrome P-450 (Buchenauer 1987). Consequently the probable mechanisms of antifungal activity of triazoles are fundamentally different from the mechanisms of teratogenicity, and it should be possible to find compounds with low or no teratogenic hazard but retaining good antifungal activity.

The selection of triazoles, from the already registered active ingredients, with high antifungal activity and minimum teratogenic hazards is the aim of further studies in this project.

REFERENCES

Barrow VM, Taylor WJ (1969) A rapid method for detecting malformations in rat fetuses. J Morph 127, 291-306

Buchenauer H (1987) Mechanism of action of triazolyl fungcides and related compounds. In: Lyr H (ed) Modern selective fungicides. Longman Scientific & Technical, England.

Flint OP, Boyle FT (1986) Structure-teratogenicity relationships among the mono- and bistriazole antifungal agents, using an *in vitro* test for teratogenic hazard. Fd Chem Toxic 24:649

Milne CM, Hasmall RL, Russel A, Watson SC, Vaughan Z, Middleton MC (1987) Reduced estradiol production by a substituted triazole results in delayed ovulation in rats. Toxicol Appl Pharmacol 90:427-435

Wickings EJ, Middleton MC, Hillier SG (1987) Non-steroidal inhibition of granulosa cell aromatase activity *in vitro*. J Steroid Biochem 26, 6:641-646

FAO Technical Papers. FAO plant production and protection papers. 42. Pesticide residues in food 1981 - Evaluations, 1982 pp 512-523

FAO Technical Papers. FAO plant production and protection papers. 61. Pesticide residues in food 1983 a - Evaluations, 1985 pp 47-57

FAO Technical Papers. FAO plant production and protection papers. 61. Pesticide residues in food 1983 b - Evaluations, 1985 pp 447-450

FAO Technical Papers. FAO plant production and protection papers. 100/2. Pesticide residues in food 1989 a - Evaluations part II: Toxicology, 1990 pp 117-139

FAO Technical Papers. FAO plant production and protection papers. 100/2. Pesticide residues in food 1989 b - Evaluations part II: Toxicology, 1990 pp 215-236

Ciba-Geigy (1992) Letter to EPA containing a summary of the report on CGA-64250 technical teratology study in rats; study number 790011, 1979.

Renhof M (1988 a) 1,2,4 -triazole; investigations into embryotoxic effects on rats after oral administration. Report no 17401 Bayer AG Fachbereich Toxicologie

Renhof M (1988 b) 1,2,4 -triazole; investigations into embryotoxic effects on rats after oral administration; supplement to study No. T 5019339. Report no 17402 Bayer AG Fachbereich Toxicologie.

Vergieva T (1990) Triazoles teratogenicity in rats. Teratology 42 (2):27A-28A

Wilson JG (1965) Methods for administrating agents and detecting malformations in experimental animals. In: Wilson JG, Warkany J (eds) Teratology, principles and techniques. University of Chicago Press, Chicago.