5. **Developmental Toxicity Study in Rabbits**

Testing Facility:

Study Numbers: Contract Lab.'s No. ML-222D
Sponsor's No. 000603

Study Dates: February to June, 1981

GLP Compliance: The study was conducted in compliance with GLP regulations.

Animals: Japanese white rabbits, about 3 months of age, obtained from were mated after a one month acclimatization period. Females showing signs of mating were assigned to 4 groups of 14 animals each (mean body weight 4.0 kg at the initiation of dosing). The confirmed day of mating was considered as Day 0 of pregnancy.

Dose Levels and Mode of Administration: 0, 30, 150 and 1000 mg/kg/day. OPC-21 (Lot Numbers 1B76 and 1C74M), suspended in 0.5% carboxymethylcellulose at appropriate concentrations, was administered daily by oral gavage at a dose volume of 5 ml/kg from days 6 to 18 of pregnancy.

[Note: It is stated that the above doses were selected based on the results of single dose and 14-day administration dose ranging studies with non-pregnant female rabbits. In the single dose study, the test drug was administered to 4 mature non-pregnant rabbits, by oral gavage, at a maximum feasible dose of 5000 mg/kg. After 7 days of observation, animals were sacrificed (on the 8th day after dosing) and the organs were examined macroscopically. In the 14-day repeat dose administration study, groups of rabbits (4/group) received the test drug by oral gavage for 14 days at 0, 500, 1000 or 2000 mg/kg/day. Clinical signs, mortality, body weight and water and food consumption were recorded daily. On the day following termination of treatment, animals were killed for post mortem examination.

Results of the above studies revealed that a single administration of the test compound, at the maximum feasible dose of 5000 mg/kg, did not cause any abnormal clinical signs or changes in body weight. After repeated administration of the test drug, body weight and food consumption tended to be higher than control at 1000 and 2000 mg/kg/day, and lower than control at 500 mg/kg/day. No macroscopic lesions were observed in the study. Based on the above

results, a dose which produces adverse effects in rabbits when administered repeatedly, was assessed to be higher than 2000 mg/kg/day. However, according to the sponsor, "administration of the test compound at such high doses was considered to be impracticable and would be tortuous to the animals; therefore, a dose of 1000 mg/kg/day was chosen as the high dose" for the definitive study.]

Observations and Measurements: The dams were observed daily for clinical signs, mortality and abortion. Body weights and food consumption were recorded once daily. Animals were sacrificed on day 28 of pregnancy, uterine contents examined and the numbers of corpora lutea, implantation sites and dead and live fetuses were counted. Fetal body weights and placental weights were recorded. Live fetuses were examined for external, visceral or skeletal abnormalities.

Data on maternal weights, food consumption, fetal and placental weights, and the numbers of corpora lutea and implantation sites were statistically analyzed using the t test. Data on the incidences of post-implantation loss, implantation rate, external, visceral and skeletal anomalies, retardation of ossification, and variation of vertebral bodies were analyzed by Wilcoxon's rank sum test.

Results:

Dams -

Accidental dosing injury or lumbar paralysis due to mis-handling was seen in 1 rabbit each from control, low and mid dose groups and 2 from the high dose group. Necropsy revealed that 2 control, 1 low dose, 3 mid dose and 2 high dose females were non-pregnant. All of the above animals were excluded from data analyses.

No treatment-related effect on body weight or food consumption was seen in the study.

One dam from the mid dose group aborted on day 28 of pregnancy. No treatment-related findings were seen macroscopically at the necropsy of this animal or of any other animals which were killed after normal maintenance of pregnancy.

Fetuses -

The numbers of corpora lutea, implantations and live fetuses, incidence of post-implantation loss, and placental and body weights

Table 22. Influence of OPC-21 on the Numbers of Corpora Lutea and Implantations, Post Implantation Loss, Body Weight of Live Fetuses, and Placental Weight in Rabbits

Group mg/kg (i.g.)	No. of mothers	Corpora lutea		Implantations		Post implantation loss		Live fetuses			Body weight (g) Mean±SD		Placental weight (g) Mean±SD	
		Group total	(Mean per mother)	Group total	(Mean per mother)	RS/DE	(% ratio to implantations)	Male/ Female	Total	(Mean per mother)	Male	Female	Male	Female
Control	11	137	(12.5)	72	(6.5)	5/4	(12.5%)	30/33	63	(5.7)	39.5 ±7.2	36.4 ±5.2	6.6 ±1.4	5.9 ±0.7
30	12	158	(13.2)	118	(9.8)*	4/7	(9.3%)	46/61	107[b]	(8.9)*	35.8 ±4.4	34.8 ±6.4	5.6 ±0.9	5.4 ±1.0
150	10-1[a]	104	(11.6)	74	(8.2)	2/2	(5.4%)	35/35	70	(7.8)	39.2 ±7.4	38.3 ±6.2	6.1 ±1.3	6.2 ±1.1
1000	10	123	(12.3)	81	(8.1)	5/2	(8.6%)	38/36	74	(7.4)	40.8 ±7.2	39.8 ±8.0	6.4 ±1.4	6.4 ±1.3

RS: Resorption sites
DE: Dead embryos
SD: Standard deviation
i.g.: intragastric

a) One mother aborted on the 28th day of gestation.
b) Ectopia of the right kidney in one fetus.
*: Different significantly from control group ($P<0.05$).

of live fetuses are presented in Table 22. No significant differences in the number of corpora lutea, post-implantation loss or placental and body weights of live fetuses were seen between treated and control groups. The numbers of implantations and live fetuses in treated groups were higher than in the control group, the difference being statistically significant at the low dose.

No external anomalies were seen in any of the fetuses. No visceral anomalies, except for ectopia of the right kidney in one low dose fetus, were seen.

The incidence of skeletal anomalies, and the numbers of ribs and caudal vertebrae observed in fetuses are presented in Table 23 and 24, respectively. No dose-dependent increase in skeletal anomalies or treatment-related difference in the numbers of ribs or caudal vertebrae was seen.

The incidence of retardation of ossification is presented in Table 25. The incidence of delayed ossification of the sternum was higher in treated groups than in the control group, the difference being statistically significant at the high dose. Although not statistically significant, the incidence of delayed ossification of metacarpus was higher in the high dose group than in the control group. However, the incidence rate of 5.4% observed in this study at the high dose is stated to be within the historical control range (2 to 9%) for the laboratory where the study was performed.

Table 23. Skeletal Anomalies in Rabbit Fetuses Treated with OPC-21

Group mg/kg	Total fetuses observed	Fetuses with skeletal anomalies						
		No.	(% ratio to total fetuses)	Sternum	Rib	Lumbar vertebrae	Lumbar and sacral vertebrae	Caudal vertebrae
Control	63	2	(3.2%)	1	-	-	1[a)]	1[a)]
30	107	3	(2.8%)	3[a)]	-	1[a)]	-	-
150	70	2	(2.9%)	-	1	-	-	1
1000	74	1	(1.4%)	1	-	-	-	-

i.g.: intragastric
a) Observed in the same fetus.

Table 24. Observation of the Numbers of Ribs and Caudal Vertebrae in Rabbit Fetuses

Group mg/kg i.g.	Total fetuses observed	No. of fetuses and / or % occurrence in each category									
		Rib configurations[a]					% occurrence of fetuses with each No. of caudal vertebrae				
		Normal	Abnormal				No. of caudal vertebrae				
		12/12	12/13	13/12	13/13	(% ratio of rib variations to total fetuses	13	14	15	16	17
Control	63	39	0	6	18	(38.1%)	0%	12.7%	55.5%	27.0%	4.8%
30	107	80	9	8	10	(25.2%)	1.9%	20.6%	53.3%	22.4%	1.9%
150	70	49	2	3	16	(30.0%)	4.3%	10.0%	57.1%	28.6%	0%
1000	74	46	1	4	23	(37.8%)	1.4%	17.6%	50.0%	31.1%	0%

i.g.: intragastric
a) Left side / Right side

Table 25. Observation of Ossification in Rabbit Fetuses

Group mg/kg i.g.	Total fetuses observed	No. of fetuses with delayed ossification and % occurrence					
		Sternum	(% ratio to total fetuses)	Middle phalanx	(% ratio to total fetuses)	Metacarpus	(% ratio to total fetuses)
Control	63	10	(15.9%)	9	(14.3%)	1	(1.6%)
30	107	27	(25.2%)	0	(0%)	2	(1.9%)
150	70	29	(41.4%)	2	(2.9%)	1	(1.4%)
1000	74	30	(40.5%)*	2	(2.7%)	4	(5.4%)

i.g.: intragastric
*: Significantly different from control group ($P < 0.05$).

6. **Perinatal/Postnatal Study in Rats**

Testing Facility:

Study Numbers: Contract Lab.'s No. ML-222F
Sponsor's No. 000900

Study Dates: December 1981 to June 1982

GLP Compliance: The study was conducted in compliance with GLP regulations.

Animals: Jcl:SD strain male and female rats (about 11 weeks old) were obtained from After a week of acclimatization, animals were mated, and females showing vaginal plugs were assigned to 4 groups (21-24 pregnant females/group; mean body weight 365 g at the initiation of dosing). The confirmed day of mating was considered as day 0 of pregnancy.

Dose Levels and Mode of Administration: 0, 30, 150 and 1000 mg/kg/day. OPC-21 (Lot No. 1F95M), suspended in 0.5% carboxymethylcellulose at appropriate concentrations, was administered daily by oral gavage at a dose volume of 10 ml/kg from day 17 of pregnancy through post partum day 27.

Observations and Measurements: Animals were observed daily for clinical signs. Body weights and food and water consumption were recorded daily until delivery and weekly thereafter.

Delivery of litters was observed, and dams were allowed to nurse their litters for 4 weeks after parturition. The nursing behavior of dams and the growth of F1 offspring were observed. The litter size per dam was limited to 10 (5 males and 5 females).

Body weights, emergence of hair, detachment of auricles, eruption of incisors and opening of the external auricular canals and palpebral fissures in F1 offspring were recorded.

General behavior of F1 offspring from 2 dams per group was observed daily and the offspring from the remaining dams were observed weekly. Behavioral assessment parameters included observation of head movement, righting reflex, rolling, creeping, walking, running, sitting, standing, grooming and social behavior.

At weaning (postpartum day 28), all dams and F1 offspring were necropsied for gross visceral examination except the offspring used

for the observations of growth, auditory function, learning ability and reproductive performance (2 animals/sex/litter).

One male and 1 female were additionally selected from 5 dams each from the control and high dose groups for the learning ability test.

The following observations/determinations were made with the F1 animals retained. (At the end of the study, all these animals were necropsied for gross visceral examination.)

a) growth and general behavior: All animals were weighed once every week; and postures, walking, drinking, eating and social behavior were observed.

b) auditory function: Test was performed in 20-23 males and females from each group at 5 weeks of age.

c) learning behavior: Five males and females each from control and high dose groups were tested in the lever pressing response and the light-dark discrimination experiments.

d) spontaneous motor activity: Five males and females each (6-7 weeks) from the control and high dose groups were tested using a motor activity measurement apparatus.

e) reproductive performance: 20 to 23 males and females from each group were paired at 12 weeks of age.

The F1 females were allowed to deliver offspring and observed 1 week for nursing behavior. The F2 offspring were observed for milk feeding behavior, reflex movement and functional activity.

Data on body weight, food and water consumption, litter size and learning ability parameters were statistically analyzed using the t-test. The mortality data were evaluated by Wilcoxon's rank sum test.

Results:

Dams -

There were no notable general behavior findings in dams during pregnancy or lactation. One high dose animal was sacrificed on postpartum day 18 due to dosing injury.

No significant difference in delivery was noted between control and treated groups. Although no treatment related effect on body weight

was noted during pregnancy, the body weights for the mid and high dose groups were lower than the control group weight during the first week of lactation, but body weights in these groups returned to control levels or higher during the latter part of lactation. Significant reductions in food consumption were noted at high and mid doses during gestational days 17 to 22 and 19 to 22, respectively.

Necropsy of dams at weaning showed calculus of the right kidney and urinary bladder and necrosis of the left kidney in one high dose animal.

F1 offspring -

Postnatal growth and survival findings for offspring, up to 4 weeks of age, are presented in Table 26. The litter size per dam at birth was not significantly different between control and treated groups. The body weights of high and mid dose F1 offspring at birth were lower than concurrent control weights. The numbers of stillborn were increased in the high and mid dose groups (statistically significant at the high dose).

External anomalies observed in high dose neonates and stillborns included vestigial tail with anal atresia in 1 liveborn, vestigial tail and anal stenosis in another liveborn, and microstomia with micrognathia and cleft palate in 1 stillborn pup. In the pup with vestigial tail and anal stenosis, ventricular septal defect and agenesis of the sacro-coccygeal vertebrae were also observed. (Note: It is not stated in the report whether external anomalies were seen in other treated and control groups.)

The mortality of pups during the postpartum period was higher in the mid dose group than in the control group, but the difference was not significant.

On necropsy, hydronephrosis and ascites were observed in one high dose pup that died at 3 days of age.

The body weights of offspring at weaning were not significantly different between control and treated groups.

Auricular detachment, hair emergence, incisor eruption, opening of the external auricular canal and palpebral fissure were similar in control and treated groups except that 1 high dose animal had a narrow fissure in the right eyelid at 21 days of age.

Necropsy of weanling offspring showed hydronephrosis in 3 mid dose animals.

Table 26. Postnatal Observation of the Rats Born from OPC-21 Treated Mothers

Group and dose (mg/kg, ig)	No. of mothers (M)	Total litter size (S) ( ):(S)/(M)	Body weight(g) at birth Mean S.D.	No. of dead at birth (BD) ( ):(BD)/(S)x100	No. of nursed (N) ( ):(N)/(M)	No. of dead 1-28th day (ND) ( ):(ND)/(N)x100	No. of weaned (W) ( ):(W)/(N)x100	Body weight(g) of weanling Mean S.D. Male	Femal
Control	23	319 (13.9)	6.3 0.6	3 (0.9%)	225 (9.8)	1 (0.4%)	224 (99.6%)	96.7 7.9	89.3 7.2
30	23	322 (14.0)	6.2 0.6	5 (1.6%)	225 (9.8)	3 (1.3%)	222 (98.7%)	95.7 7.5	88.5 6.2
150	24	340 (14.2)	5.9* 0.6	35 (10.3%)	225 (9.8)	18 (8.0%)	207(a) (92.0%)	94.2 6.8	87.2 5.4
1000	21(b)	292(c) (13.9)	5.9* 0.4	28(d) (9.6%*)	191 (9.6)	4(e) (2.1%)	187(f) (97.9%)	92.3 7.8	86.6 7.9

* : Significantly different from control, P<0.05
ig : Intragastrically
(a) 3 female : Unilateral hydronephrosis
(b) One mother was sacrificed on postpartum day 18 because of unsuccessful administration
(c) 1 female : Vestigial tail with anal stenosis, 1 female : Vestigial tail and anal atresia with ventricular septal defect
(d) 1 female : Microstomia with micrognathia and cleft palate
(e) 1 male : Bilateral hydronephrosis
(f) 1 male : Unilateral microphthalmia with anomaly of the right eyelid

No treatment-related effects were seen on post-weaning growth, auditory function, learning behavior or spontaneous motor activity.

Results of mating studies of F1 offspring are presented in Table 27. Females from all groups became pregnant after first or second mating except for one high dose female. One male animal each from the mid and high groups were found to be infertile.

F1 dams were allowed to deliver and nurse their pups for 7 days. The litter size, the number of dead and living pups and body weight of pups are presented in Table 28. The litter size was not significantly different between control and treated groups. The number of stillborn was higher in the high dose group than in other treated or control groups.

Dead offspring were necropsied and no visceral anomalies were seen. Externally, unilateral open eyelid was observed in 1 high dose pup.

The behavior of dams was normal at delivery and during lactation except for 1 high dose dam which ate all her pups after delivery.

After 7 days of nursing, the dams and their paired males were necropsied. There were no gross anomalies except for unilateral hypoplastic testes which were seen in 1 male each from the low and mid dose groups.

Table 27. Occurrence of Copulation and Pregnancy in the Rats Born from OPC-21 Treated Mothers

Group and dose (mg/kg, ig)	The first mating			The second mating			Total No. of pregnant
	No. of pairs	No. of copulation	No. of pregnant	No. of(a) observed	No. of copulations	No. of pregnant	
Control	23	23	23	0	0	0	23 (100.0%)(b)
30	23	23	22	1	1	1	23 (100.0%)
150	21	19	19	2	2	2	21 (100.0%)
1000	20	20	18	2	2	1	19 (95.0%)

(a) No. of female rats which did not become pregnant at the first mating
(b) [Total No. of pregnant / Total No. of females] x100 (%)
ig : Intragastrically

Table 28. Reproductive Performance in the Rats Born from OPC-21 Treated Mothers

Group and dose (mg/kg, ig)	No. of mothers (M)	Total Litter size (S) ( ):(S)/(M)	Body weight(g) at birth Mean S.D.	No. of dead(D) ( ):(D)/(S)x100 at birth	No. of dead(D) ( ):(D)/(S)x100 1-7th day	No. of living young at the 7th day (L) ( ):(L)/(M) Male	Female	Total	Body weight (g) at the 7th day Mean S.D. Male	Female
Control	23	309 (13.4)	6.0 0.7	8	4 (3.9%)	153	144	297 (12.9)	14.5 2.3	13.7 2.4
30	23	304 (13.2)	6.0 0.5	11	1 (3.9%)	137	148	285 (12.4)	14.7 1.8	14 1
150	21(a)	244 (12.8)	6.4* 0.6	10	6 (6.6%)	121	106	227 (11.9)	16.0* 2.3	15.6* 2.4
1000	19(b)	247 (13.7)	6.0 0.4	26	5 12.6%	110	106	216(c) (12.7)	14.6 2.5	14.0 2.6

* : Significantly different from control, P<0.05
ig : Intragastrically
(a) Data was calculated on 19 mothers
(b) Data was calculated on 18 mothers
(c) Unilateral open eyelid in one male

Genotoxicity Studies

Ames Test with Cilostazol (OPC-13013)

Testing Facility:

Study Number: 211108-0698

Study Dates: May 25 to June 18, 1981

GLP Compliance: The study was conducted in compliance with GLP regulations.

Lot Number of the Test Compound: 1C74M (purity 99.57%)

Concentrations Tested: 1, 5, 10, 50, 100, 500 and 1000 µg OPC-13013/plate (The doses were selected based on a preliminary toxicity study, under the same experimental conditions as the present study, in which 5000 µg/plate produced growth inhibition, and 1000 µg/plate caused minimum toxicity.)

Solvent: Dimethylsulfoxide (DMSO)

Tester strains: Salmonella typhimurium strains TA1535, TA1537, TA1538, TA100 and TA98, and E.coli WP2uvrA.

Metabolic Activation System: Rat liver S-9 fraction (obtained from phenobarbital/beta-naphthoflavone-treated Sprague-Dawley male rats)

Positive Control Compounds Not Requiring S-9 Mix: N-Ethyl-N-nitro-N-nitrosoguanidine (ENNG; strains TA 1535 and WP2uvrA), methyl methane sulfonate (MMS; TA100), 9-aminoacridine (ACR; TA1537) and 2-nitrofluorene (2NF; TA1538 and TA98)

Positive Control Compound Requiring S-9 Mix: 2-aminoanthracene (all strains - to test the activity of S-9 mix)

All positive control compounds were dissolved in DMSO except ENNG and MMS, which were dissolved in distilled water.