Monkeys:

Pharmacokinetics of HBW023-^{125}I After a Single I.V. Dose in Male Cynomolgus Monkeys (Study # 014769)

Methods: Cynomolgus male monkeys were given a single i.v. dose of 1 mg/kg of HBW023-^{125}I. Blood samples were collected at 0.083, 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, 96, 120, 144, 168, 192, 216/240, 312/336, 360/384 and 552 hr after the drug administration. Urine and feces were collected up to 24 hr after drug administration. In each sample radioactivity was measured by . Because of possible cleavage of methods were used for measuring r-hirudin levels in the sample.

Results: Irrespective of the assay method used the T_{max} was 5 min, and C_{max} values were similar when measured by three different methods. Hence, at T_{max} the majority of the drug is in unchanged form. However, from then on the elimination profile of r-hirudin were different depending upon the method used. The elimination profile obtained from total radioactivity produced slower elimination, mostly due to cleavage of the label ^{125}I by dehalogenases, hence should not be used to calculate pharmacokinetic parameters. The elimination profiles obtained from were similar and represents true r-hirudin kinetics. Based on the $t_{\frac{1}{2}}$ ß for r-hirudin is , plasma clearance ranged and volume of distribution ranged from . r-Hirudin and its metabolites mainly excreted in urine and fecal elimination was minimal

I.V. and S.C Pharmacokinetic Study with r-Hirudin in Rhesus Monkeys (Report No. 2.2.3.7)

Methods: Two groups of male rhesus monkeys (3/group) were administered r-hirudin at a dose of 0.5 mg/kg (dissolved in isotonic saline and given in a volume of 1 ml/kg) via either i.v. or s.c. dosing. R-hirudin concentrations in serum and urine as well as effects on TT and PTT were assessed in samples taken at 5, 10, 20, 30, 45, 60 and 90 min after dosing and then at 1 hour intervals up to 8 hours and at 24 hours after dosing. Concentrations of r-Hirudin in serum and urine were indirectly measured based on a chromogenic assay which measures the amount of unbound thrombin following incubation with bovine thrombin.

Results: Intravenous and s.c. administration of r-hirudin at a dose of 0.5 mg/ml resulted in maximal plasma r-hirudin serum concentrations of 4149.3 ± 706.4 and 290.7 ± 31.6 ng/ml at 5 min and 1.5 hours after dosing, respectively. Mean total AUC values were 2077.83 ± 509.8 and 1203.45 ± 63.5 ng/ml in the i.v. and s.c. dosed groups, respectively. Elimination of the i.v. dose appeared biphasic, whereas elimination of the s.c. dose was monophasic. Terminal elimination half lives were slightly greater following s.c. dosing versus i.v. dosing . Analysis of urine for r-hirudin showed that approximately 48% and 70% of the administered dose was excreted in the urine following i.v. and s.c. dosing, respectively. At the end of the 24 hour period, serum concentrations of 20.0 ± 6.2 and 12.0 ± 3.0 ng/ml were still measurable in the i.v. and s.c. dosed groups, respectively. PTT and TT were prolonged up to 2 and 3 hours, respectively after i.v. dosing and up to eight hours each following s.c. dosing. Finally, although TT appeared most sensitive to r-hirudin, only changes in PTT were significantly correlated to plasma concentrations.

Pharmacokinetics of r-Hirudin in Cynomolgus Monkeys After Combined I.V. Bolus and 6 Hour Infusion (Study # 134.4-07)

Methods: Cynomolgus monkeys were given i.v. bolus followed by a 6 hr i.v. infusion of r-hirudin (0.2 mg/kg i.v. bolus + 0.1 mg/kg/hr i.v. infusion or 0.4 mg/kg i.v. bolus + 0.15 mg/kg/hr i.v. infusion). Blood samples were collected at 0.25, 0.5, 1, 2, 4, 5, 6, 6.25, 6.5, 7, 9, 12, 24 and 48 hr after the start of infusion. r-Hirudin levels in plasma samples were measured by methods.

Results: r-Hirudin cleared from circulation with $t_{\frac{1}{2}}$ ß of the volume of distribution ranged indicating that drug is mainly distributed in extracellular fluid. Clearance value ranged There was no indication for change in clearance with dose.

	0.2 mg/kg + 0.1 mg/kg/hr for 6 hr.		0.4 mg/kg + 0.15 mg/kg/hr for 6 hr.	
Parameters				
$t_{\frac{1}{2}}$ ß (hr)	1.36	1.67	1.79	1.87
Cl (ml/min/kg)	4.04	3.43	3.29	2.63
V_{ss} (L/kg)	0.33	0.26	0.25	0.22

Effect of r-Hirudin On Blood Coagulation and Its Pharmacokinetics After I.V. and S.C. Dose in Rhesus Monkeys (Study # HBW023-04)

Methods: Male rhesus monkeys were given a single i.v. or s.c. dose of 0.01, 0.025, 0.05, 0.1, 0.5 or 1.0 mg/kg of r-hirudin. Blood and urine samples were collected at 5, 10, 20, 30, 45, 1, 1.5, and then each hour up to 8 hours and 24 hours after the drug administration. TT and PTT were measured in serum samples. Hirudin levels in serum and urine were also monitored.

Results: Irrespective of route of administration (i.v. or s.c.), TT and PTT increased dose dependently. In the summary sponsor indicated that there were good correlations between PTT and plasma levels of r-hirudin [r = 0.789 (i.v.) and r = 0.836 (s.c.)]. Within 24 hr 48% (i.v.) and 70% (s.c.) of the administered dose (0.5 mg/kg) were excreted renally. Following pharmacokinetic parameters were obtained at 0.5 and 1.0 mg/kg dose levels.

Pharmacokinetic Parameters in Monkeys				
	Dose (mg/kg, i.v.)		Dose (mg/kg, s.c.)	
Parameters	0.5	1.0	0.5	1.0
$t_{1/2}$ ß (hr)	0.85 ± 0.15	1.08 ± 0.06	1.21 ± 0.15	1.92 ± 0.64
Cl (ml/min/kg)	3.51 ± 1.75	4.25 ± 1.78	4.45 ± 1.71	3.95 ± 0.39
AUC (ng/ml.hr)	2078 ± 509	4456 ± 1959	2165 ± 308	4245 ± 403

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Kinetics and Metabolism of [^{125}I]-r-Hirudin following s.c. injection in Rhesus Monkeys (Report No. 01-L42-0683-93)

Methods: [^{125}I]-R-Hirudin was dissolved in sterile physiological saline and injected subcutaneously into two female rhesus monkeys at a dose of approximately 1 mg/kg in a total volume of 0.1 ml/kg. A third control monkey was mentioned, but only information regarding plasma sampling was provided for this animal. Blood samples (1 ml or 5.5 ml, marked by an asterisk,*) were collected from one treated animal at predose*, 5, 15*, 30 min, 1*, 2, 4*, 6, 24, 48, 72, 96, 120, 144, and 168 h after dosing and from the other treated and control monkeys (6 ml) at 0, 0.25, 1 and 4 hours after dosing for determination of blood and/or plasma* levels of r-hirudin. Urine samples were collected at predose and from 0-5 and 5-24 hours after dosing, daily for 12 days (animal one) and at predose, 0-5, 5-9, and 9-24 hours daily for 6 days (animal 2). Feces samples were also collected from each animal daily up to 7 days (animal 1) or 6 days (animal 2) after dosing. Radioactivity in samples of blood, plasma (marked by an asterisk,*), urine and feces was performed using a calibrated The metabolic pattern of radioactivity in plasma from the second animal was determined using HPLC compared to reference standards. Concentrations of r-hirudin were expressed as µg equivalents (i.e. the sum of parent compound and radiolabelled metabolites).

Results: Maximum blood concentrations (animal one) of 1.02 µg equivalents/ml occurred at 1 hour after s.c. administration and decreased slowly thereafter to a value of 0.08 µg equi./ml. Maximum plasma concentrations of 1.67 and 2.03 µg equivalents were also observed at 1 h after dosing in monkeys 1 and 2, respectively. The blood: plasma concentration ratio (monkey 1) rose from 8% at 15 min after dosing to 100 % at the 4 hour time point. The majority of the radioactivity was excreted via the urine within the first 24 hours after dosing (72 and 86% of the administered radioactivity), relative to a total of 77 and 93% collected in urine over the entire 168 hour collection period. The pattern of elimination in urine was biphasic with an initial rapid phase ($t_{½}$ = 5.5 h) followed by a slower phase ($t_{½}$ = 33.9 h). In turn, fecal excretion accounted for only 1-2% of the total administered dose of radioactivity, the majority again occurring during the 0-24 hour time point. Excretion was essentially complete by day 7 with total balances of radioactivity of 91.16% and 100% observed in the two monkeys. HPLC analysis of plasma samples (up to 4 hours postdosing) showed only unchanged original compound. In urine samples, the majority of radioactivity was also in the form of the parent compound, but with free iodide and smaller portions of 4 metabolites also observed. None of the metabolites were identified.

Blood Levels and Anticoagulant Activity of r-Hirudin Following Oral Administration of r-Hirudin in Rhesus Monkeys. (Report Nos. 2.2.3.8 and 2.2.3.6)

Methods: Two studies were conducted in rhesus monkeys which assessed the effects of orally administered r-hirudin (30 mg/kg) on coagulation parameters in association with measured plasma levels. In the first study, r-hirudin was dissolved in physiologic saline and water and administered by gavage at a dose of 30 mg/kg in a total volume of 8 ml/kg. In the second study r-hirudin was also administered orally at a dose of 30 mg/kg but in gastric juice resistant capsules. Blood samples were collected at predose, and again at 15 min (first study only) 30 and 60 min after dosing, then each hour for 6 hours and again at 24 hours after dosing for measurement of TT, PTT, and plasma r-hirudin concentrations. Urine samples were also collected up to 24 hours after dosing. Concentrations of r-hirudin in plasma and urine both studies were measured by incubation with bovine thrombin and measurement of remaining thrombin with a synthetic chromogenic substrate.

Results: In the first study, oral administration (by gavage) of r-Hirudin (30 mg/kg) resulted in maximal plasma concentrations of 15 and 24 ng/ml at 1 and 2 hours after dosing in the 2 monkeys tested, but had no detectable effects on either TT or PTT. In the second study plasma levels of r-hirudin following oral administration of r-hirudin (30 mg/kg) in capsule form were below the limit of detection at all sampling times, whereas, a slight prolongation of TT (from predose value of 25.8 sec up to 34.3 sec at the 2 hr time point), but no change in PTT. Thus the oral absorption of r-hirudin at doses up to 30 mg/kg appeared very low in monkeys.

Pharmacokinetics of r-Hirudin and the α-Human Thrombin-Hirudin Complex (THC) in Rhesus Monkeys (Literature report, Vol.15, pg 257)

Methods: Three monkeys were treated with a single i.v. dose of r-Hirudin (0.1 mg/kg) in week 1, followed by an i.v. dose of THC 0.067 mg/kg in the second week. Following dosing, plasma samples were collected at 0, 5, 15, 30, 60, 120, 180, 240, 360, 420, 360, 420, 480, and 560 min and 24 hours after dosing for analysis of PTT, TT, hirudin and THC concentrations. Plasma concentrations of Hirudin and THC were analyzed using chromogenic thrombin substrate assays and 2 ELISA assays. Pharmacokinetic parameters were calculated based on plasma concentration time curves which were generated.

Results: Table 4 Below shows the pharmacokinetic values for r-hirudin and THC following i.v. dosing in the rhesus monkey.

Table 4. Pharmacokinetics of r-Hirudin and Thrombin-Hirudin Complex (THC) after I.V. Dosing in Monkeys.

Parameter	Hirudin (0.1 mg/kg)	THC (0.067 mg/kg)
C_{max} (ng/ml)	736 ± 322	568 ± 39
AUC (ng·h/ml)	499 ± 143	1731 ± 685
$t_{\frac{1}{2}}\alpha$	0.19 ± 0.17	0.27 ± 0.29
$t_{\frac{1}{2}}\beta$	1.13 ± 0.086	2.49 ± 0.57
Cl_{tot} (ml/min)	27.2 ± 2.6	5.2 ± 0.57
Urinary excretion (%, 0-24 h)	50.0 ± 17.9	15.9 ± 1.9

Briefly, the data in Table 4 show that the terminal half life for THC was approximately double that of Hirudin, with an approximate 5 fold reduction in total clearance. Excretion of both hirudin and THC was biphasic in nature. Total urinary excretion of THC was approximately 3 fold less than that of unbound hirudin.

Maximal effects on PTT (increase from 32.1 sec in control up to 63.8 sec) and TT (>300 sec) occurred at 5 min and between after dosing with hirudin, respectively. In comparison THC resulted in moderate prolongation of PTT , whereas only moderate increases in TT were observed between 5 and 30 min after dosing. Since the THC is inactive, the prolonged effects of PTT apparently result from the degradation of THC thus liberating the active hirudin component.

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Plasma Levels and Effects of Low Dose r-Hirudin on Coagulation Parameters after I.V. Administration in Rhesus Monkeys. (Report No. 2.2.3.5)

Methods: Three groups of male rhesus monkeys (3/group) were administered single i.v. injections of r-hirudin at doses of 0.01, 0.025, and 0.05 mg/kg (dissolved in isotonic saline and given in a volume of 1 ml/kg). R-Hirudin concentrations in serum and urine as well as effects on TT and PTT were assessed in samples taken at 5, 10, 20, 30, 45, 60 and 90 min after dosing and then at 1 hour intervals up to 6 hours and at 24 hours after dosing. Concentrations of r-Hirudin in serum and urine were indirectly measured based on a chromogenic assay which measures the amount of unbound thrombin following incubation with bovine thrombin.

Results: Mean maximal plasma concentrations of 99.33 ± 17.24, 166.00 ± 58.64, 158.0 ± 39.7 ng/ml were observed at 5 min after i.v. injections of the 0.01, 0.025, and 0.05 mg/kg doses, respectively. R-Hirudin also produced a pronounced, dose-dependent prolongation of PTT (up to 1 h) and TT (up to 2 h) following dosing. Only changes in PTT were correlated with plasma concentrations of r-Hirudin and was thus the most suitable clotting parameter to monitor in vivo plasma concentrations. Finally, analysis of urine samples for r-hirudin showed that over a concentration of approximately of the administered i.v. dose was excreted in the urine.

Distribution

Blood Levels, Distribution, and Excretion of r-Hirudin following S.C. Injection in Rats. (Report No. 01-L42-0669-93)

Methods: Groups of Wistar rats (6/sex/group) were subcutaneously injected with ^{125}I-r-Hirudin (Batch No 1/90A,2/90) at a dose of approximately 1 mg/kg dissolved in physiological saline solution and administered at a volume of about 1.25 ml/kg. Blood samples (0.110 ml) were collected from 3 males and 3 females at approximately 0.83, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 24, and 48 hours after injection. Volume replacement was not indicated. Urine and feces samples were collected at 0, 0-5, 5-24, and 24-48 hours and at 0, 0-24, and 24 to 48 hours after dosing for determination of radioactivity content. Animals were killed at 48 hours after dosing and organs were removed for measurement of radioactivity content. Radioactivity contents in blood, urine, feces and tissue/organ samples and carcass were determined using a . Concentrations of r-Hirudin are expressed in µg equivalents and represent the sum of parent compound and or radiolabelled metabolites.

Results: Mean maximal blood levels of 1.95 µg equivalents/g in males and 1.79 µg equivalents/g in females were observed at after s.c. injection or r-Hirudin (1 mg/kg) in rats. Elimination of radioactivity in blood was biphasic with initial half life values of 3.5 hours in males and 4.2 hours in females for the initial fast phase, followed by a slow secondary phase with mean elimination half life values of 115.7 hours in females and 48.5 hours in males calculated. Generally, plasma levels of radioactivity were greater than those observed in blood by a factor of At 48 hours after dosing, approximately 81.3 and 81.9% of the administered dose was collected in the excreta (approximately of the collected radioactivity occurring in the urine and occurring in the feces).

Examination of radioactivity in tissues/organs and carcass at 48 hours after dosing showed that highest levels of ^{125}I radioactivity were observed in thyroid
of the administered dose. In males, higher values than detected in blood (0.025 µg equivalents/g) were only observed in skin (factor 2.1), plasma (2.1), stomach contents (1.3) and carcass (1.4) whereas in females, levels higher than in blood (0.025 µg equivalents/g) were detected in kidneys and liver (both factor 1.4). Apart from thyroid, no organ or tissue contained more than 0.3% (males) or 0.17% (females) of the administered dose of radioactivity at the 48 hour time point.

Distribution of HBW-023-^{125}I After I.V. Injection.

Animals: Male Wistar rats (mean body weight of 201 g; 6 to 8 weeks of age).

Methods: Eight rats were intravenously administered r-hirudin (approximately 1.2 mg/kg; 148.3 µCi/mg); vehicle was 0.09% saline solution and dosing volume was 1 mg/ml. Two animals were sacrificed at 0.083, 1, 4 and 24 h post-dosing, respectively.

One animal per dose was sacrificed by carbon dioxide asphyxiation and subjected to whole-body autoradiogram evaluation. Mean density values (optimal densities) were determined by a videodensitometric method. One animal per dose was sacrificed by carbon dioxide asphyxiation and slide sections of organs and tissues were prepared. Slide sections were exposed on
for 13 to 40 days.

Results: Whole-body autoradiogram evaluation at 0.083 h after dosing revealed that amounts of radioactivity were higher in kidneys than blood, while amounts of radioactivity were similar to that in the blood for lung, myocardium, adrenals, liver, gastrointestinal tract, skin, hair, connective tissue and eye walls. Moderate amounts of radioactivity were present in pancreas, glandular mucosa of the stomach and salivary glands. Radioactivity in bone marrow, epididymis, skeletal musculature and fat was above film background. At 1 h post dosing, radioactivity had generally decreased; however, increased radioactivity was seen in the thyroid, urine in urinary bladder, mucosa of stomach and stomach lumen. At 4 h post dosing, further decreases in radioactivity were seen; however, diffuse radioactivity was evident in the brain. At 24 h post dosing, residual radioactivity was seen in thyroid, skin/hair and kidney. Relative whole body distributions of radioactivity (radioactivity in blood was assigned a value of 1.00) are shown in the following table (from page 14 of sponsor's Report No. 01-L42-0581-90).

Data on semiquantitative distribution in different organs and tissues at several time points after a single intravenous administration of approx. 1 or 1.5 mg/kg body weight to male rats

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	Concentration ratios			
Organs / Tissues	5 min	1 h	4 h	24 h
Blood	1.00+	0.33	0.17	n.d.
Liver	0.54	0.30	0.20	n.d.
Connective tissue, skin, hairs		0.46	0.20	n.e.
Lung	0.85	0.36	0.28	n.d.
Kidney	>2.00	>2.00	0.37	
Adrenal	0.60	0.47	0.23	n.d.
Spleen	n.e.	0.24	0.19	n.d.
Salivary gland	0.30	0.23	0.16	n.d.
Fat	0.16	0.14	<0.10	n.d.
Eye (Eye lens	<0.10	<0.10 / 0.10	<0.10	n.d.
Bone marrow	0.20	0.21	0.14	n.d.
Pancreas	0.36	0.35	n.e.	n.d.
Brain	<0.10	<0.10	<0.10	n.d.
Myocardium	0.68	0.21	0.14	n.d.
Intestines (wall)	n.e.	0.32	n.e.	n.d.
Testis	<0.10	0.12	0.14	n.d.
Epididymis	0.21	0.26	0.18	n.d.
Skeletal musculature	0.15	0.11	<0.10	n.d.
Gastric mucosa	0.34		>2.00	n.e.
Thyreoidea		>2.00	>2.00	>2.00

n.d. not detectable
n.e. not estimated
+ The mean concentration in the blood 5 min p. inj. estimated by video densitometry is set to one (reference point)

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According to the sponsor, evaluations of indicated similar organ and tissue distributions of radioactivity as found in whole-body autoradiograms; sponsor did not provide detailed results for

Tissue Distribution in Rats After A Single I.V. Dose of 1 mg/kg of ^{125}I-r-Hirudin (Study # 014567)

Methods: Male rats were given a single i.v. dose of 1 mg/kg of ^{125}I-r-hirudin. Three rats each were killed at 0.083, 0.5, 1, 2, 4 and 8 hr after drug administration. Blood, plasma, and various tissues were collected and radioactivity levels were measured by gamma counter.

Preliminary study (# TEP 154/15) indicated that deiodification of the ^{125}I-r-hirudin in plasma (via dehalogenases) occurs very rapidly in rats. At 2 hr after i.v. dose, about 92% of the plasma radioactivity represented iodide (8% r-hirudin and 1% r-hirudin metabolites as peaks). In view of this, distribution study duration was limited to 8 hours.

Results: Radioactivity was distributed throughout the body. levels of radioactivity in kidneys, urinary bladder and thyroids were higher than that seen in plasma at 30 min after drug administration. Sponsor's reported pharmacokinetic parameters ($t_{\frac{1}{2}}$ ß in various organs) is irrelevant because it mainly accounts for ^{125}I. It should be noted that cleaved iodide must also be present at earlier time points (0.083 and 0.5 hr), but it represents a smaller portion (~27%; page 312, vol. 1.38) of the determined levels.

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Table 8: HBW 023-^{125}I

Distribution of radioactivity in organs and tissues at different times after intravenous administration of approx. 1 mg/kg body weight to male rats

Mean values of three animals/time

(µg equivalents/g)

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	5 min	30 min	1 h	2 h	4 h	8 h
Pancreas	0.5883	0.7393	0.7446	0.5289	0.3722	0.3497
Spleen	0.3407	0.4149	0.4235	0.4056	0.3061	0.2479
Stomach	0.6528	1.3093	2.4712	2.9988	7.0244	2.8631
Small intestine	0.3854	0.6716	1.2535	1.3419	0.6254	0.8585
Large intestine	0.4924	0.5846	0.4418	0.4184	0.3953	0.3975
Kidneys I	28.2257	25.8476	15.6391	2.9096	0.6858	0.5498
Kidneys II	27.9253	27.4029	15.5296	3.3388	0.7198	0.4416
Liver	0.5265	0.4747	0.4563	0.4561	0.3490	0.2945
Heart	0.7188	0.5221	0.3901	0.3360	0.1277	0.1937
Lung	1.3954	0.9178	0.8119	0.7155	0.5033	0.3782
Stomach contents	0.0482	2.3218	5.9553	8.6781	25.2492	9.2373
Sm. intest. contents	0.1564	0.7802	2.1469	2.1678	1.3011	2.5406
La. intest. contents	0.0868	0.1538	0.2426	0.3397	0.2970	0.6934
Urinary bladder	25.6521	3.5782	7.2661	8.0863	6.0212	3.2144
Skeletal muscle	0.3136	0.2986	0.2566	0.1835	0.1233	0.1049
Subcutaneous fat	0.5777	0.6736	0.4220	0.3067	0.2698	0.2298
Retroperitoneal fat	0.5311	0.8644	0.3727	0.1812	0.1137	0.1962
Skin	0.6329	0.9898	1.1751	1.6236	1.5987	1.4735
Brain	0.1047	0.1019	0.0809	0.0761	0.0394	0.0314
Bones	0.3680	0.4965	0.3791	0.4131	0.3403	0.3062
Bone marrow	0.1341	0.0304	0.0361	0.0329	0.0235	0.0210
Thyroid I	1.1192	27.9837	95.0920	354.2053	248.5606	702.4544
Thyroid II	1.1326	36.4826	128.3365	305.8073	197.9269	790.4822
Blood	1.8790	1.3803	1.0650	0.9285	0.6741	0.5472
Plasma	3.3325	1.9692	1.2991	1.0300	0.7294	0.5660
Faeces	0.0162	0.0373	0.1148	0.1403	0.1452	0.6128

Placental Transfer in Pregnant Rats After A Single I.V. Dose

Methods: Pregnant rats were given a single i.v. dose of 1 mg/kg of ^{125}I-r-hirudin. One-half hr later rats were sacrificed. Various organs were collected and radioactivity levels were measured by

Results: Levels of radioactivity in kidneys were higher than that seen in plasma. Radioactivity was also seen in placenta, amniotic sac and fetuses.

Radioactivity Distribution in Pregnant Rats (n=3)	
Organs	**μg Equivalent/g**
Kidney	15.64 ± 0.77
Liver	0.28 ± 0.05
Uterus	0.54 ± 0.09
Blood	0.82 ± 0.13
Placenta	0.34 ± 0.62
Amniotic Sac	0.18 ± 0.06
Fetuses	0.07 ± 0.01

Data indicated that r-hirudin and/or its metabolites crosses placental barrier.

Whole-Body Autoradiography in Male Rats After S.C. (1 mg/kg) Dose (Study # 011975)

Methods: Wistar rats were given a single s.c. (1 mg/kg) dose of ^{125}I-r-hirudin. At 5 min, 1, 4 and 24 hours after drug administration rats were sacrificed and whole-body was autoradiographed to monitor radioactivity distribution.

Results: Radioactivity was distributed throughout the body. Highest concentrations were seen in thyroid, kidneys, gastrointestinal tract, urinary tract (including bladder) and in the skin. It should be noted that radioactivity was detected early in thyroid (5 min post dosing) and gastric contents and increased as a function of time indicates that deionization of the labeled compound occurs early.

METABOLISM:

Metabolism of r-Hirudin in Rats
(Study # 134.4-02)

Methods: This is not a separate study, rather urine samples collected in study # 134.4-02 were used to determine metabolism by

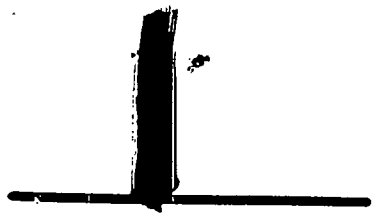
Results: No parent drug was seen in 0 - 24 hr urine sample. About 19.2% of the administered i.v. dose was excreted in urine during 0 - 24 hr period. Seven metabolites (I-50, I-52, I-56, I-53, I-51 (or 2-52), 2-51 and I-61 were identified by LC-MS methods. Metabolites I-50 (about 75% of the total urinary metabolites) and I-52 (about 24% of the total urinary metabolites) were the main metabolites in rats urine.

In another study (# 154/13), rats (3/sex/group) were given a single s.c. dose of 1 mg/kg of r-hirudin (HBW023-^{125}I). Blood samples were collected at 1 and 4 hr after drug administration. Urine and feces were collected up to 48 hr after drug administration. In all samples radioactivity were measured by and levels of the drug and its metabolites were determined by Irrespective of the sex, about 95% of the administered radioactivity was excreted in urine and about eliminated in the feces during In plasma and as well as in urine parent compound was not detected. Furthermore, most of the radioactivity in various samples were identified as ^{125}I. It should be noted that when ^{125}I is cleaved off from the ^{125}I-r-hirudin shortly after administration, then kinetic (or metabolism) study based on radioactivity is meaningless.

In Vitro Biotransformation of r-Hirudin in Kidney Fraction of Rat, Monkey and Man
(Report # 013798)

Methods: r-Hirudin (0.5 mg/ml) was incubated with 9000 g kidney fractions from rat, monkey and man. At various time intervals (rat: 0.5, 1.0 and 1.5 hr, monkey: 1.5, 2.0 and 2.5 hr and man: 3.0, 4.0 and 5.0 hr) an aliquot was removed and analyzed for r-hirudin and its metabolites by

Results: In vitro, r-hirudin biotransformed rapidly in kidney fractions of rat, monkey and man. The biotransformation in kidney fractions from monkey and man were similar and only I-62, I-63 and I-64 were identified (presumable breakdown product of carboxypeptidase), while in rat, biotransformation was extensive.

Redacted 

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HBW-023-^{125}I and Its Metabolites in Plasma and Urine After I.V. Injection.

Animals: Male and female WISKf(SPF71) rats.

Four rats (2 males and 2 females) were intravenously administered approximately 1.0 (148.3 μCi/mg) mg/kg of HBW-023-^{125}I via the tail vein; vehicle was 0.9% saline solution. Blood samples were obtained via the retrobulbar vein plexus at 0.25 and 1.0 h after injection. Six other rats (3 males and 3 females) were intravenously administered approximately 1.0 (148.3 μCi/mg) mg/kg of HBW-023-^{125}I via the tail vein; vehicle was 0.9% saline solution. Urine samples were collected for 0-5 and 5-24 h after injection.

HBW-023-^{125}I was separated from metabolites in plasma and urine specimens by Reference substances were HBW 023, iodide, and derivatives of HBW 023 shortened by either one or two amino acids at the carboxyl end; reference substances were studied in both radiolabeled and nonradiolabeled states.

Results: TLC separation data indicated that plasma specimens contained entirely HBW-023-^{125}I for up to 1 h after injection; similar data was obtained with indicated that urine samples contained mostly ^{125}I in 0-5 h samples and entirely ^{125}I in samples.

In Vivo and In Vitro Metabolism of rDNA Hirudin in Rats :
(Published report, Vol. 14, pg 73)

Methods: Male Wistar rats were injected intravenously with r-Hirudin at a dose of 1.5 mg/kg. Samples of blood and urine samples were collected at 3 hours following dosing for determination of r-Hirudin content. In the second part of the study, r-Hirudin was incubated with rat liver and kidney homogenates (1000 ng r-Hirudin/ml homogenate) at 37° C for periods of 5, 10, 15, 30, 60, 120, and 180 min or with subcellular particles (mitochondria, lysosomes, microsomes and cytosol) for 0 and 30 min. Concentrations of Hirudin were determined in samples using a chromogenic thrombin substrate assay.

Results: R-Hirudin was not detectable in plasma or tissue samples (liver, kidney, heart, lung, and muscle) at 3 hours after the injection of a 1.5 mg/kg dose, whereas approximately 13% of the administered dose was recovered in urine samples collected over the 3 hour period. In the second series of in vitro studies, r-hirudin was rapidly degraded following incubation with kidney homogenates at pH values of 4 (46% after 0.5 hours) and 8.5 (32% after 0.5 hr) and with liver homogenates at pH 4 (55% after 0.5 hr). Addition of fresh homogenates from liver and kidney induced a further degradation of r-Hirudin by about 15% within 30 min. The mitochondria and cytosol fractions had the highest enzymatic activities at pH 4.0, while at pH 8.5, degradation of r-Hirudin only occurred in kidney mitochondria and lysosome fractions.

In Vitro Degradation of Hirudin in Urine Samples and in Kidney Homogenates from Rat and Dog. (Unpublished Manuscript; Vol. 14, pg 178)

Methods: Urine samples (n=10) from rats (pH 6.2 to 9.1), dogs (pH 6.0 to 7.1), and men (pH 4.9 to 6.7) were assessed for their ability to degrade r-Hirudin (4 µg/ml) following 3, 6, and 24 hours. In addition, homogenates and cytosolic fractions from kidneys from rats and dogs were examined for their ability to degrade r-Hirudin, following incubation for 2.5, 5, 10, 15, and 30 min at 37° C, pH 4.0. Hirudin and hirudin activity equivalents in urine were measured using a

Results: At the end of 3 hours incubation, of r-hirudin was degraded in rat urine samples, whereas in dogs and humans < 3% and < 9% was degraded, respectively. After 24 hours of incubation, approximately of the r-hirudin was degraded in rat urine compared to ≤ 8% and ≤ 15.1% in dog and human urine samples, respectively. Incubation of r-Hirudin with kidney homogenates and cytosolic fractions from rats and dogs resulted in the rapid degradation of r-Hirudin by about 30% and 25%, respectively, with almost all of the degradation occurring within the first 15 min of the incubation period.

HBW-023-^{125}I and Its Metabolites in Plasma and Urine After I.V. Injection.

Animals: Two male beagle dogs (18 and 21 kg; approximately 8 years of age).

Methods: Two dogs were intravenously administered approximately and 0.6 (44.0 μCi/mg) and 1.4 (5.43 μCi/mg) mg/kg of HBW-023-^{125}I, respectively, via the saphenous vein; vehicle was 0.9% saline solution. Plasma samples, obtained at 0.25 and 1.0 h after injection in the dog receiving 0.6 mg/kg of HBW-023-^{125}I, were analyzed for HBW-023-^{125}I and its metabolites. Urine samples were collected for 0-2, 2-5, 5-8, 8-24 and 24-48 h after injection in the dog receiving 0.6 mg/kg of HBW-023-^{125}I and for 0-5 and 5-24 h after injection in the dog receiving 1.4 mg/kg of HBW-023-^{125}I; urine samples were analyzed for HBW-023-^{125}I and its metabolites.

HBW-023-^{125}I was separated from metabolites in plasma and urine specimens by Reference substances were HBW 023, iodide, and derivatives of HBW 023 shortened by either one or two amino acids at the carboxyl end; reference substances were studied in both radiolabeled and nonradiolabeled states.

Results: TLC separation indicated that plasma specimens contained entirely HBW-023-^{125}I for up to 1 h after injection; similar data was obtained with indicated that urine samples for the dog receiving 0.6 mg/kg of HBW-023-^{125}I contained entirely HBW-023-^{125}I in the 0-2 h sample and entirely ^{125}I in the 0-24 and 24-48 h samples. separation indicated that urine samples for the dog receiving 1.4 mg/kg of HBW-023-^{125}I contained mainly HBW-023-^{125}I (71%) in the 0-5 h sample and entirely ^{125}I in the 5-24 h sample.

r-Hirudin and Its Metabolites in Urine After Repeated I.V. Administration.

Animals: Wild-caught male (mean body weight of 3.6 kg; ages were not provided) and females (mean body weight of 2.4 kg; ages were not provided) cynomolgus monkeys.

Methods: Four groups of 8 monkeys (4 males and 4 females) each were intravenously administered 0, 1, 10 and 30 mg/kg/day of r-hirudin, respectively, for 13 weeks. Injections were delivered via either the saphena or cephalic veins; injections were alternated between the veins. Vehicle was sterile isotonic saline solution; dosing volume was 2 ml/kg. Urine samples were obtained during day 28 for males and day 29 for females.

Urine samples were spiked with . Determinations of r-hirudin and individual metabolites were achieved using

Results: When data were pooled for all animals, 27.3% of administered doses were accounted for in 6-h urine samples. When this 27.3% was normaliazed to 100%, 77.2% was r-hirudin, 7.1% was metabolite 1-63, 1.9% was metabolite 1-64, and 1.4% was metabolite 1-62. These metabolites result from the loss of up to 4 C-terminal amino acids.

APPEARS THIS WAY
ON ORIGINAL

EXCRETION:

Excretion of HBW-023-^{125}I After I.V. Injection.

Animals: Male and female WISKf(SPF71) rats.

Methods: Six rats (3 males and 3 females) were intravenously administered approximately 1.0 (148.3 μCi/mg) mg/kg of HBW-023-^{125}I via the tail vein; vehicle was 0.9% saline solution. Urine was collected for 24.0 h after injection.

Radioactivity in urine specimens was determined

Results: After i.v. administration of HBW-023-^{125}I, 72.88% and 71.39% of administered doses were accounted for in urine of males and females, respectively, over 24 h.

APPEARS THIS WAY
ON ORIGINAL

Involvement of Glomerular Filtration and Tubular Reabsorption in the Renal Elimination of r-Hirudin in Rats. (Unpublished Manuscript; Vol. 14 pg 201)

Methods: In order to investigate the role of glomerular filtration and tubular reabsorption in the renal elimination of r-hirudin, r-Hirudin was injected intravenously into rats at doses of 0.5, 1.0, 2.5, 5.0, 50, and 100 mg/kg. In additional studies r-hirudin (0.5 mg/kg, i.v. bolus) was injected in combination with aprotinin at i.v. bolus doses of 10 and 20 mg/kg. Twenty-four hour urine samples were collected and the urinary content of r-hirudin were determined. In other experiments, rats were intravenously injected with bolus doses of [^{125}I]-r-Hirudin at 0.1 and 1.0 mg/kg with Inulin ^{14}C used as a Urinary concentrations of r-Hirudin were determined using a

Results: Administration of increasing i.v. doses of r-Hirudin resulted in dose-dependent increases in the urinary excretion of r-Hirudin from of the administered dose at the 0.5 and 100 mg/kg doses, respectively. However, revealed only about 1.6% of the parent compound in urine. Coadministration of aprotinin with the 0.5 mg/kg dose of r-Hirudin resulted in a increased excretion of the 0.5 mg/kg dose to about 6% of the administered dose. Finally in experiments where inulin was used as a filtration marker, approximately 5% of the 0.1 mg/kg administered dose was excreted within 25 min of dosing, whereas approximately 20% of a higher dose of 1.0 mg/kg was eliminated in 25 min. Injection of 0.1 and 1.0 mg/kg doses into rats which were previously injected with 1.0 and 0.1 mg/kg doses, respectively, resulted in an approximate doubling of the urinary excretion of the dose injected last and was correlated with an increased excreted filtered load.

APPEARS THIS WAY
ON ORIGINAL

Collectively, these data suggest that glomerular filtration as well as tubular reabsorption are involved in the renal excretion of r-Hirudin. Its, reabsorption appears saturable, with very small amounts of the parent compound excreted in the urine. Aprotinin induced interference with the reabsorption of r-Hirudin also suggests an interaction at the tubular level.

APPEARS THIS WAY
ON ORIGINAL

Urinary and Biliary Excretion of HBW023 in Rats
(Study # R-Hro-1994-HBW023-03)

Sponsor has conducted two experiments:

APPEARS THIS WAY
ON ORIGINAL

Experiment 1:

Methods: Conscious rats (8 males/group) were given a single i.v. bolus dose of 0.5, 1, 2.5, 5, 50 or 100 mg/kg of r-hirudin. At twenty-four hours urine samples were collected and levels of r-hirudin were measured by three different methods

Results: Urinary excretion of r-hirudin increased dose-dependently from of the administered dose when measured by In contrast, and of the administered dose were excreted in the urine when r-hirudin levels were determined by methods respectively

Experiment 2:

Methods: Cannulated rats (n=7) were given a single i.v. dose of r-hirudin (0.5 or 5 mg/kg). Urine and bile samples were collected hourly for up to 4 hours. Samples were analyzed

Results: Summary report indicated that about and of the administered dose were excreted in the urine and bile respectively.

APPEARS THIS WAY ON ORIGINAL

Excretion of HBW-023-^{125}I After I.V. Injection.

Animals: Two male beagle dogs (18 and 21 kg; approximately 8 years of age).

Methods: Two dogs were intravenously administered approximately 0.6 (44.0 μCi/mg) and 1.4 (5.43 μCi/mg) mg/kg of HBW-023-^{125}I, respectively, via the saphenous vein; vehicle was 0.9% saline solution. Urine and feces were collected for 96 h after injection.

Radioactivity in urine and feces specimens was determined by a gamma counter.

Results: After i.v. administration of HBW-023-^{125}I, 83.9% and 90.1% of administered doses were accounted for in urine after administration of 0.6 and 1.0 mg/kg, respectively, over 96 h; 1.2% and 0.9%, respectively, in feces.

APPEARS THIS WAY ON ORIGINAL

Plasma Levels of r-Hirudin in Rats With LPS-Induced DIC and in Untreated Rats After I.V. Infusion
(Study # DN-LPS/DIC-HIRI-92)

Methods: r-Hirudin (0.125 mg/kg/hr for 4 hr) was infused in normal and DIC (LPS-induced) rats. Blood samples were collected from retroorbital vein at 5, 10, 15, 30, 60, 120 and 180 min after the termination of the infusion. r-Hirudin in each samples was measured by

Results: In control rats, r-hirudin levels in plasma were 204 ± 36 ng/ml at 5 min post infusion and reached to below detection limit at 2 hr after the termination of the infusion. In DIC rats, r-hirudin levels in plasma were 347 ± 102 ng/ml at 5 min post infusion and 190 ± 66 ng/ml at 3 hr after the stoppage of infusion. This increase level of r-hirudin in DIC rats most likely is related to impaired renal function in DIC rats.

I.M. and S.C Pharmacokinetics Study with Two Different Formulations of r-Hirudin in Rats: (Report No. V-708.1)

Methods: Four groups of rats (3/group) were administered r-DNA-Hirudin via i.m. or s.c. injection at dose levels of 10 mg/kg. For each route of administration r-Hirudin was dissolved in either 0.05 M Tris, 0.1 M NaCl, 0.1% Tween 80 (T/N/T) pH 7.5 or in 0.2 M Arginine, 0.2 M lysine, 0.1% Tween 80 (A/L/T), pH 7.5 buffer solution and administered at volumes of 1 ml/kg. Blood samples were collected via the retro-orbital sinus at 0, 0.5, 1, 2, 3, 4, 5.5, 6.5, 7, and 7.5 hours after application for determination of r-Hirudin concentrations by means

Results: Mean maximal plasma concentrations of 27.63 µg/ml (T/N/T) and of 23.88 µg/ml (A/L/T) occurred between 0.5 and 2.0 hours following i.m. injection whereas maximal plasma concentrations of 22.3 µg/ml (T/N/T) and of 6.94 µg/ml (A/L/T) were observed between 0.5 and 1 hours following s.c. administration. AUC values (available for only one animal in each of the i.m. groups) were 107.81 µg/ml·hr in the T/N/T i.m. group and 37.19 µg/ml·hr in the A/L/T i.m. group. Corresponding AUC values in the groups which received the s.c. injections (mean of 2 animals in each group) were 51.38 and 9.00 µg/ml·hr in the T/N/T and A/L/T groups, respectively. These limited results suggest a lack of differences in the maximal plasma concentrations observed following i.m. injection between the two formulations tested. However, both maximal plasma levels and AUC values for the were less than that of the T/N/T formulation following s.c. administration.

In Vitro Plasma Protein Binding in Man, Monkey, Dog and Rat
(Study # 013810)

APPEARS THIS WAY ON ORIGINAL

In vitro the plasma protein binding of r-hirudin was determined over the concentration in man, rat, monkey and dog. The plasma protein bindings in rat, dog, monkey and man were

In Vitro Plasma Protein Binding in Monkey and Human
(Study # R-61-019-616-01)

APPEARS THIS WAY ON ORIGINAL

Methods: In earlier protein binding study in which was used, the plasma protein binding was close to 80% in rat, dog, monkey and man. In the present study equilibrium dialysis method was used to determine protein binding.

Results: The plasma protein binding in monkey and man were 4% and 3% respectively. In this study plasma protein binding in non-primate (rat and dog) was not determined.

Since contradictory results were seen in studies # 013810 and # R-61-091-616-01 with respect to plasma protein binding, no conclusion shall be made at this time.

APPEARS THIS WAY
ON ORIGINAL

Absorption, distribution, metabolism and excretion studies were conducted in rats, rabbits, dogs and monkeys. Irrespective of the species $t_{\frac{1}{2}}$ ß is <2 hr (man = 1.3 hr), clearance ranged from and volume of distribution ranged . In rats, administered radio-activity was distributed throughout the body, and levels in kidneys, urinary bladder and thyroid were higher than that seen in plasma. r-Hirudin and/or its metabolites crosses placental barrier. In rats seven urinary metabolites were identified (I-50, I-52, I-56, I-53, I-51, 2-51 and I-61). I-50 and I-52 were major metabolites. In vitro, r-hirudin biotransformed rapidly in kidney 9000 g fraction of rat, monkey and man. The biotrans-formation in kidney fraction from monkey and man were similar and only I-62 (monkey), I-63 and I-64 were identified (presumable breakdown product of carboxypeptidase). Irrespective of the species, excretion is mainly via renal route and fecal excretion is negligible.

APPEARS THIS WAY
ON ORIGINAL

TOXICOLOGY:

Acute Toxicology:

Acute Toxicity Studies in Rats (i.v. and s.c.), Mice (i.v. and s.c.), and Monkeys (i.v.). (Report Nos. 134-12, 134.2-12, 134.2-12.1 and 134.4-12, i.v. in rats and 134-12.1, s.c. in rats; 134-11 and 134.2-11 and 134.4-11, i.v. in mice and 134-11.1, s.c. in mice; and 134.4-17.1, i.v. in monkeys)

Methods: Acute single dose i.v. and s.c. toxicity studies with r-Hirudin were conducted in mice and rats and an acute i.v. toxicity study was conducted in monkeys. In 3 acute i.v. toxicity studies in mice (5/sex/dose level), r-hirudin was tested at doses of 0, 0.09, 0.9, 1, 9, 10, 45, 90, 100, and 1000 mg/kg, whereas in the acute s.c. toxicity study in mice (5/sex/dose level), doses of 0, 250, 500, and 1250 were tested. In 4 acute i.v. studies conducted in rats (5/sex/dose level) r-Hirudin was tested at single i.v. bolus doses of 0, 1, 10, 100, and 1000 mg/kg, whereas in the s.c. studies in rats (5/sex/dose level), doses of 0, 50, 100, and 500 mg/kg were tested. Finally, in the acute i.v. toxicity study in monkeys (1/sex/dose level), r-Hirudin was tested at doses of 0, 1, 10, and 100 mg/kg. Rats mice and monkeys in all acute studies were evaluated for compound related

effects on mortality, clinical observations, changes in body weights over a 14-day observation period. All rats and mice underwent gross pathological analyses at the end of the observation period. However, gross pathological analyses were not conducted on monkeys due to "animal protection" and the apparent lack of clinical evidence of toxicity.

Results: Table 6 summarizes the findings from acute i.v. and s.c. toxicity studies in mice and rats and from an acute i.v. toxicity study in monkeys.

APPEARS THIS WAY
ON ORIGINAL

Table 6. Summary of preclinical toxicity studies conducted in mice, rats and dogs.

Species (No./sex)	Route	Doses Tested (mg/kg)	Minimum Lethal Dose (mg/kg)	Maximal Nonlethal Dose (mg/kg)
Mice:				
(5/sex/group)	i.v.	0.09, 0.9, 9, 45, and 90	N.L.O.	90
(5/sex/group)	i.v.	10, 100, 1000	N.L.O.	1000
(5/sex/group)	i.v.	1, 10, 100	N.L.O.	100
(5/sex/group)	s.c.	250, 500, 1250	N.L.O.	1250
Rats:				
(5/sex/group)	i.v.	1, 10, 100	N.L.O.	100
(5/sex/group)	i.v.	10,100,1000	N.L.O.	1000
(5/sex/group)	i.v.	10,100,1000	N.L.O.	1000
(5/sex/group)	i.v.	1, 10, 100	N.L.O.	100
(5/sex/group)	s.c.	50, 100, 500	100	50 TA
Monkeys:				
(1/sex/group)	i.v.	1, 10, 100	N.L.O.	100

N.L.O. = No Lethality Observed

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ON ORIGINAL

In the acute i.v. toxicity studies in rats and mice, r-Hirudin, at doses ranging produced no mortality, clinical signs of toxicity, or gross pathological effects. R-Hirudin also had no effects on body weights in rats, but produced suppressed body weight gains in mice (both sexes, at 1000 mg/kg, 88.5% in males and complete suppression in females) on Day 2 only relative to gains of 0.8 and 1.2 g in control males and females, respectively. In monkeys, i.v. doses also produced no mortality, adverse effects on body weights, or other clinical signs of toxicity, but was associated with dose-dependent effects on coagulation parameters (i.e. TEG r increased up to 9000 sec from basal range of TEG r+k increased up to up to 9000 sec from basal range of , and maximum amplitude decreased to 1 mm from basal

values of TT increased to 300 sec from basal range of ; and PTT, increased to 300 from a basal range of). Results from acute s.c. toxicity studies in mice and rats showed that r-hirudin produced common clinical signs of toxicity which included dose-dependent incidence of swelling and/or nodule formation at the injection sites at doses ≥ 500 mg/kg in mice and at all doses tested in rats. One male mouse treated at the 1250 mg/kg high dose also showed hunched posture and body weight loss (attributable to bleeding) on Day 1. Effects on body weights in rats were limited to Day 1 weight losses of 0.9 g and 2.5 g in one high dose male and in 1 mid dose female, respectively. Treatment-related mortality in the s.c. studies was limited to 1 of 5 male rats at the 100 mg/kg s.c. dose which died on day 1 due to excessive hemorrhage. Gross pathological findings in mice included: hemorrhages in the lungs (1 of 5 females at the 500 mg/kg s.c. dose and in 1 of 5 mice/sex at the 1250 mg/kg s.c. dose). In rats, the male which died at the 100 mg/kg s.c. dose showed marked hemorrhagic effects at the site of injection and clay colored kidney and heart indicative of excessive hemorrhage. In addition, hemorrhage and or blood filled nodules at the site of injection were observed 1 other male and 4 of 5 females at the mid dose, and in 1 of 5 males, but no females at the 500 mg/kg high dose. Thus, maximum nonlethal i.v. doses were 1000 mg/kg in rats and mice and 100 mg/kg in monkeys, whereas maximum nonlethal s.c. doses were 1250 in mice and 50 mg/kg in rats. Minimum lethal i.v. doses in mice, rats, or monkeys, and minimum lethal s.c. doses in mice were not established, whereas the minimum lethal s.c. dose in rats was 100 mg/kg.

Subacute/Subchronic Toxicology:

APPEARS THIS WAY
ON ORIGINAL

Rat:

Intravenous Subacute (Bolus + 72 hr Infusion) Toxicity Study in Rats. (Study No. 11589 TSR)

Study Started: April 12, 1994

Study Completed: January 24, 1995

APPEARS THIS WAY
ON ORIGINAL

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

Animals: Sprague Dawley Crl:CD(SD) BR rats; 10 weeks of age; mean body weights of 359 g for males and 246 g for females.

Drug Batch No.: 616114011

Methods: Two groups of rats (10/sex/group) were administered r-hirudin (dissolved in isotonic saline) via bolus i.v. injections at doses of 0.2, and 0.4 mg/kg in a volume of 5 ml/kg, followed by 72 hour i.v. infusions with r-hirudin at doses of 0, (vehicle 0.9% isotonic saline) 0.1 and 0.15 mg/kg/hr at an infusion rate of 1 ml/kg/hr (i.e. maximum total daily doses of 2.6 and 4.0 mg/kg). A separate group of control animals (10/sex) received equal volumes of vehicle (isotonic saline) alone. The basis of dose selection was not indicated. Following dosing rats underwent an 11 day observation period in which they were examined daily for mortality and clinical signs of toxicity.

Body weights were determined at predose, day 1 of dosing, and twice per week until the end of the observation period and food consumption was recorded twice weekly. Hematological and blood chemistry were determined in samples obtained via the orbital sinus at pretreatment, at the end of the treatment period (Day 4) and at the end of the observation period (Day 14) and urinalysis was performed on 18 hour samples obtained at the end of the observation period (Day 14). Animals which died or were sacrificed at the end of the observation period underwent complete gross examinations with determination of organ weights for adrenal, brain, heart, kidneys, liver, ovaries, spleen, testes, and thymus. Microscopic examinations were also conducted on all tissues showing macroscopic lesions in the low dose group and in all rats in the control and high dose groups.

Results:

Observed Effects: Possible treatment-related clinical signs of toxicity included: pallor of the extremities and eyes and a blackish abdomen (1 of 10 males at the 0.10 mg/kg/hr dose) and chromorhinorrhea and/or chromodacryorrhea in 2 of 10 males at the 0.15 mg/kg/hour dose (from Day 10). In addition, one of 10 females at the 0.15 mg/kg/hour dose showed pallor of the extremities and eyes, dyspnea, chromorhinorrhea, chromodacryorrhea, piloerection and round back from day 4 or 5.

Mortality: One control male died on day 9 and one 0.10 mg/kg/hr male was found dead on day 5. The latter male showed signs of pallor of the extremities and eyes and blackish abdomen prior to death.

Body Weight/Food Consumption: At the end of the treatment period control male and female rats weighed 423 ± 26.6 g and 288 ± 14.6 g, respectively. These values were not significantly different from groups treated with r-Hirudin. Similarly, r-hirudin had no adverse effects on food consumption.

Hematology/Bone Marrow: Females at the 0.15 mg/kg dose showed a slight increase in APTT (22%), relative to mean control values of 17.2 ± 1.95 sec on day 4 at the end of the infusion period. Otherwise, r-Hirudin produced no treatment-related hematological effects in mice at the doses tested herein.

Blood Chemistry: No treatment-related hematological effects were observed.

Urinalysis: Urine samples taken at the end of the observation period showed no drug-related effects.

Physical Examinations/Ophthalmic and ECG Examinations: Ophthalmic examinations were not indicated.

Organ Weights: Slight increases in absolute (18%) and relative weights (16.6%) for heart were observed for females treated at the 0.15 mg/kg/hr doses.

Gross Pathology: A limited incidence of abscess formation at the site of infusion and/or in the subcutaneous tissue was noted in both control and treated rats at similar incidence. Injection site findings not observed in control animals included: grayish nodules in 2 of 10 high dose (0.15 mg/kg) females and a hematoma in 1 of 10 low dose (0.10 mg/kg/hr) females. Other findings including enlarged lymph nodes (inguinal and iliac) were noted in a limited number of females (1-3 female rats/group) in all groups. Finally, a low incidence of enlarged spleen was seen in 1 rat/sex in the control group and in 1 male and 3 of 10 females at the high dose. The aforementioned findings did not appear to be specifically related to r-hirudin, but rather to mechanical injury associated with the infusion technique.

Histopathology: Histological correlates to gross observations at the injection site included: phlebitis, thrombophlebitis, and necrosis of the venous wall; perivenous collagen degradation, hemorrhage, fibroplasia, and inflammatory cell infiltration; and finally abscess formation at the site of injection and in the adjacent subcutaneous tissue were seen in both control and treated groups. In general, the incidence of injection site lesions was comparable between control and treated rats and thus, appeared related to mechanical injury associated with the infusion technique. Other histological findings including: plasmacytosis and/or histiocytosis of the lymph nodes occurred at equal incidence in control and treated animals and thus were not considered treatment-related.

Plasma Levels of the Drug: Measurement of plasma drug levels was not indicated.

In conclusion, administration of r-hirudin to Rats by bolus injection (0.2 or 0.4 mg/kg) immediately followed by a 72-hour infusion at 0.10 or 0.15 mg/kg/hr was well tolerated at both doses tested (i.e. maximum total daily doses of 2.6 and 4.0 mg/kg), with no evidence of test article-related toxicity and no target organs of toxicity identified. The 4.0 mg/kg dose was the no effect i.v. dose for the study.

One-Month Intravenous Toxicity Study in Rats. (Study No. 88.1049)

Study Started: July 18, 1988

Study Completed: January 24, 1989

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

Animals: Wistar rats, Hoe:WISKf(SPF171) about 5-6 weeks of age; males weights 108 g and female weights 105 g.

Drug Batch No.: U001

Methods: Two groups of rats (5/sex/group)were administered r-hirudin (dissolved in isotonic saline) at daily i.v. bolus doses of 10 and 100 mg/kg in a volume of 5 ml/kg (injected at a rate of 2 ml/min) for a period of 30 days. A separate group of control rats (5/sex) received equal volumes (5 ml/kg) of vehicle (isotonic saline) alone. Doses were selected to represent approximately 10 and 100 fold increases over the intended therapeutic dose (i.e. approximately 1 mg/kg). Rats were examined daily for mortality and clinical signs of toxicity. Body weights and food consumption were determined twice per week. Hematology and blood chemistry were performed on samples (volume not indicated) obtained via the sublingual vein at the end of the study. Urinalysis was also conducted at the end of the study. Animals were sacrificed on the day after the last day of dosing and underwent complete gross examinations, with determination of organ weights for heart lungs, liver, kidneys, spleen, adrenals, testes or ovaries, thyroid, pituitary and brain. Complete histological examinations were also conducted.

Results:

Observed Effects: Females at the 100 mg/kg dose showed thickening and bluish-red to dark blue discolorations of the tail, and increased post injection bleeding toward the end of the study. Necrosis of the tail in one 100 mg/kg high dose female was also observed.

Mortality: There were no intercurrent deaths.

Body Weight/Food Consumption: At the end of the treatment period control male and female rats weighed 248 ± 14 g and 170 ± 8 g, respectively. These values were not significantly different from groups treated with r-Hirudin. Similarly, r-hirudin had no adverse effects on food consumption.

Hematology/Bone Marrow: Treatment-related hematological changes included: in males at the 10 and 100 mg/kg doses; slight reductions in erythrocytes (7.5 and 6.7%, respectively, relative to control values of 7.86 x 10^9/ml) and in females at the 100 mg/kg dose; moderate reductions in erythrocytes (31%, relative to control values of 7.82 x 10^9/ml), Hb (27%, relative to control values of 146 g/l) and Hct (26%, relative to control values of 45%) and increased reticulocyte counts (3 fold, relative to control values of 20%).

Blood Chemistry: No changes in serum chemistry attributable to treatment were observed.

Urinalysis: An increase incidence of hemoglobin in the urine was observed in rats treated at the 100 mg/kg dose (3 of 5 males and 2 of 5 females, versus 1 of 5 males and no females in the control group, respectively). No adverse effects on other urinalysis parameters were observed.

Physical Examinations/Ophthalmic and ECG Examinations: No adverse effects on physical parameters or on the eyes were reported.

Organ Weights: Treatment-related changes in organ weights included increases in absolute weights (34 and 50%) and relative weights (30 and 44%) for spleen in females at the 10 and 100 mg/kg doses, respectively, with less dramatic increases also observed in males (up to 20%, for both indices at the 100 mg/kg dose).

Gross Pathology: Macroscopic observations of enlarged and reddened iliac lymph nodes were observed in 1 of 5 males and in 3 of 5 females at the 100 mg/kg high dose. One of the aforementioned females also showed thinned and light colored blood and a light colored liver grossly.

Histopathology: Microscopic findings included: sinal catarrh (i.e. mature cellular sinus histiocytosis) of the lymph nodes in the iliac lymph nodes at both the 10 mg/kg (1 of 5 males and 4 of 5 females) and 100 mg/kg doses (4 of 5 males and all 5 females). The high dose female which had necrosis of the tail also presented with hematopoiesis of the liver, spleen, and bone marrow.

Plasma Levels of the Drug: Plasma drug levels were not determined.

In conclusion, administration of r-hirudin to rats by bolus i.v. injection (10 or 100 mg/kg) for a period of 30 days was well tolerated, with no mortality or effects on body weights or food consumption observed. Observed effects on spleen; increased weights (both doses) and hematopoiesis in the spleen and liver (one high dose female), along with enlarged iliac lymph nodes (sinal catarrh) coincided with increased bleeding and probably reflect secondary changes due to the hemorrhagic effects of r-hirudin. As such no target organs of toxicity were identified. With the exception of the findings of the sinal catarrh of the lymph nodes, the 10 mg/kg dose could be considered the no effect dose for the study which provides an adequate margin of safety for the proposed clinical dose of 0.4 mg/kg.

13-Week I.V. Toxicity Study in Rats
(Study # 134.4-52)

Study Started: January 30, 1995

Study Completed: November 29, 1995 (report date)

GLP Requirements: A Statement of Compliance with GLP regulations was included.

Animals: Wistar (Crl:(WI)BR) rats (about 6 weeks old;

Drug Batch No.: 115011

Methods: Groups of rats (12/sex/group) were given daily i.v. doses of 1, 10 and 100 mg/kg/day of r-hirudin for 91 days. The control group rats were given vehicle (saline) in similar fashion. The volume of administration was fixed at 2 ml/kg.

Additionally, 6 rats/sex were included in control and high dose groups and used for 30-day recovery study. All rats were observed for clinical signs daily. Body weights and food intakes were recorded weekly. Blood samples were collected at pre-test, before third treatment in week 2, 5 and 9, 24 hr after the last dose and at the end of 30-day recovery period for hematology and serum chemistry tests. Ophthalmic examinations were performed on all rats at pre-test, after week 4 and 12 of the study and at the end of the study period. At the end of study/recovery period all rats were sacrificed and subjected to complete necropsy. Histopathological examinations were limited to control and high dose groups and all gross lesions. Furthermore, if microscopic changes were seen in high dose group then examination was extended to the next lower dose.

Results:

1. Observed Effects: Hair loss and eschar formation was seen in one control rat, one low dose treated rat and 3 high dose treated rats.

2. Mortality: A total of 12 rats (one male from control group, one male and one female from mid dose group and 4 males and 5 females from high dose group) died or killed in moribund state during study period. Cause of death in mid and high dose groups were most likely treatment related (hemorrhages).

3. Body Weight/Food Consumption/Water Consumption: In males body weight gains were decreased by 7.9%, 5.7% and 8.4% at low, mid and high dose respectively, when compared to control values (control: mean initial body weight = 169.95 g and mean final body weight = 473.2 g). In females body weight gains were decreased by 11.5% and 7.6% at mid and high dose respectively (control: mean initial body weight = 147.4 g and mean final body weight = 272.65 g). Food intakes were not affected by the treatment (control: mean daily food consumptions were 24.2 g/rat/day for males and 17.5 g/rat/day).

4. Hematology/Coagulation/Bone Marrow: No treatment related effects were seen except fibrinogen levels were increased by 16.9% and 23% in males and 9.0% and 57% in females at mid and high dose respectively, compared to their respective control values.

5. Blood Chemistry/Urinalysis: No treatment related effects were seen except, A/G ratio were decreased by 15% and 27% in high dose treated males and females respectively, compared to control values.

6. Vital Signs/Physical Examination/Ophthalmic Examination: No treatment related effects were seen.

7. Organ Weights: No treatment related effects were seen.

8. Gross Pathology: Enlarged and reddened lymph nodes were seen in some of the mid dose (5/24) treated rats and in most of the high dose treated rats (18/24). Anemia, hemoperitoneum and/or hemorrhages were seen in treated rats which died/killed during the course of the study period.

9. Histopathology: Histopathological examinations revealed dose dependent decrease n hemosiderin deposits in spleen (males: control = 11/12, low dose = 10/12, mid dose = 6/12 and high dose = 2/12; females: control = 12/12, low dose = 12/12, mid dose = 11/12 and high dose = 4/12) and increase erythropoiesis in spleen of high dose treated male rats (control = 2/12, low dose = 2/12, mid dose = 3/12 and high dose = 6/12). Increased erythropoiesis was also seen in bone marrow (both sexes: control = 0/24, low dose = 0/23, mid dose = 1/23 and high dose = 7/23). Hemorrhage and inflammatory reaction at the injection sites with sinushistiocytes in the iliac lymph node were seen in treated rats (incidences and severity increased with increasing dosages). Some of the above mentioned findings were still present at the end of recovery period. The above mentioned histopathological findings are related to exaggerated pharmacodynamic effect of the drug.

In this study no target organ of toxicity was identified. Low dose level can be considered as well tolerated dose. Dose levels ≥10 mg/kg/day produced lethality due to exaggerated pharmacological effects (hemorrhages).

One Month S.C. Toxicity Study in Rats
(Study # 134.4-42)

Testing Laboratories: Behringwerke AG
Marburg, Germany

Study Started: June 26, 1996

Study Completed: November 21, 1996 (report date)

GLP Requirements: A Statement of Compliance with GLP regulations was included.

Animals: Wistar (Crl:WI BR) rats (about 6-7 weeks old.

Drug Batch No.: 118011

Methods: Groups of rats (20/sex/group) were given s.c. doses of 1, 10 and 100 mg/kg/day for 28 consecutive days. The control group rats received isotonic saline in similar fashion. The volume of administration was fixed at 2 ml/kg. All rats were observed daily for clinical signs and mortality. Body weights and food intakes were recorded weekly. Blood samples were collected at pre-test, before first treatment in week 2 and 24 hr after the last treatment for hematology and blood chemistry tests. Ophthalmic examinations were performed on all rats at pre-test and just before sacrifice. At the end of treatment period rats were sacrificed and subjected to complete necropsy. Histopathological examinations were limited to control and high dose groups and all gross lesions.

Results:

1. **Observed Effects**: Hematoma and swelling were seen in all treated rats.

2. **Mortality**: One female from mid dose group and 3 males and 3 females from high dose group died/killed during the study period. These deaths were treatment related.

3. **Body Weight/Food Consumption/Water Consumption**: At high dose body weight gains were reduced by 32% and 19.5% in males and females respectively, when compared to control values (control mean initial and final body weights in males were 152.4 g and 329.9 g respectively and in females the corresponding weights were 156.9 g and 220.7 g respectively). Food intakes were not affected by the treatment (control: mean daily food consumptions were 23.2 g/rat/day for males and 17.45 g/rat/day)

4. **Hematology/Bone Marrow**: In both sexes at high dose, decreases in red blood cells , hemoglobin and hematocrit were seen. In high dose treated males leucocytes counts were increased by 36%.

5. **Blood Chemistry/Urinalysis**: Increase in thrombin time (males: control = 50.5 sec, low dose = 57.0 sec, mid dose = 64.1 sec and high dose = 59.0 sec; females: control = 47.5 sec, low dose = 57.7 sec, mid dose = 123.6 sec and high dose = 53.5 sec). At high dose serum fibrinogen levels were increased by 23% in males.

6. **Ophthalmic Examinations**: No treatment related effects were seen.

7. Organ Weights: At high dose spleen and thymus weights were increased by respectively (both sexes).

8. Gross Pathology: Enlarged and reddened lymph nodes, hemorrhages in thymus and injection sites were seen in some of the mid and most of the high dose treated rats.

9. Histopathology: Hemorrhage in thymus, injection sites were seen in all treated rats. Additionally, slight increase in erythropoiesis in 1 rat from mid dose group and 2 rats from high dose group were seen. Slight to moderate hemorrhages in all high dose treated rats and 4/20 mid dose group rats were also seen. One high dose treated rat had hemorrhages in skeletal muscle and large intestine.

In this study no target organ of toxicity was identified. Low dose level can be considered as well tolerated dose. Dose level ≥10 mg/kg/day produced lethality due to exaggerated pharmacological effects (hemorrhages).

3-Month Subcutaneous Toxicity Study with r-Hirudin in Rats.
(Study No. 89.0648)

Testing Laboratories: Pharma Research, Toxicology and Pathology
Hoechst AG, D-6203 Frankfurt(M) 80

Study Started: June 27/29 1989

Study Completed: May 7, 1990

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

Animals: Immature Wistar Rats; Hoe::WISKf(SPF71); about 5-weeks of age; Males (107 g) and Females (99 g).

Drug Batch No.: C 003

Methods: Groups of Wistar rats (15/sex/group) were administered either vehicle (2 ml physiological saline) or r-Hirudin dissolved in physiological saline and administered subcutaneously at doses of 0.4, 2.0, or 10 mg/kg in volumes of 2 ml/kg daily for 90 days. Five rats/sex from all groups underwent an additional 29-day recovery period. The 10 mg/kg was selected as the high dose for the study since it was reportedly the maximum administrable dose, determined on the basis of hematomas formed at the site of injection (Note: the referenced study for the basis of dose selection was not cited). Animals were observed daily for mortality and clinical signs of toxicity, with body weights and feed consumption determined twice weekly. Venous blood samples (taken from sublingual vein, volume not indicated) were collected from 10 rats/sex after about 7 weeks and at the end of the study and after recovery of 29 days for analysis of hematology (including coagulation parameters). After about 2 months,

sublingual venous blood samples (volume not indicated) were obtained at 10, 30, and 60 min, or 6 and 24 hours after dosing for determination of glucose, GOT, GPT, alkaline phosphatase, and levels of r-Hirudin. Other vena cava blood samples (volume not indicated) were also taken at the time of sacrifice (at the end of 90 days and at the end of the recovery period) for analysis of clinical chemistry values. Urinalysis was conducted on overnight samples collected after 7 weeks of dosing and at the end of the experiment. At either the end of treatment or the end of the recovery, animals were sacrificed and underwent complete gross and histological examinations, with determination of absolute and relative organ weights.

Results:

Observed Effects: Hematomas and/or marked swellings due to hematomas and discharge of blood in the area of the injection sites were observed at the 2.0 and 10.0 mg/kg doses, with pale eyes due to excessive hemorrhaging also seen occasionally at the 10.0 mg/kg high dose.

Mortality: Two of 15 rats/sex died at the 10.0 mg/kg dose (each after 51 to 55 doses), with death attributable to excessive hemorrhagic effects which occurred subsequent to blood withdrawal.

Body Weight/Food Consumption: At the end of the treatment period control male and female rats weighed 416 ± 28 g and 183 ± 15 g, respectively. These values were not significantly different from groups treated with r-Hirudin. R-Hirudin also had no adverse effects on food consumption.

Hematology/Bone Marrow: Hematological changes included: treatment-related reductions in erythrocytes, HB and Hct in 10 mg/kg high dose males at the intermediate time point and at the end of the study and in 10 mg/kg high dose females at the end of the study. Compensatory increases in reticulocytes were also observed in high dose males at the intermediate time point (64%) and in males at all doses and high dose females (3.35 times control values) at the end of the study. No differences in the various coagulation factors (always measured at 24 hours after the last dose) were observed, with the exception of thrombin times which showed a slight, but dose-dependent increase (up to 9% in males and 26% in females at the 10 mg/kg dose).

Blood Chemistry: Treatment related changes in clinical chemistry were limited to a increased values for inorganic phosphorus (27.4%) in the 10 mg/kg high dose males at the end of the study.

Urinalysis: No drug related effects were observed.