Fifty-two-Week Oral Toxicity Study in Beagle Dogs

Testing Facility:

Study Number: 001267 (Report Number - 001997)

Study Dates: September 9, 1982 to May 26, 1984

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

Animals: Beagle dogs USA), 4/sex/group and housed individually in stainless steel cages, were about 8 months old (body weight ranged 9.4-12.0 kg for males and 7.0-10.7 kg for females) at the initiation of dosing.

Dose Levels and Mode of Administration: 0, 6, 12, 30 and 150 mg/kg/day. The drug was orally administered once daily in gelatin capsules. Control animals received empty capsules. [It is stated that the doses were selected based on the results of a 13-week oral toxicity study (0, 30, 150 and 750 mg/kg/day) in beagle dogs. In that study, dose-dependent coronary arteritis and periarteritis, and intimal thickening of coronary artery were seen at 150 and 750 mg/kg/day doses. No significant histopathological findings were noted at 30 mg/kg/day. Therefore, 150 mg/kg/day was selected as the high dose for the present study. Six mg/kg/day, which is twice the dose showing pharmacological activity in dogs, was selected as the low dose, and 12 and 30 mg/kg/day were chosen for intermediate doses.]

Observations/Measurements: Dogs were observed twice daily for clinical symptoms. Body weights were recorded before the initiation of treatment and once weekly throughout the study. Food consumption was determined daily. Water consumption was recorded pretest and at the end of 13, 26, 39 and 52 weeks of treatment. Urinalyses were conducted at the same time intervals as water consumption measurements. Hematological [erythrocytes, leucocytes (total and differential), platelet and reticulocyte counts, hemoglobin, hematocrit, prothrombin time, activated partial thromboplastin time, MCV, MCH and MCHC] and blood chemistry (GOT, GPT, LDH, CPK, leucine aminopeptidase, cholinesterase, alkaline phosphatase, total bilirubin, glucose, total protein, serum protein fractions, triglycerides, phospholipids, total cholesterol, urea nitrogen, creatinine, uric acid, allantoin, sodium, potassium, chloride, calcium and inorganic phosphorus) evaluations were conducted at

pretest and during treatment weeks 13, 26, 39 and 52. EKG and heart rates were recorded for all control and high dose animals before the initiation of treatment, and immediately before and 2 hr after dosing during treatment weeks 12, 25, 38 and 51. Rectal temperature and respiration rates were also recorded for control and high dose animals at the above time intervals. Ophthalmologic examinations were done on all animals (all groups) pretest and after 12, 25, 38 and 52 weeks of treatment. Hearing tests were done on all animals at the initiation of the study, and high dose and control animals were also tested during treatment weeks 12, 25 38 and 52. Indocyanine green (ICG) excretion tests (ICG was injected iv at 0.5 mg/kg and the amount retained in the blood at 30 min post-dose was determined) were conducted on high dose and control animals at the termination of the study.

Blood samples were collected from all treated animals immediately before and at 2 and 4 hr after dosing on the last day of treatment for plasma drug level determinations.

At the end of the study, complete necropsies were performed, all organs were grossly examined, and brain, pituitary, submandibular glands, thyroid, thymus, heart, lungs, liver, spleen, pancreas, adrenals, kidneys, testes, prostate, ovaries and uterus were weighed. In addition to these organs, samples of the following tissues were also preserved in 10% neutral buffered formalin: gall bladder, lymph nodes, (submandibular, retropharyngeal and mesenteric nodes), aorta, trachea, larynx, tongue, salivary glands, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, ureter, epididymis, vagina, skeletal muscle, mammary gland, skin, spinal cord, sciatic nerve and bone marrow (sternum, ribs and femur). The eye balls were fixed in Davidson's fluid. The tissues were processed by routine histologic procedures and stained with H&E and Masson-trichrome. Additionally, the heart and aorta sections were stained with Elastin van Gieson, the brain and spinal cord with Kluver-Barrera, and liver and kidneys with PAS staining procedures. Tissues from all animals were examined histologically.

Data were evaluated statistically by the analysis of variances, followed by Student's t test or by Welch's test.

Results: Mild diarrhea was observed in high dose animals throughout the treatment period. No treatment-related deaths occurred in the study. One control female dog was sacrificed *in extremis* on Day 156.

No significant treatment-related effects on body weight or food consumption were seen.

Table 3. Gross abnormalities of electrocardiographs
-52 weeks oral toxicity study of OPC-13013-

Dose mg/kg	Animal No.	Sex	Pretreatment	Hours after dosing							
				12weeks		25weeks		38weeks		51weeks	
				pre	2	pre	2	pre	2	pre	2
0	001	M									
	002	M									
	003	M	B		B	B	B	B	B	B	B
	004	M								E	
	021	F	E	E							
	022	F									
	023	F				–	–	–	–	–	–
	024	F									
150	017	M			T			T	T		D
	018	M									
	019	M								B	B
	020	M									
	037	F									
	038	F								A	
	039	F					E		E		
	040	F									

A : Atrioventricular blocks B : Bradycardias
D : Depression of the S-T segment E : Elevated negative T waves
T : Tachycardias – : Not examined

Urinalysis showed the presence of occult blood in both treated and control groups, the incidence being slightly higher in the treated animals than in controls (not dose-dependent). No significant hematological findings or serum enzyme elevations were seen. Total cholesterol and phospholipid levels were slightly, but significantly, increased (not dose-dependent) in the 12, 30 and 150 mg/kg/day female dose groups only during treatment week 39. No treatment-related findings were seen for the hepatic function test.

The electrocardiographic findings observed in the high dose and control animals are presented in Table 3. Bradycardia (less than 70 beats/min) was observed in one of the four high dose males at treatment week 51 and in one of the four control males at pretest and throughout the treatment period. An increase in the amplitude of the negative T-wave was seen in one male and one female from the control group and in one high dose female. Tachycardia and ST-segment depression was noted in one high dose male. One high dose female showed atrioventricular block (grade II) during week 51. There were no treatment-related changes in R-R, P-P, PQ, QRS and QT intervals.

Hearing tests and ophthalmologic examinations revealed no significant findings.

The mean plasma drug levels (OPC-13013), determined before dosing (0 hr) and at 2 and 4 hr post-dose on the last day of treatment, are given below.

Mean Plasma Concentrations (ng/ml)

Dose (mg/kg/day)	Male			Female		
	Time Intervals (hr)					
	0	2	4	0	2	4
6	7	89	240	10	704	225
12	6	142	86	31	452	256
30	8	266	268	210	872	472
150	39	306	125	108	1380	860

Mean plasma concentrations were generally higher at 2 hours than at 4 hours. The plasma drug levels were consistently higher in females than in males at all dose levels tested.

Macroscopically, treatment-related findings were seen only in the heart. Two males at 12 mg/kg/day had a small hematocyst at the tricuspid valve, and one of them also had focal hemorrhage at the bicuspid and aortic valves. One female at 30 mg/kg/day showed focal hemorrhage at the apex of the papillary muscle of the left ventricle. In the 150 mg/kg/day group, one male and one female had yellow linear endocardial thickening and focal hemorrhage in the septum of the left ventricle. In addition, the male had focal hemorrhage at the bicuspid and aortic valves. Another male from this group showed hemorrhage at the apex of the left ventricular papillary muscle.

Absolute and relative adrenal weights of 30 mg/kg/day male group were significantly higher than control weights. There were no significant weight differences observed in other treated groups.

Treatment-related histopathological findings were limited to the heart and coronary arteries. These cardiovascular lesions included focal endocardial fibrous thickening with hemorrhage in the left ventricle and intimal thickening and arteritis and/or periarteritis of the coronary arteries. The incidences of these lesions are given below.

Lesions	Number of Dogs with Lesions Dose (mg/kg/day)				
	0	6	12	30	150
Males					
Focal endocardial fibrosis with hemorrhage	0	0	0	0	2
Intimal thickening - coronary artery	1	0	0	1	3
Arteritis/periarteritis	1	0	0	1	2
Swelling of tunica intima and/or media	2	2	3	3	2
Females					
Focal endocardial fibrosis with hemorrhage	0	0	0	1	1
Intimal thickening - coronary artery	0	1	1	1	4
Arteritis/periarteritis	0	1	1	1	1
Swelling of tunica intima and/or media	0	2	2	3	0

N = 4 dogs/group

According to the sponsor, 12 mg/kg/day was considered to be the non-toxic dose for this study. (Note: It is uncertain whether a no-toxic effect dose was established in this study, especially in females, since all treated groups showed cardiovascular lesions, while the female control group had no lesions. In males, 6 mg/kg/day may be considered as a non-toxic dose level since the incidence of intimal lesions in this group was similar to that of the control group.)

It is noted that an evaluation of individual animal data showed no correlation of clinical pathology, histopathology, and systemic exposure to cilostazol in this study.

In the control female sacrificed <u>in extremis</u>, macroscopically, ruptured epicardial blood vessels and retention of blood in the pericardial cavity were noted. Yellowish white or brown nodules were scattered in the myocardium, lungs and kidneys. A small swelling with ulceration was seen on the rear surface of the right

forelimb. Histologically, abscess or inflammatory cell infiltration was observed in liver, kidneys, spleen, lungs and heart.

[Note: The significant findings observed in a 13-week oral toxicity study in dogs (Study No. 000762; 0, 30, 150 and 750 mg/kg/day) were limited to the heart and coronary arteries. The incidences of these lesions (microscopic) are given below.

Lesions	Number of Dogs with Lesions Dose (mg/kg/day)			
	0	30	150	750
Males				
Endocardial hemorrhage, brown pigmentation and fibrosis of the left ventricle	0	0	1	4
Arteritis and periarteritis of coronary artery	0	0	2	3
Thickening of tunica intima of coronary artery	0	0	2	4
Swelling of the tunica intima and/or media of coronary artery	0	2	1	3
Females				
Endocardial hemorrhage, brown pigmentation and fibrosis of the left ventricle	0	0	0	1
Arteritis and periarteritis of coronary artery	0	0	0	1
Thickening of tunica intima of coronary artery	0	0	1	3
Swelling of the tunica intima and/or media of coronary artery	1	0	0	3

N = 4 dogs/group

The 30 mg/kg/day dose is considered, by the sponsor, to be the no-toxic effect dose in this 13 week study. However, the above data

indicate that a no effect dose level for cardiotoxicity was not established in males.

No significant serum enzyme elevations were seen in the 13 week study.

Cardiovascular lesions were also observed in the high dose males and females in a 5-week oral toxicity study (with 5-week recovery period) in dogs (0, 30, 150 and 750 mg/kg/day) when animals were sacrificed at the end of the treatment period. These lesions included swelling of the tunica intima and/or media of the coronary arteries, hemorrhage of the coronary arterial wall, necrosis of the smooth muscle cells of the coronary arteries and hemorrhage of the right atrial and left ventricular walls. No lesions were seen after the recovery period.

Although plasma CPK levels in the above 5-week study were found elevated in 3 of the 6 high dose males during treatment week 5, very slight or no histopathological lesions were seen in these animals, indicating a lack of correlation between enzyme elevation and lesion development.]

Other Relevant Toxicity Studies in Dogs

1. A 2-week oral toxicity study was conducted in male beagle dogs to determine whether coronary arteritis is a lesion specific to OPC-13013, by comparing the test drug with two other drugs, trapidil and cinepazide maleate, both of which have an antiplatelet aggregation or a vasodilating effect. Groups of dogs (5/group) were treated with oral doses of OPC-13013 (450 mg/kg/day), trapidil (90 mg/kg/day) or cinepazide (48 mg/kg/day) for two weeks. Control dogs received empty gelatin capsule. At the end of the treatment period, the animals were necropsied and tissues examined histologically.

Coronary arteritis was observed in 2 of 5 dogs each from the OPC-13013 and trapidil treated groups and 1 of 5 animals each from the cinepazide and control groups. Cardiac lesions of varying severity were present in treated and control groups.

2. OPC-13015, a main human metabolite of OPC-13013, was tested for its cardiotoxic potential in dogs. OPC-13015 was orally administered to 4 male dogs at 600 mg/kg/day for 2 weeks. Macroscopic examination of animals showed endocardial hemorrhage of the left ventricle in all animals. Histologically, swelling of the tunica media of the coronary arteries, hemorrhage of the arterial wall and thickening and hemorrhage of endocardium were seen in 3 of the 4 animals. Coronary arteritis was seen in one dog.

3. A 5-week oral toxicity study was done in male beagle dogs to investigate the effects of other classes of drugs (calcium antagonist, beta-blocker or vasodilator) on the cardiotoxicity of OPC-13013. Groups of 5 dogs each were given oral administration of OPC-13013 (450 mg/kg/day) alone or in combination with nicardipine (3 mg/kg/day), propranolol (9 mg/kg/day) or dilazep (9 mg/kg/day) for 5 weeks. Results indicate that concomitant administration of the above drugs had no effect on the occurrence of cardiovascular lesions induced by OPC-13013 in dogs.

Thirteen-Week Oral Toxicity Study in Monkeys

(The major goal of the study was to examine the cardiotoxic potential of the test drug in monkeys.)

Testing Facility:

Study Number: 002569 (Report Number - 002600)

Study Dates: April 11, 1985 to December 12, 1985

GLP compliance: The study was conducted in accordance with GLP regulations of the Japanese Ministry of Health and Welfare.

Animals: Male cynomolgus monkeys
6/group and housed individually, were about 2.6 to 3.6 years old and weighed 2.4 to 3.2 kg at the initiation of treatment.

Dose Levels and Mode of Administration: 0, 300, 900 and 1800 mg/kg/day. OPC-13013 (lot number 3E96M) was suspended weekly at required concentrations in sodium carboxymethylcellulose. The preparations were stored at room temperature and were tested to be stable and homogeneous for one week.

The test solutions were administered once daily by oral intubation at a dosage volume of 9 ml/kg.

(The doses were selected based on a previous ex vivo study in male cynomolgus monkeys, in which inhibition of platelet aggregation was noted at an oral dose of 300 mg/kg/day. Therefore, the 300 mg/kg/day was selected as the low dose and the highest feasible volume dose of 1800 mg/kg/day was selected as the high dose for the present study. The intermediate dose was set at 900 mg/kg/day.)

Observations/Measurements: Animals were observed twice daily for general condition. Body weights were recorded weekly. Hematological (erythrocytes, leucocytes and platelet counts, hemoglobin, hematocrit, MCV, MCH and MCHC) and blood chemistry (CPK, LDH, GOT and GPT) evaluations were performed during study weeks 5 and 13. Platelet aggregation test was done 2 hours after dosing on the first and third day of treatment and during study weeks 4, 8 and 13 (to determine the inhibitory effect of the test drug on platelet aggregation induced by ADP, collagen and arachidonic acid). Blood was collected before and 2, 4, 8 and 24 hours after dosing on the

first day of treatment and during weeks 4, 8 and 13 for plasma drug level (parent drug and its metabolites) determinations. Electrocardiographic evaluation and heart rate monitoring were done 2 hours after dosing during weeks 5 and 12.

At the termination of the study, animals were necropsied, examined grossly, and heart weights were determined. The following tissues were fixed in 10% neutral buffered formalin: brain, pituitary, thymus, heart, lungs, spleen, liver, pancreas, kidneys, adrenals, thyroid, stomach, small and large intestines, testes, prostate, aorta, and mesentery and mesenteric lymph nodes. Heart sections were stained with H&E, Masson-trichrome and elastica-van Gieson, stomach and colon with H&E, liver with H&E and Oil Red, kidneys with H&E and PAS, and lungs with H&E, toluidine blue, Alcian blue-PAS and Oil Red.

All data, except that of the platelet aggregation test, were evaluated using the Pitman test. The platelet aggregation data were analyzed using t-test with F-test for homogeneity of variance.

Results: Soft stool and/or diarrhea was seen in all treated groups. Feces containing blood was observed in mid and high dose animals.

One animal from the high dose group was sacrificed in a moribund state on Day 77. This animal became hypoactive, cyanotic and was found laying on its side. Although another animal from the same group became hypoactive, had diarrhea and did not consume food for 2-3 days (Days 73-75), it recovered.

Group mean body weights and food consumption were comparable between treated and control groups. The WBC counts were decreased in the high dose group, compared to the concurrent control, during treatment week 5 but not in week 13. Blood chemistry evaluations showed no drug-related findings. The test drug either completely inhibited or reduced the platelet aggregation induced by collagen, arachidonic acid and ADP in all drug treated groups. An increase in heart rate was seen at 900 mg/kg/day in treatment weeks 5 and 12 (10-31%) and at 1800 mg/kg/day in week 12 (36%). Shortening of the Q-T interval was observed at both dose levels during weeks 5 and 12.

The mean peak plasma concentrations of OPC-13013 and its metabolites, observed 2 hours after dosing (Cmax at 2 hr post-dose), are given below.

Plasma Concentrations of OPC-13013 and its Metabolites:
Mean Peak Plasma Concentrations (ng/ml)

	Day 0 2 hr	4 wk 2 hr	8 wk 2 hr	13 wk 2 hr
OPC-13013				
300	47	60	69	99
900	87	54	75	176
1800	106	104	88	179
OPC-13213				
300	203	301	274	398
900	184	210	203	300
1800	239	346	299	308
OPC-4C3C				
300	70	70	61	79
900	72	46	45	57
1800	95	72	59	60
OPC-13015				
300	60	68	55	81
900	82	50	84	90
1800	70	85	87	80
OPC-13269				
300	17	41	34	45
900	19	31	29	45
1800	24	59	45	34
OPC-13326				
300	11	36	48	44
900	11	30	42	48
1800	15	41	43	43

A dose-dependent increase in OPC-13013 plasma concentrations was observed on Day 0. The plasma concentrations of OPC-13013 at week 13 were higher than at other time points; however, no difference in week 13 plasma concentration was noted between mid and high dose groups. The major human metabolite (OPC-13213) concentrations were not dosage dependent but were higher than the concentrations of the parent or other metabolites at all time points.

There were no treatment-related differences in heart weights.

Macroscopically, petechial hemorrhage of the papillary muscle and/or septum of the left ventricle was noted in one animal each from the control and high dose groups and in two animals each from the low and mid dose groups. No treatment-related findings were observed in other organs.

The incidences of the cardiovascular histopathological findings are given below.

Lesions	Number of Animals with Lesions Dose (mg/kg/day)			
	0	300	900	1800
Hemorrhage/left ventricular endocardium	1	1	2	1
Hemorrhage/right atrial endocardium	0	0	0	1
Hemorrhage/myocardium	0	2	0	0
Fibrosis, focal/myocardium	0	0	1	0
Angiectasis	0	1	0	0
Inflammatory cell infiltration/myocardium	5	3	5	3
Inflammatory cell infiltration/epicardium	1	4	2	0
Inflammatory cell infiltration/endocardium	2	2	1	0
Intimal thickening/artery	4	5	5	5

N = 6/group except the high dose group which only had 5 animals.

It is noted that although some of the lesions were found both in control and treated groups, atrial endocardial hemorrhage, myocardial hemorrhage and fibrosis, and angiectasis were limited to the treated groups; however, no dose dependency was found for these lesions. The incidence of intimal thickening of the coronary artery was higher in treated groups than in control (67, 83, 83 and 100% for control, low, middle and high dose groups, respectively.) No coronary arteritis was observed in this study.

Other histopathologic lesions included aspiration pneumonia-like lesions of the lungs (lungs filled with macrophages containing large and small vacuoles in the pulmonary alveoli) in one high dose animal. No other notable histological lesions were seen in the study.

[Note: The high dose animal that was sacrificed in extremis showed

decreased WBC count, increased CPK and GOT levels and increased heart weight. Macroscopically, endocardial and/or epicardial hemorrhage of left and right atria and ventricles, yellowish white foci of the epicardium of the left ventricle and adhesions of the lung lobes were seen. Histologically, hemorrhage of the endocardium, myocardium and epicardium, necrosis of the papillary muscle, ventricular septum and right atrium, edema at the periphery of the coronary artery, necrosis or absence of the epithelium of the alveoli and bronchioles and complete obstruction of the alveoli and bronchiolar spaces were observed. The lung lesions were characteristic of aspiration pneumonia. The debilitating condition of this animal, therefore, was attributed to the erroneous administration of the test compound.]