Skeletal alterations of F_1 fetuses derived from pregnant F_0 female rats that received hirulog by the subcutaneous route at dose of 0, 10, 50, and 150 mg/kg/day from days 7 to 17 of gestation (F_0 female subjected to caesarean section on day 20).

Parameter	0	10	50	150
Skull:				
Nasals, small Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Premaxillae, fused Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Maxillae, fused Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Zygomatics, fused Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Eye socket, small Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Tympanic rings, fused Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Sphenoid, incompletely ossified Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Mandibles, not ossified Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Vertebrae:				
Thoracic. Centrum, Bifid Fetuses/Litters	0	0	4 (2.8%)* /3 (14.3%)	2 (1.4%) 72 (9.5%)
Thoracic, Arch, Incompletely Ossified Fetuses/Litters	0	0	0	1(0.7%) /1 (4.8%)
Lumbar, Arch, Incompletely Ossified Fetuses/Litters	0	0	0	1(0.7%) /1 (4.8%)

Ribs:				
Incompletely Ossified (Hypoplastic) Fetuses/Litters	0	0	2 (1.4%) /2 (9.5%)	1 (0.7%) /1 (4.8%)
Wavy Fetuses/Litters	0	2 (1.2%) /2 (8.7%)	2 (1.4%) /2 (9.5%)	1 (0.7%) /1 (4.8%)
Cervical rib at the 7th cervical vertebra Fetuses/Litters	0	1 (0.6%) /1 (4.3%)	1 (0.7%) /1 (4.8%)	0
Sternal centra:				
Sternal centra summarization (incompletely ossified + not ossified) Fetuses/Litters	2 (1.2%)/ 1 (4.5%)	6 (3.6%) /4 (17.4%)	6 (4.1%) 5 (23.8%)	1 (0.7%) /1 (4.8%)
Sternal centra, incompletely ossified Fetuses/Litters	2 (1.2%) /1 (4.5%)	6 (3.6%) /4 (17.4%)	5 (3.4%) /4 (19.0%)	1 (0.7%) /1 (4.8%)
Sternal centra, not ossified Fetuses/Litters	0	0	1 (0.7%) /1 (4.8%)	0
Pelvis:				
Pubes, incompletely ossified Fetuses/Litters	4 (2.4%) /3 (13.6%)	9 (5.5%) /5 (21.7%)	5 (3.4%) /5 (23.8%)	3 (2.1%) /3 (14.3%)
Ischia, incompletely ossified Fetuses/Litters	2 (1.2%) /2 (9.1%)	7 (4.3%) /3 (13.0%)	3 (2.1%) /3 (14.3%)	3 (2.1%) /3 (14.3%)

In a Segment II toxicity study, potential teratogenic effects of hirulog were evaluated using Sprague Dawley rats. Pregnant F_0 female rats received hirulog by the subcutaneous route at doses of 0, 10, 50, and 150 mg/kg/day from days 7 to 17 of gestation. Hirulog was not teratogenic.

injection were given when necessary, to attain the appropriate dosage for each animal. Rabbits were examined for clinical signs of toxicity, abortions, premature deliveries, and death before dosing and approximately 1 hr after dosing from days 6 to 18 of gestation. Animals were observed once daily from days 19 to 29 of gestation. Body weights were measured on gestational days 0, 6 through 19, and 24 through 29. Food consumption was measured daily throughout the study. Pregnant female rabbits were sacrificed on day 29 of gestation and subjected to a gross examination. Gross lesions were preserved for possible future examination. The number of corpora lutea in each ovary was counted. The uterus was removed and examined for pregnancy, number and distribution of implantations, early and late resorptions, and live and dead fetuses. Each fetus was weighed and examined for gross external alterations. Live fetuses were sacrificed and examined internally to identify sex and any visceral alterations. All cavitated organs, including the brain were examined by dissection. Fetal gross lesions were preserved for possible future examination. All fetuses were eviscerated, processed, and examined for skeletal alterations. Late resorptions were examined to the extent possible.

Results: One pregnant female rabbit in the 15 mg/kg/day group spontaneously aborted on day 27. Examination of the uterus from this animal found 8 resorbed fetuses. There were no treatment-related effects on body weight gain or food consumption. There were no treatment-related deaths, abortions, or premature deliveries. Litter parameters were unaffected. Hirulog at doses ≤150 mg/kg/day caused no gross external, soft tissue, or skeletal malformations or variations.

Litcer parameters for pregnant female rabbits that received hirulog by the subcutaneous route cf administration at doses of 0, 15, 50, and 150 mg/kg/day from days 6 to 18 of gestation.

Parameter	0	15	50	150
Pregnant rabbits at day 29	18	19	20	20
Corpora lutea/dam	9.9	9.9	10.3	9.8
Implantations/dam	7.6	8.5	8.7	8.5
Litter size/dam	7.0	8.3	8.3	8.1
Live fetuses/dam	7.0 (127/18)	8.3 (157/19)	8.3 (166/20)	8.1 (162/20)
Resorptions -total -early -late	 0.6 0.3 0.3	 0.2 0.1 0.1	 0.4 0.2 0.2	 0.4 0.2 0.2
%Live male fetuses	49.5% (65/127)	60.6% (96/157)	48.7% (80/166)	52.2% (83/162)
Fetal body weight, g -male -female	 45.28 43.91	 43.74 41.63	 44.61 42.56	 44.54 43.53

Gross external alterations for fetuses derived from pregnant female rabbits that received hirulog by the subcutaneous route of administration at doses of 0, 15, 50, and 150 mg/kg/day from days 6 to 18 of gestation.

Parameter	0	15	50	150
Fetuses/Litters	127/18	158[A]/19	166/20	162/20
Snout: short Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0

A. One fetus from the total of 158 was dead when evaluated.

APPEARS THIS WAY
ON ORIGINAL

Soft tissue alterations for fetuses derived from pregnant female rabbits that received hirulog by the subcutaneous route of administration at doses of 0, 15, 50, and 150 mg/kg/day from days 6 to 18 of gestation.

Parameters	0	15	50	150
Fetuses/Litters	127/18	158[A]/19	166/20	162/20
Brain:				
Moderate dilation of lateral ventricles Fetuses/Litters	0	0	4 (2.4%) /1 (5.0%)	0
Marked dilation of lateral ventricles Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0
Vessels:				
Subclavian, absent Fetuses/Litters	0	0	0	1 (0.6%) /1 (5.0%)
Lungs:				
Intermediate lobe, agenesis Fetuses/Litters	2 (1.6%) /2 (11.1%)	8 (5.1%) 6 (31.6%)	2 (1.2%) /2 (10.0%)	4 (2.5%) /3 (15.0%)
Kidneys:				
Left, absent Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0

A. One fetus from the total of 158 was dead when evaluated.

Skeletal alterations for fetuses derived from pregnant female rabbits that received hirulog by the subcutaneous route of administration at doses of 0, 15, 50, and 150 mg/kg/day from days 6 to 18 of gestation.

Parameters	0	15	50	150
Fetuses/Litters	127/18	158[A]/19	166/20	162/20
Skull, Irregular ossification				
Nasals, Internasal Fetuses/Litters	2 (1.6%) /2 (11.1%)	1 (0.6%) /1 (5.3%)	2 (1.2%) /2 (10.0%)	4 (2.5%) /3 (15.0%)
Nasals, Irregular Suture Fetuses/Litters	0	3 (1.9%) /2 (10.5%)	0	1 (0.6%) /1 (5.0%)
Nasals, fused Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0
Nasals, short Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0
Nasals, midline suture displaced Fetuses/Litters	3 (2.4%) /3 (16.7%)	9 (5.7%)/ 7 (36.8%)	12 (7.2%) /10 (50%)	9 (5.6%) /8 (40.0%)
Nasals-Frontals, Irregular suture Fetuses/Litters	2 (1.6%) /2 (11.1%)	5 (3.2%) /4 (21.0%)	6 (3.6%) /5 (25.0%)	9 (5.6%) /6 (30%)
Frontals, Interfrontals Fetuses/Litters	1 (0.8%) /1 (5.6%)	2 (1.3%) /2 (10.5%)	1 (0.6%) /1 (5.0%)	2 (1.2%) /1 (5.0%)
Frontals, Intrafrontals Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	1 (0.6%) /1 (5.0%)
Frontals, Irregular Suture Fetuses/Litters	4 (3.1%) /4 (22.2%)	1 (0.6%) 1 (5.3%)	4 (2.4%) /2 (10%)	7 (4.3%) /6 (30%)
Frontals, fused Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0
Parietal, Intraparietal Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0
Maxilla, incompletely ossified Fetuses/Litters	0	0	1 (0.6%) /1 (5.0%)	0

Vertebrae:				
Cervical, Centrum, unilateral ossification Fetuses/Litters	0	0	0	1 (0.6%) /1 (5%)
Thoracic, Centrum, unilateral ossification Fetuses/Litters	0	1 (0.6%) /1 (5.3%)	0	1 (0.6%) /1 (5%)
Thoracic, Centra, Fused Fetuses/Litters	0	0	0	1 (0.6%) /1 (5%)
Thoracic, Arch, Small Fetuses/Litters	0	0	0	1 (0.6%) /1 (5%)
Ribs:				
Fused Fetuses/Litters	0	1 (0.6%) /1 (5.3%)	0	0
Small Fetuses/Litters	0	1 (0.6%) /1 (5.3%)	0	0
Cervical rib at 7th cervical vertebra Fetuses/Litters	0	1 (0.6%) /1 (5.3%)	0	0
Sternal Centra:				
Incompletely ossified Fetuses/Litters	0	1 (0.6%) /1 (5.3%)	0	1 (0.6%) /1 (5%)
Scapulae Fetuses/Litters	0	1 (0.6%) /1 (5.3%)	0	0

A. One fetus from the total of 158 was dead when evaluated.

In a Segment II tetratogenicity study, pregnant female rabbits received hirulog by the subcutaneous route of administration at doses of 0, 15, 50, and 150 mg/kg/day from days 6 to 18 of gestation. At doses ≤150 mg/kg/day, hirulog was not teratogenetic in rabbits.

Rats

A Study of the Effects of Hirulog Administered Subcutaneously on Pre- and Postnatal Development, Including Maternal Function in the Rat (Project No. P8967-97-01).

Testing Laboratory: [redacted]

Study Started: August 25, 1997

Study Completed: April 23, 1998

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Animals: Pregnant female Crl:CD®(SD)BR rats were used in this study. Female rats were approximately 13 weeks old and had a minimum weight of 220 g at the time of cohabitation with male rats of the same strain and source. Body weights for pregnant female rats on day 0 of gestation ranged from 229 to 270 g.

Drug Batch: Hirulog, Lot No. 67A03Q (modified homogenous phase commercial scale).

Methods: In a Segment III pre- and postnatal study, pregnant female rats received hirulog by the subcutaneous route of administration at doses of 0, 10, 50, and 150 mg/kg/day from days 6 to 19 of gestation and days 2 to 20 of lactation. Dams were not treated from day 20 of gestation through day 1 of lactation due to the possibly of excessive hemorrhaging during parturition, because hirulog is an antithrombin. For administration of the test article, the needle was inserted into the subcutis over the scapular or lumber regions. The site of injection was alternated on a daily basis, clockwise among four distinct sites in the left and right scapular and lumbar regions. The dosing volume was 3 mL/kg. Control animals received 0.9% NaCl. There were 25 females per group. Animals were monitored twice daily for mortality and moribundity. Clinical signs of toxicity were observed daily at the time of dosing and approximately 1 hr after dosing for each F_0 female until necropsy, with particular attention toward sites of drug administration for signs of irritation and hemorrhaging. Body weights for F_0 dams were measured on days 0, 6, 9, 12, 15, 18, and 20 of gestation and days 1, 4, 7, 14, and 21 of lactation. Food

consumption for each F_0 dam was measured daily during the periods of gestation and lactation. F_0 dams were allowed to naturally deliver and rear their offspring to postpartum day (PND) 21. During the period of expected parturition, dams were observed twice daily for initiation and completion of parturition and signs of dystocia. The day that delivery was judged to be completed was designated PND 0. At completion of parturition, numbers of stillborn and live pups were sexed and examined for gross malformations. The duration of gestation was recorded. All F_0 dams that delivered offspring were sacrificed on lactation day 21 and subjected to a gross examination. Numbers of former implantation sites were counted. Maternal tissues were collected and preserved for possible histopathological analysis based upon gross findings. F_0 female rats that did not deliver were sacrificed on post-mating day 25 and subjected to a gross examination. Each F_1 litter was monitored daily for pup mortality. To reduce variability among F_1 litters, eight pups/litter (or 4 pups/sex/litter, if possible) were randomly selected on PND 4. Culled pups were weighed, sacrificed, and discarded. F_1 litters were examined daily for clinical signs of toxicity. Each F_1 pup received a detailed physical examination on PND 1, 4, 7, 14, and 21, and at weekly intervals thereafter until sacrifice. Any abnormalities in nesting and nursing behavior by F_0 dams were noted. F_1 pups were weighed individually on PND 1, 4, 7, 14, and 21, and at weekly intervals thereafter until sacrifice. F_1 pups were individually sexed on PND 0, 4, and 21. Twenty-five pups/sex/group were randomly selected for the F_1 generation between days 4 and 9 of gestation. From these selected pups, 10 pups/sex/group were selected for neurobehavioral evaluation. Each dam and litter remained together until weaning at PND 21. The 25 F_1 pups/sex/group were allowed to attain sexual maturity for assessment of development and reproductive performance. All other F_1 pups were sacrificed on PND 21 and subjected to gross examination. Male F_1 pups were monitored for balanopreputial separation beginning on PND 40. Female F_1 pups were monitored for vaginal perforation beginning on PND 30. An auditory startle response (i.e., mean latency to peak, response duration, average response, and peak amplitude) was measured for 10 F_1 rats/sex/group on PND 21 and 60. Motor activity (i.e., total and ambulatory activity) was measured for 10 F_1 rats/sex/group on PND 13, 17, 21, and 60. Swimming ability, learning, and memory were assessed for 10 F_1 rats/sex/group using a water-filled, eight-unit T-maze during two testing intervals that were initiated on PND 20-23 and PND 59-62, respectively. Each testing interval was composed of three phases: swimming ability on the first day of testing, maze acquisition (learning) on the second through fifth day of testing, and after a three-day rest period, each animal was retested in the maze (day 6

of testing) for memory. F_1 rats were monitored twice daily for mortality and moribundity and detailed physical examinations were performed weekly until sacrifice. F_1 male rats were weighed weekly from PND 28 until sacrifice. F_1 female rats were weighed weekly from PND 28 until cohabitation with F_1 male rats. Following cohabitation with F_1 male rats, body weights for pregnant F_1 female rats were measured on days 0, 6, 9, 12, 16, and 20 of gestation. Gravid uterine weight and net body weight for F_1 female rats were determined on day 20 of gestation at scheduled laparohysterectomy. F_1 male and female rats were paired on a 1 to 1 basis within each treatment group, such that sibling matings were avoided. When evidence of copulation was not detected after 10 days of pairing, the female was placed with another male of the same treatment group, that had previous evidence of mating, for an additional 5 days. With detection of mating, F_1 female rats were placed in individual cages until day 20, when they were sacrificed and subjected to a gross examination. The uterus and ovaries were removed and the number of corpora lutea on each ovary was counted. The uterus was weighed, opened, and numbers of F_2 fetuses, early and late resorptions, and implantation sites were counted. Positions of fetuses within the uterus were also recorded. F_2 fetuses were sexed, weighed, and examined for any external abnormalities. Approximately one-half of the F_2 fetuses were prepared for possible soft tissue analysis, while the other half was prepared for possible skeletal tissue examination. F_1 female rats that failed to mate and F_1 male rats, after completion of laparohysterectomies on pregnant F_1 female rats, were sacrificed and subjected to gross examination.

Results:

1. **Observed Effects for F_0 Dams**: The number of pregnant F_0 female rats in the 0, 10, 50, and 150 mg/kg/day groups that delivered offspring were 23, 23, 24, and 25, respectively. The only clinical observation attributed to hirulog treatment was hemorrhaging at the injection site. The incidence of hemorrhage occurred in dose-related manner. The number of F_0 dams in the 0, 10, 50, and 150 mg/kg/day groups observed with hemorrhage at the injection site were 2, 6, 12, and 22, respectively. The incidence of hemorrhage was highest in the right and left lumbar regions.

2. **Body Weight and Food Consumption for F_0 Dams**: Body weight gain for F_0 dams that received hirulog at doses of 10, 50, and 150 mg/kg/day was unaffected during the periods of gestation and lactation as compared to respective controls. Food consumption for F_0 dams that received hirulog at doses of 10, 50, and 150 mg/kg/day was unaffected during the periods of gestation and lactation as compared to respective controls.

3. **Gestation, Parturition, and Lactation for F_0 Dams**: The length of gestation for pregnant F_0 dams that received hirulog at doses of 10, 50, and 150 mg/kg/day was unaffected as compared to controls. Parturition was unaffected by treatment with hirulog. The number of implant sites/F_0 dam were unchanged between control and treatment groups.

4. **Gross Pathological Findings for F_0 Dams**: All F_0 dams survived to necropsy. The only significant finding after necropsy of F_0 dams in the 0, 10, 50, and 150 mg/kg/day groups on day 21 of lactation was hemorrhage at the injection site for 1, 7, 10, and 21 rats, respectively. Hemorrhage was attributed to the pharmacological properties of hirulog, which is an antithrombotic agent.

5. **Pre- and Postnatal Development of F_1 Rats**: Number of live pups/litter, % males/litter, and stillbirth indexes were unaffected by treatment with hirulog. F_1 pup viability and body weight during days 1 to 21 of lactation were unchanged between control and treatment groups. Pup deaths during lactation displayed no relationship to treatment. F_1 pup development as monitored by balanopreputial separation for male pups and vaginal perforation for female pups were unaffected by hirulog treatment. The auditory startle response on post-parturition days 21 and 60 was unaffected by hirulog treatment. Motor activity (i.e., total and ambulatory activity) on post-parturition days 13, 17, 21, and 60 was unaffected by hirulog treatment. Swimming ability, learning, and memory assessed during two periods, post-parturition days 20-23 and post-parturition days 59-62, were unaffected by hirulog treatment. There were no clinical signs of toxicity for F_1 male and female rats. Body weight gain for F_1 male and female rats were unaffected by hirulog treatment. Body weight gains for pregnant F_1 female rats during gestation were unaffected by hirulog treatment. Fertility and reproductive performance of F_1 male and female rats were unaffected by hirulog treatment. Litter parameters for F_1 dams (i.e., corpora lutea/dam, pre-implantation loss/dam, implant sites/dam, post-implantation loss/dam, viable fetuses/dam, male/female ratio, and fetal body weight) were unaffected by hirulog treatment. There were no significant gross pathological findings for F_1 male and female rats.

6. **External Abnormalities for F_2 Fetuses**: The male to female ratio and body weights for F_2 fetuses were unaffected by hirulog treatment. There were no external abnormalities for F_2 fetuses that displayed any relationship to treatment.

Litter parameters for F_0 dams that received hirulog by the subcutaneous route at doses of 0, 10, 50, and 150 mg/kg/day. Implant sites were counted at necropsy on day 21 of lactation.

Parameter	0	10	50	150
Implant sites/dam	14.7	14.7	13.8	15.0
Number born/dam	14.0	13.5	12.9	13.8
Unaccounted sites/dam	0.7	1.2	1.0	1.2
Live births/dam	13.4	13.2	12.7	13.5
Stillbirth Index, % per litter	4.85	2.11	1.57	2.05
Sex at birth, % males per litter	54.8	52.1	56.2	51.0

Viability and body weight for F_1 pups during days 1 to 21 of lactation.

Parameter	0	10	50	150
Died/Sacrificed during days 1 to 21 of lactation -found dead -missing (cannibalized) -moribund sacrifice	 16 2 0	 9 2 0	 6 5 0	 10 6 1
Survival (%), birth to day 4 of lactation	93.7	97.0	96.5	95.9
Survival (%), days 4 to 21 of lactation	100	99.5	100	98.5
F_1 male body weight, g -day 1 -day 4 (before selection) -day 21	 6.9 9.9 52.3	 6.9 10.1 52.7	 7.1 10.2 51.5	 7.0 10.1 53.5
F_1 female body weight, g -day 1 -day 4 (before selection) -day 21	 6.4 9.3 49.6	 6.5 9.4 49.5	 6.7 9.7 50.1	 6.6 9.5 50.6

Litter and reproductive parameters for F_1 male and female rats derived from F_0 dams that received hirulog by the subcutaneous route at doses of 0, 10, 50, and 150 mg/kg/day.

Parameter	0	10	50	150
F_1 male mating index, %	96.0 (24/25)	92.0 (23/25)	96.0 (24/25)	92.0 (23/25)
F_1 female mating index, %	96.0 (24/25)	96.0 (24/25)	100 (25/25)	100 (25/25)
F_1 male fertility index, %	88.0 (22/25)	92.0 (23/25)	92.0 (23/25)	80.0 (20/25)
F_1 female fertility index, %	88.0 (22/25)	96.0 (24/25)	96.0 (24/25)	84.0 (21/25)
Corpora lutea/dam	16.5 (363/22)	15.8 (379/24)	15.2 (365/24)	15.6 (311/21)
Pre-implantation loss/dam	2.3 (51/22)	1.6 (38/24)	1.5 (35/24)	1.5 (30/21)
Implant sites/dam	14.2 (312/22)	14.2 (341/24)	13.8 (330/24)	14.2 (298/21)
Post-implantation loss/dam -total -early -late	 0.9 (20/22) 0.8 (18/22) 0.1 (2/22)	 0.7 (17/24) 0.7 (17/24) 0	 1.0 (24/24) 1.0 (24/24) 0	 0.6 (12/21) 0.6 (12/21) 0
Dead fetuses/dam	0	0	0	0
Viable fetuses/dam	13.3 (292/22)	13.5 (324/24)	12.8 (306/24)	13.6 (286/21)
Sex -number of males -number of females	 138 154	 171 153	 153 149	 141 145
Fetal body weight, g -male -female	 3.5 3.3	 3.5 3.3	 3.5 3.3	 3.5 3.3

In a Segment III pre- and postnatal study, pregnant F_0 female rats received hirulog by the subcutaneous route of administration at doses of 0, 10, 50, and 150 mg/kg/day from days 6 to 19 of gestation and days 2 to 20 of lactation. F_0 dams were not treated from day 20 of gestation through day 1 of lactation due to the possibly of excessive hemorrhaging during parturition, because hirulog is an antithrombin. Pre- and postnatal development for F_1

rats were not affected by hirulog treatment. There were no clinical signs of toxicity or mortality for F_0 dams at doses ≤150 mg/kg/day. The male to female ratio and body weights for F_2 fetuses were unaffected by hirulog treatment. There were no external abnormalities for F_2 fetuses that displayed any relationship to treatment.

Genotoxicity

Ames/Salmonella-E. Coli Liquid Pre-Incubation Assay on Hirulog (Biogen Study No. P90-19).

Testing Laboratory: [redacted]

Study Started: May 14, 1990

Study Completed: July 31, 1990

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Drug Batch: Hirulog, Lot No. 67W01W (solid phase peptide method).

Methods: The genotoxic potential of hirulog was examined with tester strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98, and TA 100) and Escherichia coli (WP2 uvrA) using the liquid pre-incubation method. The S9 metabolic activation mix was prepared from the livers of rats treated with Aroclor-1254. Positive controls in the absence of S9 metabolic activation mix were sodium azide (10.0 µg/plate) for TA1535 and TA1537, 9-aminoacridine (150 µg/plate) for TA1537, 2-nitrofluorene (5 µg/plate) for TA1538 and TA98, and ENNG (2 µg/plate) for WP2 uvrA. The positive control in the presence of S9 metabolic activation mix was 2-aminoanthracene at 2.50 µg/plate for all Salmonella typhimurium strains and at 80 µg/plate for WP2 uvrA. In a preliminary toxicity assay, hirulog was evaluated at concentrations of 50, 167, 500, 1670, and 5000 µg/plate with TA1538, TA100, and WP2 uvrA. Mutation assays were performed with all 6 tester strains. Following a 48 hr incubation, revertant colonies were counted. A positive result was defined as a statistically significant, dose-dependent increase in the number of histidine-independent revertants with at least one dose level inducing a revertant frequency that is two-fold the spontaneous solvent control value.

Results: In a dose range finding assay, hirulog at concentrations ranging from 50 to 5000 µg/plate in the absence of S9 was not toxic to either TA1538, TA100, or WP2 uvrA. In the mutation assay, hirulog was evaluated at concentrations of 16.7, 50, 167, 500, 1670, and 5000 µg/plate. In the initial assay, poor growth was observed with TA1537, TA1538, TA98, and WP2 uvrA in the presence or absence of S9. In a second assay, hirulog was found to have no mutagenic potential with TA1535, TA1538, TA100, TA98, or WP2 uvrA. With TA1537, concentrations of 16.7 and 50 µg/plate in the absence of S9 produced revertant counts of 11 and 12, respectively, as compared to a control value of 7. However, with concentrations ≥167 µg/plate, revertant counts were ≤10. This slight increase over the control value was <2-fold and not dose-dependent. Hirulog was not genotoxic with tester strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98, and TA 100) and Escherichia coli (WP2 uvrA) using the liquid pre-incubation method.

Hirulog was not genotoxic with tester strains of Salmonella typhimurium (TA1535, TA1537, TA1538, TA98, and TA 100) and Escherichia coli (WP2 uvrA) using the liquid pre-incubation method.

Evaluation of a Test Article in the Salmonella typhimurium/ Escherichia coli Plate Incorporation Mutation Assay in the Presence and Absence of Aroclor-Induced Rat Liver S-9 (Biogen Study No. P8967-93-09).

Testing Laboratory: [redacted]

Study Started: December 3, 1993

Study Completed: March 25, 1994

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Drug Batch: Hirulog, Lot No. 67Z17S (modified homogenous phase commercial scale).

Methods: The genotoxic potential of hirulog was examined with tester strains of Salmonella typhimurium (TA1535, TA1537, TA98, and TA 100) and Escherichia coli (WP2 uvrA) using the plate incorporation technique. The S9 metabolic activation mix was prepared from the livers of rats treated with Aroclor-1254.

Positive controls in the absence of S9 were sodium azide (1 µg/plate) for TA100 and TA1535, 2-nitrofluorene (5 µg/plate) for TA98, 9-aminoacridine (50 µg/plate) for TA1537, and methyl methanesulfonate (4000 µg/plate) for WP2uvrA. The positive control in the presence of S9 was 2-aminoanthracene at 1.25 µg/plate for all Salmonella typhimurium tester strains and at 10 µg/plate for WP2uvrA. To select appropriate hirulog concentrations for the mutation assay, a range finding assay was performed with TA100 and WP2uvrA at hirulog concentrations ranging from 0.1 to 5000 µg/plate in the presence and absence of S9. A response was considered positive with TA98 or TA100 if a dose produced a mean reversion frequency that was ≥ 2times the mean reversion frequency of the control. A response was considered positive with TA1535, TA1537, or WP2uvrA if a dose produced a mean reversion frequency that was ≥3 times the mean reversion frequency of the control. Further, the response must be dose-dependent or increasing concentrations of the test article must show increasing mean reversion frequencies.

Results: Based upon results of the range finding assay, hirulog concentrations selected for the mutation assay were 250, 500, 1000, 2500, and 5000 µg/plate in the presence and absence of S9. No precipitation was observed with any hirulog concentrations. In the mutation assay, hirulog was found to have no genotoxic potential with tester strains of Salmonella typhimurium (TA1535, TA1537, TA98, and TA100) and Escherichia coli (WP2uvrA). No toxicity was observed with a hirulog concentration of 5000 µg/plate.

Hirulog was found to have no genotoxic potential with tester strains of Salmonella typhimurium (TA1535, TA1537, TA98, and TA100) and Escherichia coli (WP2uvrA) using the plate incorporation technique.

Test of Hirulog Induction of Gene Mutation at the HGPRT Locus in Cultured Chinese Hamster Ovary (CHO) Cells With and Without Metabolic Activation (Biogen Study No. P8967-94-04).

Testing Laboratory: [redacted]

Study Started: June 28, 1994

Study Completed: July 13, 1995

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Drug Batch: Hirulog, Lot No. 67A03Q (modified homogenous phase commercial scale).

Methods: Hirulog was evaluated for its potential to induce forward gene mutation at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in cultured Chinese hamster ovary cells (CHO-K1-BH_4) in the presence and absence of metabolic activation. The S9 metabolic activation system was prepared from the livers of rats treated with Aroclor-1254. Positive controls in the presence and absence of metabolic activation were 7,12-dimethylbenz(α) anthracene and ethyl methanesulfonate, respectively. In range finding test, hirulog was examined at concentrations of 0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000, and 5000 µg/mL. For tests with or without metabolic activation, CHO cells were incubated with hirulog concentrations for 4 hr. For determination of cytotoxicity, cells were transferred to new plates at 200 cells/60 mm dish and allowed to grow for 7 days. After 7 days, cells were fixed, stained, and counted to determine to relative cloning efficiency as referenced to the solvent control. For the mutation assay, cells were treated with hirulog at concentrations of 50, 100, 500, 1000, and 5000 µg/mL for 4 hr in the presence or absence of metabolic activation. Following hirulog treatment, cells transferred for mutant selection and determination of cytotoxicity. For expression of 6-thioguanine-resistant mutants, cells were subcultured at 3 days intervals for a total of 8 days prior to selection of the mutant phenotype. After the 8 day expression period, cells were transferred to plates containing 10 µM 6-thioguanine and incubated for 7 days. After 7 days, colonies were fixed and stained to determine the cloning efficiency and thioguanine-resistant mutants. The test results were considered positive if a dose showed more than a 2-fold increase in the mean number of mutants per 1 x 10^6 cells surviving cells. Mutant data from test doses that produced a >90% reduction of the relative cloning efficiency were not included in the results. Further, the mutant frequency should be >15 x 10^6 surviving cells.

Results: In the range finding test, concentrations ≤5000 µg/mL in the absence of metabolic activation were nontoxic. With metabolic activation, 5000 µg/mL reduced the relative cloning efficiency to 89% of the control, while lower doses were nontoxic. In the mutation assay in the presence or absence of metabolic activation, there was no significant change in the mutation frequency per 10^6 cells following hirulog treatment as compared to the solvent control.

Hirulog was negative in the forward gene mutation test at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in cultured Chinese hamster ovary cells (CHO-K1-BH_4) in the presence and absence of metabolic activation.

Test of Hirulog for Chemical Induction of Chromosome Aberration in Cultured Human Peripheral Blood Lymphocytes With and Without Metabolic Activation (Biogen Study No. P8967-94-03).

Testing Laboratory: [redacted]

Study Started: June 24, 1994

Study Completed: July 13, 1995

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Drug Batch: Hirulog, Lot No. 67A03Q (modified homogenous phase commercial scale).

Methods: Hirulog was evaluated for its potential to induce chromosomal aberrations in cultured human peripheral blood lymphocytes. Peripheral blood lymphocytes were collected from a healthy 42 year old male volunteer and a healthy 28 year old female volunteer. The S9 metabolic activation mix was prepared from livers of rats treated with Aroclor-1254. Positive controls in the absence and presence of metabolic activation were mitomycin C and cyclophosphamide, respectively. Lymphocytes were activated in medium containing 1% phytohemagglutinin (M-form). In a range finding assay, the cytotoxicity of hirulog was assessed by the relative mitotic index at concentrations ranging between 1 and 5000 µg/mL. Lymphocytes were incubated with different hirulog concentrations for 18 hr in the absence of metabolic activation. In the presence of metabolic activation, cells were incubated with different concentrations of hirulog for 3 hr, followed by replacement with fresh medium and incubation for an additional 15 hr. Cells were treated with colcemid for 2 hr prior to harvest to induce metaphase arrest. Cells were collected, swelled in 0.075 M KCl for 4 min, fixed, and stained with 5% Giemsa to allow determination of the mitotic index at each hirulog concentration. In a separate assay, the average generation time for each hirulog concentration in the presence or absence of S9 metabolic activation

was determined by incorporation of 5-bromo-2-deoxyuridine. Based upon results of the range finding assay, the chromosomal aberration assay was performed with hirulog concentrations of 625, 1250, 2500, and 5000 µg/mL in either the presence or absence of metabolic activation. Cells were treated with hirulog in the presence or absence of metabolic activation as described above for the range finding assay; although, treatment with KCl was extended to 10 min. One hundred well-spread metaphases were scored for chromosomal aberrations from the 3 highest doses of hirulog in addition to the solvent and positive controls. The percentage of polyploid and endoreduplicated cells were determined separately by scoring 100 metaphases from each of the duplicate cultures. The test article was considered to have caused a positive response if there was a positive dose-response trend and a statistically significant increase over that of at one or more dose levels.

Results: Hirulog at 5000 µg/mL reduced the mitotic index by only 21% in the non-activated system and 11% in the activated system. The solvent control had an average generation time of 17.4 hr. For lymphocytes treated with hirulog in the range of 500 to 5000 µg/mL, the average generation time was 18.1 hr in the non-activated system and 18 hr in the activated system. Harvest time for the non-activated system was 18 hr and for the activated system was 21 hr (3 hr of treatment time plus 18 hr of incubation). Due to minimal toxicity, chromosomal aberrations were scored at hirulog concentrations of 1250, 2500, and 5000 µg/mL. The incidence of chromosomal aberration found in response to hirulog treatment was not significantly different from the solvent control either in the presence or absence of metabolic activation.

The incidence of chromosomal aberrations in human peripheral blood lymphocytes induced by treatment with hirulog was not significantly different from the solvent control.

In Vivo Test for Hirulog Induction of Micronucleated Polychromatic Erythrocytes in Rat Bone Marrow Cells (Biogen Study No. P8967-94-05).

Testing Laboratory: [redacted]

Study Started: August 2, 1994

Study Completed: April 29, 1997

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Animals: Male and female Sprague Dawley rats were used in this study. Animals were 7 to 8 weeks old at time of treatment and body weight ranges were 208-240 grams for male rats and 181-223 g for female rats.

Drug Batch: Hirulog, Lot No. 67A03Q (modified homogenous phase commercial scale).

Methods: Hirulog was evaluated for its potential to induce micronucleated polychromatic erythrocytes in the bone marrow of rats. In the dose range finding study, 3 rats/sex/group received hirulog by the subcutaneous route of administration at doses of 100, 250, and 500 mg/kg/day for 2 days. There was no mortality or significant clinical signs of toxicity over a 3 day observation period. For the micronucleus assay, 5 rats/sex/group received hirulog by the subcutaneous route of administration at doses of 0, 250, 500, and 1000 mg/kg/day for 2 days. Another group of rats received the positive control, triethylenemelamine, which was administered once by the intraperitoneal route at a dose of 1 mg/kg. Animals were sacrificed 24 hr after the last dose. Bone marrow cells were collected from the femur, placed on slides, fixed, and stained with acridine orange. The number of polychromatic erythrocytes (PCE) and normochromatic erythrocytes in 1000 erythrocytes was determined. The number of micronucleated polychromatic erythrocytes was determined. The test article is considered to have caused a positive response if a positive dose response-trend and a 3-fold increase in the number of micronucleated polychromatic erythrocytes over the vehicle at ≥1 dose level are found. If the vehicle-control value is unusually low, the historical control value may be consulted. If there is not positive dose-response trend, at least 2 consecutive dose levels should show a 3-fold increase in the number of micronucleated polychromatic erythrocytes as compared to the vehicle- or historical-control.

Results: The number of micronucleated polychromatic erythrocytes in 1000 polychromatic erythrocytes was not significantly different between hirulog treatment groups and the vehicle-control. The polychromatic erythrocyte to normochromatic erythrocyte ratio was not significantly different between hirulog treatment groups and the vehicle-control.

Hirulog had no genotoxic potential in rat micronucleus test.

Rat Hepatocyte Primary Culture/DNA Repair Test on Hirulog (Biogen Study No. P90-20).

Testing Laboratory: [redacted]

Study Started: May 21, 1990

Study Completed: July 31, 1990

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Animals: One male Fisher 344 rat with a weight of 172 g was used in this study.

Drug Batch: Hirulog, Lot No. 67W03T (solid phase peptide method).

Methods: Hirulog was evaluated for its ability to induce unscheduled DNA repair in rat hepatocytes in vitro. Hepatocytes were isolated from a male Fisher 344 rat. Hepatocytes (1×10^5) were seeded into wells and allowed to attach for 2 hr. New medium containing hirulog at concentrations of 0, 8, 80, 160, or 250 µg/mL or the positive control, 2-acetamidofluorene at 10^{-7} M, plus ^{3}H-thymidine at 10 µCi/mL were added to hepatocytes for a 18-20 hr incubation. The hirulog concentration of 250 µg/mL was the limit of maximum solubility. Cells were processed and after a 7 day exposure time, autoradiographs were developed. Unscheduled DNA repair synthesis, as evidenced by a net increase in black silver grains over the nucleus, was quantified by determining nuclear and cytoplasmic grain counts. A total of 150 cells/dose point were counted for autoradiographic unscheduled DNA synthesis. The test article is considered positive when the minimum net grain count of 5 per nuclei is consistently observed in triplicate wells.

Results: Net nuclear grain counts (nuclear grain counts - cytoplasmic grain counts) between hirulog treated hepatocytes and vehicle control-treated hepatocytes were not significantly different.

Hirulog treatment did not induce unscheduled DNA repair in rat hepatocytes in vitro.

Special Toxicity Tests

Irritation Studies in Rabbits with Hirulog (Biogen Study Numbers P91-123, P8967-92-05, and P8967-93-12).

Testing Laboratory: [redacted]

Study Start and Completion Dates: August 13 - October 25, 1991
July 3 - October 22, 1992
December 8, 1993 - March 7, 1994

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Animals: New Zealand White rabbits (SPF) were used in these studies. On the first day of treatment, body weight ranges were 2.0 to 3.0 kg for female rabbits and 2.3 to 2.8 kg for male rabbits.

Drug Batch: Hirulog prepared by homogenous phase pilot scale and modified homogenous phase commercial scale were used in these studies.

Methods: In one study, five formulations of hirulog were evaluated for the potential to cause irritation and/or corrosion to the intact skin of the rabbit following a single, subcutaneous injections. Seventy-two hr after dosing, animals were sacrificed and skin from the hirulog- and control-treated sites (4 x 4 cm) were collected for determination of macroscopic (erythema/edema) and microscopic lesions (inflammation, necrosis, and hemorrhage). In two other studies, hirulog was evaluated for local irritation effects to the skin and subcutis during daily subcutaneous injections to male or female rabbits for 5 days. There were 5 males or females/group. Hirulog was administered daily for 5 days by subcutaneous injection (25 or 50 mg in 1 mL/dose site) in a defined area of shaved skin. Each injection was made into a fresh skin site with the day 1 injection being nearest the head and the day 5 injection being farthest from the head. On day 6, following the last observations of local irritation at injection sites, animals were sacrificed and injection sites were removed, processed, and subjected microscopic examination for inflammation, necrosis, and hemorrhage.

Results: An examination of five hirulog formulations found that hirulog at 25 mg/mL in NaOH + Mannitol was the least irritant and hirulog at 75 mg/mL in NaOH + Mannitol was the most irritant. Irritation produced by subcutaneous administration of hirulog to female rabbits at 25 or 50 mg/injection site for 5 days was not significantly different from the control. Lesions of gross erythema (hemorrhage) and microscopic lesions of hemorrhages and focal to locally diffuse chronic inflammation are characteristic of any subcutaneous injection and are not considered related to the test article or dose. In contrast, results for male rabbits indicated increasing mean irritation scores for 25 and 50 mg/injection groups of 4.6 and 6.2, respectively, as compared to 1.8 for the control. The sponsor attributed increasing scores to an increased prevalence of minimal irritation findings in the hirulog-treated rabbits and not due to an increased severity of skin lesions between hirulog-treated and saline control animals. They again indicated that irritation observed was characteristic of any subcutaneous injection and not related to hirulog. However, the number of injection sites with macroscopic/microscopic changes for the control, 25 mg/injection, and 50 mg/injection groups were 2, 3, and 3, respectively. Further, severity at injection sites 3 and 4 was increased for the 25 mg/injection and 50 mg/injection groups as compared to any injection sites for the control group.

The potential irritant effects of hirulog were evaluated with rabbits. An examination of five hirulog formulations for the potential to cause irritation and/or corrosion to the intact skin of the rabbit following a single, subcutaneous injection found that hirulog at 25 mg/mL in NaOH + Mannitol was the least irritant and hirulog at 75 mg/mL in NaOH + Mannitol was the most irritant. Hirulog was evaluated for local irritation effects to the skin and subcutis during daily subcutaneous injection to male or female rabbits for 5 days. Irritation produced by subcutaneous administration of hirulog to female rabbits at 25 or 50 mg/injection site for 5 days was not significantly different from the control. In contrast, results for male rabbits indicated increasing irritation scores for the 25 and 50 mg/injection groups as compared to the control. The number of injection sites with macroscopic/microscopic changes for the control, 25 mg/injection, and 50 mg/injection groups were 2, 3, and 3, respectively. Further, severity at injection sites 3 and 4 were increased for the 25 mg/injection and 50 mg/injection groups as compared to any injection sites for the control group.

Hirulog: Hemolysis and Plasma Protein Flocculation of Human Blood In Vitro (Biogen Study No. P8967-94-08).

Testing Laboratory: [redacted]

Study Started: July 6, 1994

Study Completed: August 30, 1995

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Drug Batch: Hirulog, Lot No. 67A03Q (modified homogenous phase commercial scale).

Methods: Red blood cell hemolysis and protein flocculation were examined in a 10 mg/mL solution of hirulog. Blood samples were obtained from 4 healthy, non-smoking human volunteers aged between 20 and 30 years.

Results: No evidence of hemolysis was found following incubation of whole blood in a 10 mg/mL solution of hirulog at concentrations of 1, 3, or 10% (v/v) blood as compared to the control incubation (0.9% NaCl). No evidence of protein flocculation was found following incubation of plasma in a 10 mg/mL solution of hirulog at concentrations of 1, 3, or 10% (v/v) plasma. Trichloroacetic acid at concentrations of 1 and 5% were used as a positive control for protein flocculation studies.

There was no evidence of red blood cell hemolysis or plasma protein flocculation in a 10 mg/mL solution of hirulog.

Antigenicity Study in Guinea Pigs (Biogen Study No. P90-024).

Testing Laboratory: [redacted]

Study Started: August 16, 1990

Study Completed: October 10, 1990

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Animals: Male and female Hartley guinea pigs were used in this study. Body weight at the initiation of the study ranged from 300 to 500 g.

Drug Batch: Hirulog, Lot No. 67W08T (solid phase peptide method).

Methods: The antigenic potential of hirulog was examined in male and female guinea pigs and compared with hirudin. The test article was prepared in vehicle or vehicle containing [redacted] Guinea pigs were treated with either the vehicle + alum, hirulog + alum, hirudin + alum, or hirulog without alum once per week by the subcutaneous route for 3 weeks. The dose was 1 mg/kg and the dose volume was 2 mL/kg. There were 3 animals/sex/group. At week 4, animals were challenged by intravenous administration of hirulog, hirudin, or the vehicle. Animals were observed immediately after challenge and at 1, 2, 4, and 24 hr after challenge. Blood was collected prior to challenge and 24 hr after challenge.

Results: One female treated with hirulog + alum was found moribund prior to challenge due to blood collection and sacrificed. There were no clinical signs of toxicity or mortality in any group. Hirudin did not produce an antigenic response; however, this protein has been found to be immunogenic in several *in vitro* and *in vivo* test, which places the credibility of the present test in some doubt.

Hirulog was not antigenic in guinea pigs treated with hirulog by the subcutaneous route once per week for 3 weeks followed by challenge with hirulog by the intravenous route.

Subacute Subcutaneous Study in Rabbits (Biogen Study No. P90-023 and P91-009).

Testing Laboratory: [redacted]

Study Start and Completion Dates: June 19 - September 17, 1990
April 11 - July 31, 1991

GLP Requirements: A statement of compliance with the GLP regulations and quality assurance unit was included with the exception of blood collection procedures.

Animals: Albino New Zealand White rabbits were used in this study. At the start of treatment, mean body weights were 2740 g for male rabbits and 3058 g for female rabbits.

Drug Batch: Hirulog, Lot No. 67W01W (solid phase peptide method).

Methods: Local tissue tolerability at subcutaneous injection sites were assessed in rabbits that received hirulog by daily subcutaneous injection of hirulog at a dose of 12.5 mg/kg/day for 5 or 7 days. In the 5 day study, the vehicle [redacted] was administered at 0.5 mL/kg/day for 5 days. For the 7 day study, vehicle was administered on day 8. Each injection was administered at a naive injection site. Male rabbits were inadvertently administered hirulog twice on day 4 at the same site. These males were not treated on day 5. Female rabbits received appropriate injections on days 4 and 5. Rabbits were observed for 30 min after each dose. All rabbits were sacrificed 24 hr after the last injection. Injection sites were analyzed microscopically.

Results: For the 5 day study, there was no significant development of erythema or edema at control or hirulog-treated sites during a 30 min period after dosing. Microscopic examination did not found any significant subcutaneous changes in either control or hirulog-treated injection sites. Tissue reactions at earlier injection sites appeared to be resolving over the 5 day period. For 7 day study, slight edema and/or purple discoloration were observed at injection sites during a 30 min period after dosing. Slight erythema and/or purple discoloration at the last injection sites were observed 24 hr post dose. Comparison of hirulog-treated areas with the control area was extremely variable. The sponsor concluded that tissue reactions produced by injection of hirulog were not serious enough to produce permanent damage.

Hirulog administered by the subcutaneous route to rabbits at 12.5 mg/kg/day for 5 or 7 days was well tolerated at the injection site.

PROPOSED TEXT OF THE LABELING FOR HIRULOG.

The label (see Appendix I) is according to 21 CFR 201.50, Subpart B (April 1, 1998). However, the following changes should be incorporated:

1. Carcinogenesis, Mutagenesis, Impairment of Fertility:

Sponsor's Version:

"In vitro and in vivo mutagenicity studies have not demonstrated any mutagenic effects. In rats, adverse effects on mating and fertility indices were observed at doses that caused exaggerated pharmacological effects. Long-term studies in animals have not been performed to evaluate the carcinogenic potential of Hirulog®."

Evaluation: The text is not in accord with 21CFR, 201.50, Subpart B (April 1, 1998).

Proposed Version: No long-term studies in animals have been performed to evaluate the carcinogenic potential of Hirulog®. Hirulog® displayed no genotoxic potential with the *in vitro* Salmonella typhimurium/Escherichia coli gene reverse mutation test, the *in vitro* forward gene mutation test at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in cultured Chinese hamster ovary cells, the *in vitro* human peripheral blood lymphocyte chromosomal aberration assay, the *in vitro* rat hepatocyte unscheduled DNA repair assay, and the *in vivo* rat micronucleus assay. Fertility and general reproductive performance in rats were unaffected by subcutaneous doses of Hirulog® up to 150 mg/kg/day (900 mg/m^2/day), which is up to 1.6 times the dose, on a body surface (mg/m^2) basis, for a 50-kg person of average height (1.46 m^2 body surface area) given the recommended human dose of 15 mg/kg (555 mg/m^2).

2. Pregnancy:

Sponsor's Version:

"Pregnancy Category B: Reproduction studies in rats and rabbits at doses up to five times the human dose and have revealed no evidence of impaired fertility or harm to the fetus due to Hirulog®. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used in pregnancy only if clearly needed."

"Reproductive and developmental toxicity: Studies conducted in rats and rabbits have demonstrated no evidence of tetratogenicity or adverse effects on fetal development. Hirulog® had no direct effects on fertility or embryo-fetal development. Developmental toxicity (adverse fetal effects) was observed at maternally toxic doses. Hirulog® treatment during gestation had no effects on peri- and post-natal development."

Evaluation: The text is not in accord with 21CFR, 201.50, Subpart B (April 1, 1998). There is no section entitled "Reproductive and developmental toxicity."

Proposed Version: Pregnancy Category B: Teratogenicity studies have been performed in pregnant rats at subcutaneous doses up to 150 mg/kg/day (900 mg/m^2/day, 1.6 times the recommended human dose based on body surface area) and pregnant rabbits at subcutaneous doses up to 150 mg/kg/day (1800 mg/m^2/day, 3.2 times the recommended human dose based on body surface area) and have revealed no evidence of impaired fertility or harm to the fetus due to Hirulog®. There are, however, no adequate and well-controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

3. Nursing Mothers:

Sponsor's Version:

"It is not known whether Hirulog® is excreted in human milk or absorbed systemically after ingestion."

Evaluation: The text is not in accord with 21CFR, 201.50, Subpart B (April 1, 1998).

Proposed Version: It is not known whether this drug is excreted in human milk. Because many drugs are excreted in human milk, caution should be exercised when Hirulog® is administered to a nursing woman.

4. Overdosage:

Sponsor's Version:

"Discontinuation of Hirulog® leads to rapid reduction in pharmacologic effects. There has been no experience of overdosage in human clinical trials. In case of overdose, Hirulog® should be discontinued and the patient should be closely monitored for signs of bleeding. There is no known antidote to Hirulog®"

Evaluation: The text is not in accord with 21CFR, 201.50, Subpart B (April 1, 1998). Data from acute toxicity studies with mice and monkeys should be included.

Proposed Version: Discontinuation of Hirulog® leads to rapid reduction in pharmacologic effects. There has been no experience of overdosage in human clinical trials. In case of overdose, Hirulog® should be discontinued and the patient should be closely monitored for signs of bleeding. There is no known antidote to Hirulog®. Single intravenous administration of Hirulog to mice at 200 mg/kg (600 mg/m^2, 1.1 times the recommended human dose based upon body surface area) and monkeys at 100 mg/kg (1200 mg/m^2, 2.2 times the recommended human dose based upon body surface area) did not produce significant clinical signs of toxicity or mortality.

SUMMARY AND EVALUATION

Hirulog, a 20 amino acid peptide, is a direct thrombin inhibitor that was designed using the leech protein, hirudin, as a model. The formation of the Hirulog-thrombin complex has been postulated to involve a 4 step mechanism. The first step with hirulog involves binding of its COOH-terminal hirudin-like region to the anion-binding exosite to form EI^1 (enzyme-inhibitor complex^1) with a dissociation constant of 0.75 µM. Second, an intramolecular conformational change is induced by this binding to form, EI^2 with a rate constant of 300 s^{-1}. In the third step, the P_1 arginine of hirulog interacts with the primary specificity pocket in a very rapid step to form EI^3. Finally, there is a second intramolecular conformational change to form EI^4 with a rate constant of 30 s^{-1}. Active site interactions of Hirulog, described by the formation of EI^3 and EI^4, increase the stability of the Hirulog-thrombin complex by 400-fold. The K_i for Hirulog was determined to be 1.9 nM. Hirulog possesses pharmacological properties that distinguish it from heparin and hirudin. Hirulog inhibits human α-thrombin activity in a concentration-dependent manner. This inhibition is of a mixed competitive/non-competitive nature. In contrast, hirudin binds to thrombin in an irreversible manner. Hirudin has been found to be immunogenic in vivo, while it appears that hirulog is not immunogenic. Hirulog (0.1-1 µM) produced a concentration-dependent inhibition of free thrombin-mediated release of fibrinopeptide A (FPA). Unlike heparin, hirulog produced a concentration-dependent inhibition of clot-bound thrombin-mediated release of FPA. The activity of heparin may be reduced by products of platelet activation, while Hirulog was unaffected by these products. Hirulog specifically interacts with thrombin, while heparin interacts with antithrombin III.

Hirulog is indicated for use as an anticoagulant in patients undergoing percutaneous transluminal coronary angioplasty (PTCA). For a 50-kg person of average height (1.46 m2 body surface area), the recommended dosage of Hirulog is a 4 hr IV infusion at a rate of 2.5 mg/kg/hr (92.5 mg/m^2/hr) with an IV bolus dose of 1.0 mg/kg (37 mg/m^2) administered immediately after initiation of the infusion, followed by an IV infusion at a rate of 0.2 mg/kg/hr (7.4 mg/m^2/hr) for up to 20 hr as clinically warranted. The total dose for 24 hr is equivalent to 15 mg/kg or 555 mg/m^2. Treatment with Hirulog should be initiated just prior to PTCA. The dose of Hirulog may need to be reduced, and anticoagulation status monitored, in patients with renal impairment.

In support of the proposed clinical use of hirulog, the sponsor has submitted the following preclinical studies: pharmacology; absorption, distribution, metabolism, and excretion studies in rats, rabbits, baboons, and monkeys; acute toxicity studies by intravenous bolus and subcutaneous administration to mice and rats; acute toxicity studies by intravenous bolus administration to monkeys; an acute toxicity study of hirulog and its major impurities in rats; an acute toxicity study of two hirulog formulations administered by a 4 hr intravenous infusion to female dogs; an acute toxicity study of two hirulog formulation administered by 4 hr intravenous infusion to female cynomolgus monkeys; a 14 day toxicity study of hirulog administered by continuous infusion to rats; a 14 day intravenous toxicity study of hirulog and partially degraded hirulog in rats; a 14 day intravenous toxicity study of PB19-6, a potential hirulog impurity, in rats; a 28 day intravenous toxicity study in rats; a 28 day intravenous infusion toxicity study of hirulog in rats with a 14 day recovery period; a 7 day pilot intravenous toxicity study in cynomolgus monkeys; a 28 day subcutaneous toxicity study in cynomolgus monkeys; a 28 day intravenous infusion toxicity study in cynomolgus monkeys with a 14 day recovery period; reproductive toxicology studies that included a specialized Segment I fertility and reproductive performance study in rats, a specialized Segment II tetratogenicity study in rats, a Segment II tetratogenicity study in rabbits, and a Segment III perinatal and postnatal development study in rats; genotoxicity studies that included a Salmonella typhimurium/Escherichia coli gene reverse mutation assay using a pre-incubation method, a Salmonella typhimurium/Escherichia coli gene reverse mutation assay using a plate incorporation method, a forward-gene mutation test at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in cultured Chinese hamster ovary cells, a human peripheral blood lymphocyte chromosome aberration assay, a rat hepatocyte unscheduled DNA repair assay, and a rat micronucleus test; and special toxicity tests that included subcutaneous irritation studies in rabbits, a study of hemolysis and protein flocculation with human blood in vitro, an antigenicity study in guinea pigs, and local tissue tolerability studies at subcutaneous injection sites in rabbits.

The absorption, distribution, metabolism, and excretion of hirulog were examined in rats, rabbits, baboons, and monkeys. Plasma drug parameters (i.e., C_{max}, AUC) in animal studies were equal to or exceeded concentrations found in humans (See table at end of ADME section). With a 4 hr continuous intravenous infusion of hirulog (homogenous phase commercial scale) to rats (1.04 mg/kg/hr), monkeys (1.7 mg/kg/hr), and humans (0.5 mg/kg/hr), AUC values were 6970, 12054, and 6721 ng*hr/mL, respectively. Dose normalized AUC values (AUC values divided by dose in mg/kg/hr) for rats, monkeys, and human were 6702, 7090, and 13442 ng*hr/mL, respectively. Hirulog was resistant to degradation in citrated plasma from human and human whole blood treated with citrate or heparin. Plasma half-lives of hirulog in rats, monkeys, and humans with a 4 hr continuous intravenous administration were 0.4-2.2, 0.45-0.48, and 0.61-1.05 hr, respectively. The volume of distribution of hirulog in rats following intravenous or subcutaneous administration exceeded blood volume suggesting distribution into tissues. The distribution and metabolism hirulog was examined in rats following intravenous or subcutaneous administration. The two amino acid portion of the N-terminal end, phenylalanine-proline, was cleaved off the peptide and excreted into the urine. The remaining 18 amino acid portion of hirulog was degraded into individual amino acids, which were subsequently incorporation into new protein. Less than 25% of this remaining 18 amino acid portion was excreted into the urine and feces. Hirulog C_{max} values following intravenous dosing exceeded values found with subcutaneous dosing; however, AUC values for subcutaneous dosing appeared to exceed those found with intravenous dosing. Metabolism and excretion studies in cynomolgus monkeys yielded similar results as described for rats. Following continuous intravenous infusion of hirulog at 250 mg/kg/day for 28 days to male rats, the activity of CYP4A was elevated, which may be reflective of increased peroxisomal proliferative activity.

The acute toxicity of hirulog was examined in mice, rats, and cynomolgus monkeys. Hirulog administered by either the intravenous or subcutaneous route to mice at doses between 0 and 200 mg/kg did not produce any mortality or signs of toxicity. Hirulog administered by the subcutaneous route to rats at doses between 12.5 and 200 mg/kg produced no mortality or clinical signs of toxicity. Acute intravenous administration of hirulog to cynomolgus monkeys using a dose escalation design at doses of 5, 15, 50, and 100 mg/kg produced no clinical signs of toxicity or mortality. When hirulog was administered by the intravenous route to rats, the maximum nonlethal and minimum lethal intravenous doses were 25 and 50 mg/kg, respectively. Pulmonary distress followed by death was observed in rats when hirulog was administered at doses ≥50 mg/kg

by the intravenous route at high dose volumes in saline and possibly dextrose, but not in vehicle (composition was not clearly specified). Pulmonary distress and subsequent death appeared to be due to high dose volume and unrelated to hirulog. Acute intravenous toxicity studies with hirulog in rats do not appear to be characteristic of hirulog toxicity.

The acute intravenous toxicity of hirulog and its major [redacted] at a single dose of 50 mg/kg were examined in male and female rats. There were no significant clinical signs of toxicity or mortality.

In a 14 day study, rats received hirulog by continuous intravenous infusion at doses of 80 and 1000 mg/kg/day. Significant deviation in dosing occurred during the course of this study and results from this study should probably be disregarded. Attention should be directed toward the 28 day continuous intravenous infusion study with hirulog in rats followed by a 14 day recovery period that is discussed below.

The toxicity of hirulog and partially-degraded hirulog [redacted] were evaluated in rats following intravenous bolus administration at 36 mg/kg/day (216 mg/m^2/day) for 14 days. There was no mortality or treatment-related clinical signs of toxicity. The liver was the target organ of toxicity for rats treated with hirulog or [redacted] The incidence of biliary hyperplasia was increased following treatment with either [redacted] or hirulog as compared to the control. The incidence of biliary hyperplasia in [redacted]-treated female rats was higher as compared to hirulog.

[redacted] hirulog, was evaluated in rats by intravenous bolus administration into a caudal vein at a dose of 36 mg/kg/day (216 mg/m^2/day) for 14 days. The sponsor neglected to include a positive control group receiving hirulog to allow an assessment of the toxicity of [redacted] relative to hirulog. There was no mortality or clinical signs of toxicity. Target organs of toxicity with [redacted] appeared to be the liver, kidney, heart, and lungs. Treatment-related liver changes consisted of biliary hyperplasia, portal inflammation, and hepatocellular necrosis. The sponsor attributed these changes to intravenous administration of a protein. Further, they claimed that these changes were reversible; although, a recovery period was not included in the study. There was an increased incidence of chronic inflammation of the heart for rats treated with [redacted] The sponsor claimed that chronic inflammation of the heart is typical for rats >4 months old and the test article either accelerated or enhanced the development of these changes in rats sacrificed at 9 weeks of age; however, no historical data was included to support this claim. Changes in the heart were not observed for other studies with hirulog in rats.

In a 28 day tudy, rats received hirulog by the intravenous route of administration at doses of 0, 4, 12, and 36 mg/kg/day. The no effect level was 36 mg/kg/day (216 mg/m^2/day). A target organ of toxicity was not identified.

In a 28 day study, rats received hirulog by continuous intravenous infusion at doses of 0, 25, 75, and 250 mg/kg/day. Rats from the control and 250 mg/kg/day groups entered a 14 day recovery following the treatment period. The no effect level was 25 mg/kg/day (150 mg/m^2/day). Treatment-related mortality occurred at 250 mg/kg/day. The liver was the target organ of toxicity. Sinusoidal histiocytosis and centrilobular and midzonal necrosis were observed for the liver at doses of 75 and 250 mg/kg/day. An increased incidence of pancreatitis was found at 250 mg/kg/day; although, it was not test article-specific. Hemorrhage was observed in the thymus and optic nerve at all dose levels. Hemorrhage was also found in the lung at 250 mg/kg/day group. The severity of hemorrhage appeared to be low as there were no changes of red blood cell counts, hemoglobin levels, or hematocrit at any dose level. The severity of phlebitis at the infusion site was increased in treatment groups in a dose-related manner. Increased extramedullary hematopoiesis in the spleen and increased myelopoiesis in the bone marrow were found and attributed by the sponsor to an influx of inflammatory cells at the infusion site. With the exception of periphlebitis, no treatment-related changes were observed at the end of the recovery period. On day 28, plasma hirulog levels were proportional to dose.

In a 7 day pilot study, cynomolgus monkeys received hirulog by intravenous bolus administration at doses of 0, 12, and 36 mg/kg/day. The maximum tolerated dose was 36 mg/kg/day (216 mg/m^2/day). Skeletal muscle inflammation was observed in both the 12 and 36 mg/kg/day groups. There was no evidence for the development of anti-hirulog cross-reacting antibodies in monkeys that received hirulog by the intravenous route at 12 or 36 mg/kg/day for 7 days.

In a 28 day study, cynomolgus monkeys received hirulog by the subcutaneous route of administration at doses of 0, 4, 12, and 36 mg/kg/day. The maximum tolerated dose was 36 mg/kg/day (432 mg/m^2/day). There was no target organ of toxicity. Bruising was observed at sites of drug administration. The severity of bruising was proportional to the hirulog dose. An increased incidence of histopathological changes were identified for the parathyroid glands, sciatic nerve, stomach, and bronchial lymph nodes; however, these changes generally did not occur in a dose response related manner and were not test article specific. There was no evidence for the development of anti-hirulog cross-reacting antibodies in monkeys that hirulog by the subcutaneous route at doses of 4, 12, or 36 mg/kg/day for 28 days.

In a 28 day study, cynomolgus monkeys received hirulog by continuous intravenous infusion at doses of 0, 15, 45, and 150 mg/kg/day. Two animals/sex from the control and 150 mg/kg/day groups entered a 14 day recovery period following the treatment period. The no effect dose was 45 mg/kg/day (540 mg/m^2/day). APTT values were increased at all dose levels during the treatment period. Target organs of toxicity included the heart and skeletal muscle. For the 150 mg/kg/day group, 1 male and 1 female were observed with myocardial degeneration/necrosis and hemorrhage. Skeletal muscle degeneration and/or necrosis was observed for 2 females of the 150 mg/kg/day group. The incidence of thymic hemorrhage was increased in the 45 and 150 mg/kg/day groups; although, there was not a dose response relationship and the effect was not test article specific. Plasma hirulog levels were determined on day 29 at the termination of the infusion. Concentration values at steady state increased with dose; although, the value at 15 mg/kg/day was lower than expected based upon values at 45 and 150 mg/kg/day.

For reproductive toxicology studies described below, the sponsor used the subcutaneous route rather than the intravenous route, which is the intended clinical route of administration. The sponsor has not provided substantive justification for using the subcutaneous route, particularly with regard to bioavailability, as agreed to at the pre-NDA meeting held on August 4, 1997. However, in the Segment I fertility and reproductive performance study with rats, a subcutaneous dose of 500 mg/kg/day produced dose limiting toxicity in terms of mortality and impairment of body weight gain. The use of the subcutaneous route for reproductive toxicity studies, while not completely justified, does not present an obstacle to a recommendation for approval of the NDA. Further, clinical use of hirulog in human is limited to a 24 hr period and issues of reproductive toxicity are most likely a minor issue.

In a Segment I study, the effects of hirulog on fertility and reproductive performance were evaluated in male and female rats. Male and female rats received hirulog by the subcutaneous route of administration at doses of 0, 50, 150, and 500 mg/kg/day. Male rats received hirulog for 4 weeks prior to mating and through the mating period. Female rats received hirulog for 2 weeks (15 days) prior to mating, through the mating period, and from days 0 to 17 of gestation. Mating and general reproductive performance were unaffected at doses ≤150 mg/kg/day (900 mg/m^2/day). Mortality and significant impairment of body weight gain were observed for male and female rats that received hirulog at 500 mg/kg/day. At 500 mg/kg/day, the mating and fertility indexes were slightly reduced. Evaluation of sperm number and viability was not performed; although, testicular toxicity was not evident in any intravenous

toxicity studies with rats or cynomolgus monkeys. At 500 mg/kg/day, the number of corpora lutea, implantations, litter sizes, and live fetuses were reduced. The number of late resorptions were increased at 500 mg/kg/day. There was an increased incidence of skeletal variation at 500 mg/kg/day. Fetal skeletal variations observed at 500 mg/kg/day included an increased incidence of right cervical rib at the 7th cervical vertebra and delayed sternal ossification. Decreased numbers of live fetuses and an increased incidence of skeletal variations found at 500 mg/kg/day are most likely due to maternal toxicity (i.e., mortality and impairment of body weight gain) observed at this dose.

In a Segment II toxicity study, pregnant female Sprague Dawley rats received hirulog by the subcutaneous route at doses of 0, 10, 50, and 150 mg/kg/day from days 7 to 17 of gestation. Hirulog at doses ≤150 mg/kg/day (900 mg/m^2/day) was not teratogenetic in rats.

In a Segment II tetratogenicity study, pregnant female rabbits received hirulog by the subcutaneous route of administration at doses of 0, 15, 50, and 150 mg/kg/day from days 6 to 18 of gestation. At doses ≤150 mg/kg/day (1800 mg/m^2/day), hirulog was not teratogenetic in rabbits.

In a Segment III pre- and postnatal study, pregnant F_0 female rats received hirulog by the subcutaneous route of administration at doses of 0, 10, 50, and 150 mg/kg/day from days 6 to 19 of gestation and days 2 to 20 of lactation. F_0 dams were not treated from day 20 of gestation through day 1 of lactation due to the possibility of excessive hemorrhaging during parturition, because hirulog is an antithrombin. Pre- and postnatal development for F_1 rats were not affected by hirulog at doses ≤150 mg/kg/day (900 mg/m^2/day). There were no clinical signs of toxicity or mortality for F_0 dams at doses ≤150 mg/kg/day. The male to female ratio and body weights for F_2 fetuses were unaffected by hirulog treatment. There were no external abnormalities for F_2 fetuses that displayed any relationship to treatment.

Hirulog was found to have no genotoxic potential either <u>in vitro</u> using the Salmonella typhimurium/Escherichia coli gene reverse mutation test (Ames test), the forward gene mutation test at the hypoxanthine-guanine phosphoribosyl transferase (HGPRT) locus in cultured Chinese hamster ovary cells, the human peripheral blood lymphocyte chromosomal aberration assay, and the rat hepatocyte unscheduled DNA repair assay, or <u>in vivo</u> using the rat micronucleus assay.

There was no evidence of red blood cell hemolysis or plasma protein flocculation in a 10 mg/mL solution of hirulog. Red blood cells and plasma proteins were obtained from whole human blood.

The antigenic potential of hirulog was examined in male and female guinea pigs and compared with hirudin. Hirulog was not antigenic in guinea pigs treated with hirulog by the subcutaneous route once per week for 3 weeks followed by challenge with hirulog by the intravenous route.

In humans, hirulog will be will be administered by continuous intravenous route for a 24 hr period. The sponsor has conducted sufficient preclinical intravenous general toxicology studies in rats and monkeys. In 28 day studies in which hirulog was administered by continuous intravenous infusion, the no effect doses for rats and monkeys were 25 mg/kg/day (150 mg/m^2/day) and 45 mg/kg/day (540 mg/m^2/kg), respectively. For rats, the target organ of toxicity was the liver. Sinusoidal histiocytosis and centrilobular and midzonal necrosis were observed for the liver at doses of 75 and 250 mg/kg/day. An increased incidence of pancreatitis was observed at 250 mg/kg/day; although, it was not test article-specific. Hemorrhage was observed in the thymus and optic nerve at all dose levels, which may be related to the anticoagulant activity of hirulog. For monkeys, target organs of toxicity included the heart, and skeletal muscle. Degeneration and/or necrosis were found in the heart and skeletal muscle with a dose of 150 mg/kg/day. Hemorrhage, related to the anticoagulant activity of hirulog, was found in the thymus and heart. The sponsor has adequately characterized hirulog and conducted sufficient intravenous toxicology studies in different species for the anticipated use of this drug. From a preclinical standpoint, the application is recommended for approval.

The label is not according to 21 CFR, 201.50 Subpart B (April 1, 1998), and changes in text as outlined in the review portion are needed.

RECOMMENDATION:

From a preclinical standpoint, the application is recommended for approval with a provision that labeling be changed as outlined in the review portion.

/S/
Timothy W. Robison, Ph.D. 1-23-98
Date

cc:
Orig NDA 20,873
HFD-180
HFD-181/CSO
HFD-180/Dr. Choudary
HFD-180/Dr. Robison
HFD-345/Dr. Viswanathan

R/D Init.: J. Choudary 6/12/98

TWR/hw/7/10/98 & 7/23/98

/S/
7/27/98