CENTER FOR DRUG EVALUATION AND RESEARCH

Application Number 21-271

PHARMACOLOGY REVIEW(S)

PHARMACOLOGIST'S REVIEW OF NDA 21-271
(Amendment # B2 dated October 11, 2000)

SPONSOR & ADDRESS: Aventis Pharmaceuticals Inc.,
Parsippany, NJ.

REVIEWER: Yash M. Chopra, M.D., Ph.D.
Pharmacologist, HFD-180

HFD-180 RECEIPT DATE: October 12, 2000

DATE OF REVIEW: November 30, 2000

DRUG: Desirudin/ ——— Injection

PHARMACOLOGICAL CATEGORY: Antithrombotic Agent

SUBMISSION CONTENTS: Sponsor's response to the preclinical requests: (1) 1-month subacute s.c. toxicity study in monkeys and, (2) Batch # (by the method production) used in preclinical studies submitted with the initial application.

These are reviewed below by first giving the Agency request followed by the sponsor's response and Reviewer's Comments:

Agency Request 1: Conduct a subacute 1-month sc toxicology study in monkeys and provide a final report of the study for review and evaluation. In this study, desirudin should be administered sc twice daily and at least 3 doses should be tested with 4 animals/sex/dose group. Please monitor the toxicokinetic and immunogenicity of desirudin.

Sponsor's Response: Desirudin was used by subcutaneous route in over 2100 patients in Europe and there was sufficient data on the immunogenicity potential of the drug. In submitted summaries of clinical study #RH/A02 (conducted in 1989) and 3 Phase I clinical studies (RH/ET6 by subcutaneous route, RH/ET5 and RH/ET14 by intravenous route of administration), Hirudin specific antibodies IgE, were detected in the study conducted by subcutaneous route of administration at 2 occasions and 1 month apart. In studies # RH/ET5 and RH/ET/6, three out of 200 volunteers on desirudin showed allergic reactions with the 2nd dose of given 28 days apart. In a few line summary report on the comparison of skin reactions produced by lepirudin and Bivaluriden (different from desirudin by only

2 N-terminal amino acids), low immunogenicity reaction was developed by these compounds. In 3-month subchronic iv toxicity study in dogs, the common adverse reactions were bleeding; non-dose dependent immune related arteritis. These reactions were not observed in 3-month rat study. The special immunogenicity studies in the rabbit, dog, baboon and guinea pig indicated a low titre of specific antibodies in dogs but no titre in rabbits and baboons. None of the guinea pigs showed anaphylaxis in the absence of Freund's complete Adjuvant (FCA). Based on these observations, sponsor had asked if the available clinical data was sufficient to replace the requested 1-month toxicity study in monkeys.

Reviewer's Comments: In the submitted NDA, desirudin has been recommended at a subcutaneous dose of 0.3 mg/kg (6034.2 antithrombin units/kg) twice a day or i.e., 15 mg bid (based on 50 kg body weight of an adult human) at least 5 to 15 min before surgery and postoperatively for 9 to 12 days or till the patient becomes fully ambulatory. To support the clinical trials and marketing of the compound for 9 to 12 days duration, 1-month toxicity studies in rodent and non-rodent species are needed. The 1-month toxicity in rats was submitted earlier and acceptable but 1-month toxicity study in non-rodent species was not conducted/submitted by sponsor. Sponsor was asked to conduct 1-month sc toxicity study in monkeys by selecting the same route and frequency of administration of desirudin as proposed in the clinical application, i.e., desirudin should be administered subcutaneously and in 2 daily divided doses. The submitted human imunogenicity data from European clinical trials could not give information on the general toxicity of the compound in the animals. Thus the data could not replace the requested study. Sponsor should again be requested to conduct the suggested study. The toxicokinetics and immunogenicity potentials of the compound should also be monitored during the study.

Agency Request 2: Please provide information on the specific batches (by the production Method I, II and III) of desirudin employed in the preclinical testing for each study and immunogenic response elicited.

Sponsor Response: The information on the specific batch #s of desirudin employed in the special immunogenicity studies in rabbit, dog, guinea pig and baboon, was submitted.

Immunogenicity studies in rabbit, dog and baboon were done by employing desirudin prepared by method I and batch #13/615/1, 13/615/1 and 13/616/1 respectively. The immunogenicity study in guinea pig was conducted by using desirudin prepared by method II (batch #810189). The compound synthesized by method # I (year 1988) or II (year 1988-90) was used in research, preclinical and early clinical studies.

Reviewer's Comments: Sponsor had used 3 different methods (MI, II and III) for the synthesis of desirudin and preclinical studies were conducted using method # M I or II. The compound synthetized by using these methods of synthesis (M1, M2 or M3) contained about [redacted] desirudin. The remaining fraction of the impurities might be different in their chemical and toxicological characters. Thus, the impurities in samples could exert different toxicity or immunotoxicity profile. Sponsor was asked to send information on the batch number used in the tests conducted for the determination of toxicity and/or immunogenicity of the compound. Sponsor submitted only a partial list of immunogenicity studies and the details about the method of synthesis of the compound used in assessing pharmacology; general toxicity and other preclinical toxicity studies were not given. The provided information could not give replace the suggested 1-month toxicity study in a non-rodent species. In the initial submission, sponsor submitted details indicated that desirudin (batch #MI) was used in vitro (protein binding study), acute iv toxicity studies in mice, rats and monkeys, acute sc, 10-day, 1-month and 3-month sc toxicity in rats, 7-day and 1-month iv study toxicity studies in dogs, 14-day iv study in monkeys, iv Segment II. Developmental toxicity study (including the repeat study) in rabbits, special local irritation toxicity studies in rabbits, dogs and baboons. Desirudin prepared by M2 procedure was used in Segment I, II (including repeat Segment II) and III studies in rats, Modified IV Teratology study in rabbit. None of the preclinical studies were conducted by using the compound synthesized by method M3.

Sponsor should conduct suggested 1-month toxicity study in monkeys. The study should be done by using desirudin prepared by method M3 and administering the daily dose in 2 divided doses (as proposed in the clinical study).

SUMMARY AND EVALUATION:

In the present amendment, sponsor sent their response to the Division letter of September 1, 2000. Division had asked the sponsor to conduct 1-month subacute s.c. toxicity study in monkeys and to provide batch # of Hirudin used in preclinical immunogenicity studies.

In response to the suggestion of the conduct of 1-month subcutaneous toxicity study in 4 groups of monkeys (at least 3 dose levels of desirudin and by giving the drug to animals as proposed in humans), sponsor stated that there was considerable human immunogenicity data from European clinical studies and the data could address the Agency concern. There was no need to conduct suggested 1-month sc toxicity study in monkeys. In the submitted NDA, desirudin would be used at a subcutaneous dose of 0.3 mg/kg (6034.2 antithrombin units/kg) twice a day and the same treatment can be repeated postoperatively for 9 to 12 days or till the patient becomes fully ambulatory. To support such clinical trials and marketing of the compound of 9 to 12 days duration, 1-month toxicity studies in rodent and non-rodent species are needed. The 1-month toxicity study in rats was submitted earlier and acceptable but 1-month toxicity study in non-rodent species was not conducted/submitted by sponsor. Sponsor was asked to conduct such a study in monkeys by selecting the route and frequency of administration of the drug to be the same as proposed in the clinical application, i.e., in 2 divided doses/day.

The submitted tabulated information of the batch number, production method (# I, II or III) of 4 immunogenicity studies only (one each in rabbits, dogs, baboons and guinea pigs) and their results can not replace the suggested 1-month toxicity study in monkeys. The toxicity profile of the compound in non-rodent species could not be derived from the information. The 1-month toxicity study in monkeys may now be conducted by using the lot synthesized as mentioned above and the toxicokinetics and immunogenicity of the compound should be monitored during the study.

RECOMMENDATIONS:

Sponsor should again be asked to conduct the suggested 1-month toxicity study in 4 groups of monkeys by administering the compound in 2 daily divided doses and monitoring the toxicokinetics and immunogenicity potentials of the compound. The results of the study should be submitted to the Division for the review.

Yash M. Chopra, M.D., Ph.D.
Pharmacologist

COMMENTS:

J.B. Choudary, B.V.Sc., Ph.D.
Supervisory Pharmacologist,HFD-180

Original NDA
HFD-180
HFD-181/CSO
HFD-180/Dr. Chopra
HFD-180/Dr. Choudary

R/D Init.: J. Choudary 11/13/00

YC/Deg: 11/30/00
C:\DATA\PHARM\N21271011.0YC

APPEARS THIS WAY
ON ORIGINAL

/s/

Yash Chopra
12/1/00 09:42:53 AM
PHARMACOLOGIST

Jasti Choudary
12/4/00 09:28:29 AM
PHARMACOLOGIST
The present review pertains only to NDA 21,271 Amendment dated October 11, 2000.

NDA 21-271 Review #1

SPONSOR & ADDRESS: Aventis Pharmaceuticals Inc.,
Collegeville, PA.

REVIEWER: Yash M. Chopra, M.D., Ph.D.,
Pharmacologist

DATE OF SUBMISSION: June 28, 2000

HFD-180 RECEIPT DATE: June 29, 2000

DATE OF REVIEW: May 3, 2001

REVIEW AND EVALUATION OF PHARMACOLOGY AND TOXICOLOGY DATA
Original Summary

DRUG: Recombinant Desulfato-Hirudin/Rec-Hirudin/R-hirudin/
Desirudin/CGP-39393/RPR205511, 15 mg Injection

DRUG CATEGORY: Thrombin Inhibitor

CHEMICAL NAME: A single 65 amino acid chain polypeptide with 3 disulfide chain bridges.

MOLECULAR WEIGHT: 6963.52

MOLECULAR FORMULA: $C_{287}H_{440}N_{80}O_{110}S_6$

STRUCTURAL FORMULA:

Val - Val - Tyr - Thr - Asp - Cys - Thr - Glu - Ser - Gly[10]

Gln - Asn - Leu - Cys - Leu - Cys - Glu - Gly - Ser - Asn[20]

Val - Cys - Gly - Gln - Gly - Asn - Lys - Cys - Ile - Leu[30]

Gly - Ser - Asp - Gly - Glu - Lys - Asn - Gln - Cys - Val[40]

Thr - Gly - Glu - Gly - Thr - Pro - Lys - Pro - Gln - Ser[50]

His - Asn - Asp - Gly - Asp - Phe - Glu - Glu - Ile - Pro[60]

Glu - Glu - Tyr - Leu - Gln[65]

FORMULATION: Each single dose vial contains 15 mg lyophilized powder of r-hirudin mixed with 0.5 ml of 3% sterile non-pyrogenic mannitol in water for injection (0.5 ml ampoule) before use. The composition of each vial is given below:

Ingredients	Quantities (w/v) per Vial
Recombinant Hirudin (R-hirudin)	[redacted]
Magnesium Chloride,	[redacted]
Sodium Hydroxide Solution for Injection	[redacted]

RELATED IND: IND 34,046.

MARKETING INDICATION:

[redacted]

DOSE: It is recommended at a subcutaneous dose of 0.3 mg/kg twice a day or i.e., 15 mg bid (based on assumed average body weight of an adult = 50 kg). The treatment can be repeated for 9 to 12 days.

PRECLINICAL STUDIES AND TESTING LABORATORIES

Type of Study	Study #	Name of Laboratory	Drug Batch #	Review Page #
I. Pharmacology	--	--	--	--
II. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION:				
1. Comparison of 2 analytical methods, TCA and ELISA for measuring CGP 39393 in plasma	B/13/1991	Ciba Geigy Research, Basle, Switzerland	--	16
2.Pharmacokinetic of single I.V. Dose in Rats and Dogs	B55/1989	,,	5-7014	17
3. Half Life of Thrombin-Hirudin Complex in rats	4/91	,,	r-HH5-7018	17
4.Bioavailability & Pharmacokin. In Rats After I.V. or S.C. Administration	B19/1991	,,	14/194/1	18
*5.Pharmacokin. Study of Hirudin and Heparin in normal and Nephrectomized rats.	Res. Pub.	[redacted]	--	18
*6.Pharmacokin. of a slow bolus I.V. Dose in Dogs	BPK1994/057	[redacted]	16/623/1	19
DISTRIBUTION:				
*7.In Vitro Serum Binding Study in Human Serum	BPK(F) 1994/30 & B383	Ciba Geigy Research, Basle, Switzerland	15/039/1 15/532/1 16/447/1	19
*8. Immunocytochem Detection in Rat Tissues	Biol Rep. # 3/94(18/B17/12	,,	RHH5-ZB-7056	20
*9. Placental Transfer in Rabbits.	Res.Publ:	Pharmazie 43:203-207, 1988	--	20
METABOLISM AND EXCRETION:				
*10.Biotransformation of a Single I.V. Dose in Rats	DMET(EU)2/1995	,,	15/039/1	21

*11. In Vitro Metabolism In Liver and Kidney of rats, baboons, and man.	DMET(EU)3/1995	,,	14/787/3 14/194/1	22
*12.Elimination & Biotrans. In Situ Perfused Rat Kidney	DM(EU)9/1994	,,	810389	24
*13.Renal Excretion of a Single I.V. Dose from Dog Kidney	1/1995	,,	16/623/1	26
III. **TOXICOLOGY:**				
Acute Toxicity Studies:				
i.v.				
1a. Rat	87-6176	,,	810189	27
1b. Dog	87-6179	,,	rHH57087	27
2a. Mouse	90063	,,	810189	28
2b. Rat	90052	,,	810189	28
3. Monkey	91-6016	,,	15/317/1	29
Subacute/Subchronic Toxicity Studies:				
Rat				
1. 1-Month S.C. with 1-month Recovery.	87-6256	,,	13/175/1	30
2. 3-Month S.C. with 1-month Recovery.	89-6052	,,	13/995/1	32
Dog:				
3. 7-Day Continuous I.V. Infusion 24/day in Beagle Dogs	90077	[redacted]	810189	34
4. 1-Month I.V.	87-6257	[redacted]	13/175/1	37
5. 3-Month I.V. with 1-month Recovery	89-6052	[redacted]	13/996/1 14/021/1 14/023/1	39
Monkeys:				
6. 14-Day Continuous I.V. Infusion 24hr/day	91-6017	[redacted]	15/317/1	43
Reproductive Toxicity Studies:				
1. Subcutaneous Segment I. Fertility and Reproductive Performance Study in Rats	94055	Ciba-Geigy, Basle	810192	46
Segment II. Teratology Study:				
2. SC Rats	89-6087	,,	14/787/3	48
3. Repeat SC-Rat	92099	,,	810191	52
4. I.V. Rabbits	89-6312/ 89-6074	,,	14/091/1	53
5.Modified I.V. Segment II Study in Rabbits	94028	,,	14/019/1	56
6. Subcutaneous Segment III. Perinatal and Postnatal Study in Rats	92106	,,	810192	59
IV. MUTAGENICITY:				
1. Ames Test	90-6187	,,	14/019/1	61

2. Gene Mutation in CHO V79 cells	93-6153	,,	810192	62
3. In Vitro, Chromosomal Aberration Assay in CHO cells	93-6152	,,	810192	63
4. In Vivo, Rat Micronucleus Test	91-6086	,,	810492	67
V. SPECIAL STUDIES:				
1. Immunotoxicity in Rabbits	88-6128	,,	13/615/1	68
2. Immunological Study In Dogs	88-6097	Ciba Geigy Ltd.	13/615/1	68
3. Antigenicity Study in Guinea pigs	90015/AG-1089	[redacted]	810189	69
4. 5-Day Local Subcut. Irritation Study in Rabbits	89-6059	Ciba Geigy Ltd.	--	70
5. Local Irritation & Tolerance Study in Rabbits	93-6221	,,	SH 46, 47 & 48	71

The preclinical studies with r-hirudin were submitted under IND 34,046 and reviewed as Initial application (review date January 18, 1990), amendment dated March 20, 1990 & August 17, 1990 (review dated October 23, 1990); amendment dated December 27, 1990 (March 12, 1991), amendment dated February 11, 1991 (March 12, 1991); amendments dated June 6, July 12 and July 30, 1991 (review date September 4, 1991), amendments dated September 27, 1991, October 10, 1991, March 16, 1992, May 1, 1992 and September 1, 1992 (review dated October 2, 1992); amendments dated August 7, 1992, January 4, 1993 and February 22, 1993 (review dated August 9, 1993); amendment dated December 6, 1993 (review dated December 27, 1993); amendment January 7, 1994 (review dated March 20, 1994); amendment dated October 6, 1994 (review dated February 3, 1995); amendment dated May 3, 1995 (review dated June 28, 1995) and; amendment dated January 24, 1996 (review dated April 15, 1996). These studies were incorporated in the present review. New studies submitted in this NDA are indicated by asterisk (*) in the above table.

GOOD LABORATORY PRACTICE & QAU REGULATIONS:

Sponsor has included the statements that all preclinical studies were conducted in compliance with GLP and QAU regulations.

PHARMACOLOGY:

Desirudin (r-hirudin) a single chain 65-amino acid recombinant polypeptide with 3 disulfide bridges is identical to the naturally occurring leech hirudin. It lacks a sulfate group in hirudin

structure and was claimed by sponsor to be a selective and more potent thrombin inhibitor.

Primary Activity

1. Mechanism of Action: One molecule of r-hirudin binds with 1 molecule of thrombin to form a stable non-covalent 1:1 molar complex (Ki = 231 fM) and it blocks the enzymatic activity of thrombin. It acts as an anticoagulant by selectively inhibiting free circulating and clot bound thrombin. Activated partial thrombolplastin time (APTT) of human plasma was increased in a dose dependent manner and doubled at the concentration of 0.08 uM r-hirudin. It does not require a cofactor anti-thrombin III (AT) and did not exert any effect on plasma factor Xa, plasmin, kallikrein, serine proteases and the digestive enzymes, tyrosine and chymotryspin up to a concentration of 22 uM r-hirudin.

2. Characterization of CGPP 39393 Activity on Proteolytic Enzymes in Vitro Inhibition of Thrombin Activity: CGP 39393 is a relative specific inhibitor of thrombin, having K_i values as follows:

231 fM (Braun et al., Biochemistry 27: 6517-6522, 1988)
19 pM (Dodt, et al., FEBS Lett. 229 (1): 87-90, 1988)
200 PM (Ambler et al., CIBA-GEIGY, Biology report 1/89, 5/89).

The Ambler et al value is an underestimate of the true Ki because in their experiment the concentration of inhibitor is similar to that of the enzyme, and one could not use Michaelis-Menten Kinetics.

3. Effect on Other Proteolytic Enzymes: Other enzymes of the hemostatic system, such as factor Xa, plasmin, Kallikrein, and the digestive enzymes, trypsin and chymotryspin were not inhibited by CGP 39393 at a concentration of 22 uM (Ambler, et al., CIBA-CEIGY (Horsham) Biology Report, 1/89, 5/89). CGP 39393 at 25 uM had no effect on the esterolysis activity of purified serine proteases (Clr and Cls) of the classical pathway of complement activation. At 1 uM there was only 5% inhibition of C_{5a} (activated complement factor 5) generation in human serum. CGP at 27 uM inhibited 16% the complement induced hemolysis via classical pathway. At 6.3 uM, it inhibited the complement-induced hemolysis via alternative pathway by 7%. Thus suggesting that CGP 39393 is a relative specific inhibitor of human alpha-thrombin.

4. Characterization of Effect on Platelet Aggregation in Vitro of Human Platelet Rich Plasma (PRP):
CGP 39393 inhibited platelet aggregation induced by thrombin with an A_2 (presumably EC_{50}) value of 3 nM. CGP 39393 had little effect on platelet aggregation induced by other agents such as platelet activating factor (PAF) and collagen at concentrations greater than two orders of magnitude higher than those that inhibited thrombin

induced aggregation, and these effects were not even concentration dependent (Ambler, et al., CIBA-GEIGY (Horsham) Biology Report, 1/89, 5/89).

5. Effect on in Vitro Clotting Times: CGP 39393 inhibits plasma (human or rat) clotting irrespective of whether it is induced by intrinsic (activated partial thromboplastin time-APTT), or extrinsic (prothrombin time-PT) rout, or directly by thrombin (thrombin time-TT). The TT measurement is the most sensitive to r-hirudin, since a concentration of 0.005 uM doubles the clotting time in human plasma. However this method was dependent on the thrombin concentration used and the stability of thrombin in solution. The PT measurement was the least sensitive to r-hirudin since a concentration of 1.14 uM is required to double the clotting time in human plasma. The APTT in human plasma is doubled by approximately 0.08 uM r-hirudin. For the reason of simplicity, the APTT was chosen to be monitored in further studies. In vitro the dose response curve was shallower than that for heparin. Thus by increasing the CGP 39393 concentration it is possible to attain controlled anticoagulation up to 4 times control APTT. On the other hand heparin dose response curve is undesirably steep, furthermore it works via antithrombin III and inhibits several enzymes in coagulation pathway.

6. The Effect of Recombinant desulphatohirudin (CGP 39393), in Combination with Heparin, on Coagulation Parameters (APTT) In Rats: Prolongation of APTT by 1.61 and 2.37 times over the control APTT values (16.8 $\pm$ 1.2 sec and 17.4 $\pm$ 1.5 sec) were seen in rats after the i.v. bolus administration of 0.03 mg/kg and 0.3 mg/kg of CGP 39393 respectively. Sixty minutes after the CGP 39393 administration APTT values returned to baseline values. Heparin (20 IU/kg, i.v. plus 0.4 IU/kg/min, i.v.) by itself prolonged APTT by about 2.48 times over the control values (18.6-19.5 sec) in rats, When a single i.v. bolus dose of CGP 39393 (0.03 or 0.3 mg/kg) was given to rats who are already receiving heparin (20 IU/kg, i.v. plus 0.4 IU/kg/min, i.v.) then APTT was further prolonged in dose dependent fashion. APTT values were prolonged by 1.61 and 3.50 times over the heparin baseline values (48.5 4.7 sec). The effect on APTT is of synergistic nature, and APTT returned to heparin baseline values by 20 min at low dose and by 60 min at high dose of CGP 39393. Thus the time to return to baseline APTT was not affected by concomitant administrations of heparin and CGP 39393.

7. Characterization of Ex-Vivo Effects on Clotting Times: CGP 39393 was given i.v. or s.c. to groups of five rats and blood samples were taken to measure ex-vivo APTT clotting times at various doses and time intervals. The anticoagulant effects were dose dependent. The dose of 0.1 mg/kg by i.v. and approximately 2 mg/kg by s.c. doubled the APTT. The dose response curves for CGP

39393; heparin and fragmin based on ex-vivo coagulation (APTT) indicate that irrespective of the route of administration (i.v. or s.c.), CGP 39393 inhibited coagulation with linear relationship over a greater range of concentration than heparin and fragmin. When intravenous infusion study was conducted in anesthetized rat, it was found that a steady state approximately 2 times greater than the control APTT was achieved over 20-60 min by infusing CGP 39393 at a concentration of 14 ug/kg/min. The effect on APTT, after 60 min infusion, was linear with respect to dose up to approximately 4 times control APTT values. The duration of action of CGP 39393 (by APTT measurement) is approximately 1 hr after i.v. administration (0.3 mg/kg) and 2-4 h after s.c. administration (3.0 mg/kg). In nephrectomized rats the anticoagulation effect of CGP 39393 (i.v.) was greater than 4 hr. This indicated that in the absence of normal clearance the compound exerted significant pharmacological activity for an extended period of time, and kidneys played a greater role in elimination of the compound in this species.

In Vivo Studies:

Antithrombotic Effects

8. Venous Thrombosis Model: The effects of CGP 39393, heparin and fragmin on venous thrombosis were assessed in the rat. The test compound was administered either by i.v. or s.c. route. CGP 39393 reduced the thrombus weight with an ID_{50} of 0.01 mg/kg i.v. or 0.45 mg/kg s.c. and, total inhibition at 0.03 mg/kg, i.v. and 1.0 mg/kg s.c.. Heparin and fragmin were also very potent in this model with ID_{50}s of 3.0 IU/kg i. v. and 110 IU/kg s.c. for heparin and 0.03 mg/kg i.v. and 0.45 mg/kg s.c. for fragmin. It should be noted that in this model total inhibition of thrombus formation is achieved at doses of all three compounds, which have only small effect on APTT.

9. Shunt Thrombosis Model: In the shunt thrombosis model in rat, CGP39393 completely inhibited the thrombus formation at 3.0 mg/kg (i.v.) or 10 mg/kg (s.c.). Doses of 0.3 mg/kg i.v. and 1 mg/kg s.c. inhibit shunt thrombus formation in the rat by 50%. Similar activity is only found with heparin at a dose causing excessive anticoagulation (Ambler, et al., CIBA-GEIGY (Horsham) Biology Report, 1/89, 5/89).

10. Antithrombin efficacy v/s Anticoagulant Effect: In arteriovenous shunt model in rat, 71% and 96% reduction in thrombus weight were achieved by r-hirudin at approximately 2 times and 3 times control APTT values, respectively. Heparin, in contrast required anticoagulant level of 6 and greater than 15 times the control APTT to produce comparable reductions in thrombus weight. Fragmin showed a similar correlation between anticoagulant and antithrombotic effects as CGP 39393.

11. Arterial Thrombosis Model: In the arterial thrombosis model in rat the inhibitory effect on thrombus formation is measured by platelet and fibrinogen accumulation on the damaged vessel. CGP39393 showed a potent antithrombotic effect with approximately 60-70% inhibition of platelet and fibrinogen accumulation at 1 mg/kg i.v. or 6 mg/kg s.c. increasing the doses to 15 mg/kg s.c. produced 80-90% inhibition of thrombus formation.

In summary, different dose levels of CGP 39393 (and heparin and fragmin) are required to inhibit thrombosis in three different experimental models, with the venous model being most sensitive, followed by the shunt model and arterial model being the least sensitive to treatment. Thus for example, CGP 39393 (1.0 mg/kg, s.c.) completely inhibited the venous thrombosis with only a small anticoagulant effect (APTT = 1.6 x control) in the shunt model total inhibition (CGP39393 at 10 mg/kg s.c.) is associated with APTT value of 3.7 x control. In the arterial model the same level of anticoagulation is correlated with over 70% inhibition of thrombus formation.

Heparin (300 IU/kg, s.c.) completely inhibited the venous thrombosis with only a small anticoagulant effect ($APTT_{\Delta}$ = 3.6 x control). However, in the shunt model excessive anticoagulation (APTT greater than 15) is necessary to produce substantial inhibition of thrombus formation, and furthermore a dose (600 mg/kg s.c.) causing greater than 15 X APTT values had no significant inhibition of thrombosis in the arterial model.

The results obtained with fragmin were similar to those of CGP 39393 in venous and shunt thrombosis. However it had very little efficacy in the arterial thrombosis model, with only 50% inhibition of thrombus formation producing greater than 15 times control APTT values.

Thus it seems that CGP 39393 has advantages over heparin and fragmin on at least two counts 1) r-hirudin displayed considerable efficacy in arterial thrombosis, where as heparins are ineffective, 2) a higher antithrombotic activity with lower effect on coagulation parameters.

12. Prevention of Coronary Reocclusion After Thrombolysis in Dogs with High-grade Residual Stenosis: Coronary thrombosis was induced by electrical stimulation of the endothelium in conscious dogs with distal stenosis induced previously with a tygon constrictor (85% reduction in the lumen). Reocclusion occurred within 2 hr after thrombolysis (t-PA 1 mg/kg, over 60 min.) in 5 out of 6 dogs in control group, 5 out of 5 dogs in aspirin (5 mg/kg) and 4 out of 4 in hirudin (1.5 mg/kg) treated dogs. However, no reocclusion was evident in 4 dogs which were treated with hirudin and aspirin.

Thus combination of hirudin and aspirin prevented early reocclusion after thrombolysis despite high-grade residual stenosis, and individual agents were not effective in this experiment. Therefore both antiplatelet and antithrombin agents are necessary for the maintenance of patency after thrombolysis therapy in the presence of severe coronary stenosis.

13. (a) Effect of Rec-hirudin or Heparin on Thrombus Formation in the Carotid Artery After Deep Vein Arterial Injury in the Pigs: CGP 39393 (0.3, 0.7 and 1.0 mg/kg) or heparin (50 u/kg) were administered to groups of pigs (n=10) as a bolus followed by infusion of the same dose per hour, 10 min after the start of infusion, the animals were subjected to bilateral carotid angioplasty, and they were sacrificed 15 min after the last balloon inflation. Heparin and hirudin (0.7 mg/kg) both prolonged the APTT time to twice the control value, Heparin did not prevent significantly macroscopic mural thrombosis (control = 19/25, test 13/23) or platelet deposition (control = 54 $\pm$ 21 X $10^6/cm^2$, test 33 $\pm$ 9x10^6cm^2) however fibrinogen deposition were reduced (control = 54 $\pm$ 24 x 10^6cm^2, test = 19 $\pm$ 2 x 10^6cm^2). Hirudin significantly reduced macroscopic mural thrombosis (control = 19/25, low dose = 13129, mid dose = 0/24, high dose = 0/24), dose dependent decreases in platelet deposition in areas of deep arterial injury (control = 54 $\pm$ 21 x 10^6cm^2, low dose = 22 $\pm$ 6 X10^6cm^2, mid dose = 8 $\pm$ 1 x 10^6cm^2, high dose = 7 $\pm$ 1 x 10^6cm^2) and fibrinogen deposition (control = 54 + 24 x $10^{12}cm^2$, low dose = 28 + 6 x $10^{12}cm^2$, mid dose = 13 $\pm$ 2 x $10^{12}cm^2$, and high dose = 12 $\pm$ 1 X $10^{12}cm^2$). Thus hirudin is more effective than heparin in preventing acute arterial platelet rich thrombosis at doses that produce a similar prolongation in the APTT (2-3 time control).

(b) Hirudin Markedly Enhances Thrombolysis with rt-PA (Report# 90-169):

In this experiment platelet rich thrombi were produced in the left carotid artery of pigs. Thirty minutes after thrombus formation the pigs were given placebo (not identified), i.v. rt-PA (0.3 mg/kg bolus plus 3 mg/kg over 90 min, then 1 mg/kg over 120 min), hirudin (1 mg/kg bolus then 0.7 mg/kg/hr) and rt-PA plus hirudin (same dosing regimen as mentioned above). During the 210 min study period only 1/6 animals treated with rt-PA reperfused while 7/7 animals treated with rt-PA plus hirudin reperfused [no reperfusion was evident in control group animals (n=3)]. Platelet deposition in the area of arterial injury in rt-PA plus hirudin treated animals were also reduced significantly (control = 335 x 10^6cm^2, rt-PA = 123 x 10^6cm^2 and rt-PA + hirudin = 13 x 10^6cm^2). Thus Hirudin markedly enhances thrombolysis by rt-PA after deep injury-induced arterial occlusion in the pigs.

(c) Does a Short Term Hirudin Infusion Reduce Thrombosis 48 Hr After Arterial Injury (Report # 90-189)

Administration of 1 mg/kg bolus plus 1 mg/kg/hr infusion of hirudin for 75 min, starting just before deep arterial injury in pig did not reduced platelet thrombus formation at 48 hour after the injury, and hirudin did not reduce stretch associated smooth muscle cell proliferation.

14. Angiographic and Pathologic Study of the effect of Rec-Hirudin on Restenosis Following Balloon Angioplasty in Rabbits:

In this experiment, twenty-nine rabbits with focal femoral atherosclerosis were used. Focal femoral atherosclerosis was induced by air desiccation endothelial injury followed by 28 days of high cholesterol (2%) diet. At the time of balloon angioplasty one group of rabbits (n = 16) were given heparin (150 u/kg i.v. bolus) and another group of rabbits (n=13) were given hirudin (1 mg/kg bolus, 1 mg/kg infusion for Ist hour and then 0.5 mg/kg during 2nd hour). Angiography was done before and after balloon angioplasty (BA) and prior to sacrifice. Rabbits were sacrificed 2 hr and 28 days after BA. No intergroup differences in mean luminal diameter (LD) and histological findings were seen in animals sacrificed at 2 hr after BA. However at day 28, rec-hirudin produced less angiographic restenosis than heparin.

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	Mean Luminal Diameter (mm)		
	Pre-BA	2-hr Post BA	28-day Post BA
Heparin	1.18	1.86	0.94
Hirudin	1.14	1.68	1.37

15. Effect of CGP 39393 on Acute Platelet Thrombus Deposition During Angioplasty in Pigs: CGP 39393 (1 mg/kg) or heparin (doses from 35-250 u/Kg) was administered to groups of pigs as a bolus dose followed by infusion of the same dose per hr. 20 min after the start of infusion, the animals were subjected to bilateral carotid angioplasty, and they were sacrificed approximately 1 hr later. The APTT values were 3 x the baseline values for the lowest heparin concentration used, as well as the group treated with CGP 39393. In the CGP 39393 treated group, platelet and fibrinogen deposit/cm^2 of deep medial arterial injury were significantly lower than the heparin treated group ($P < 0.001$). CGP 39393 even reduced platelet and fibrinogen deposition more effectively than the

highest dose heparin group (Heras et al., Circulation 79: 539-544, 1986).

16. Antithrombotic Activity in Baboons: In baboons, CGP 39393 infusion (5 or 20 nmole/kg/min) produced a dose-dependent prolongation of bleeding times and clotting times (APTT), and inhibited fibrin and platelet accumulation and prevented the rapid occlusion of a dacron vascular graft or collagen-coated tubing in femoral arteriovenous shunt. The blood markers (BTG, PF4 and FPA) were reduced to basal levels at doses greater than 5 mmole/Kg/min (Kelly, et al., Circulation 78: (4 part 2), 1988).

17. Prevention of Microthrombosis in Rat Lungs: Hirudin infusion from a dose of 200 AT-U/kg administered 1 hr before thrombin infusion, prevented thrombin induced microthombi and accumulation of ^{125}I-fibrin. At this dose the blood levels were 0.6 - 0.3 AT/ml after 60 min of the administration.

18. Reversal of Effect of CGP 39393 by Factor VIII or DDAVP: Factor VIII and 1-deamino-8-arginine vasopression (DDAVP) both are used in patients with Factor VIII insufficiency (e.g. Haemophilia A) and Von Willebrands disease. Butler et al. (CIBA-GEIGY, Horsham, Biology Reports, 7/88, 10/88) used the above mentioned compounds to reverse the effects of CGP 39393 in both in vitro and in vivo test systems. In vitro, CGP 39393 (0-0.78 um) induced activation of APTT in human plasma (1-10 x the control APTT) was markedly inhibited by factor VIII (1-51 u/ml) in a dose dependent fashion. The duration of anticoagulant action of CGP 39393 (3 mg/kg IV) after a single bolus injection in the rat was significantly reduced by the administration of factor VIII (120 IU/kg, i.v.) given 2 or 10 min post CGP 39393. Similar results were obtained after CGP 39393 i.v. infusion (results were not remarkable). In humans, an infusion of DDAVP (0.3 ug/Kg) over a 15 min time period resulted in a significant increase (approx. 3 x the baseline value at 30 min post infusion) in the plasma levels of factor VIII. CGP 39393 consequently was less effective at increasing the degree of anticoagulation in vitro in human plasma samples. It should be noted that at no time factor VIII or DAAVP completely block the effect of CGP 39393 as seen in Butler et al. study.

19. Reversal of Effects of CGP 39393 by Recombinant factor VIII, Recombinant Factor VIIa, Epsilon-Aminocaproic Acid [EACA], desmopressin (DDAVP) and Vueffe: All five agents (factor VIII, factor VIIa, EACA, DDAVP and Vueffe (low molecular weight peptide fragment from bovine factor VIII) have been used to treat a number of clinical bleeding disorders. Administration of CGP 39393 (30 mcg/kg/min for 40 min) to rats resulted in prolongation of rat tail bleeding time at 0 and 30 min after the stoppage of CGP 39393 infusion (post 60 min bleeding time returned to normal).

Administration of DDAVP (i.v. infusion: 1 mcg/kg over 15 min.), recombinant factor VIII (i.v. bolus: 100 or 200 IU/kg), and Vueffa (i.v. bolus: 3 mcg/kg) immediately following cessation of the infusion of CGP 39393 significantly reduced the bleeding time and bring back to normal values. In this experiment factor VIIA and EACA were not effective. All five agents had no effect on bleeding time in control rats (not exposed to CGP 39393).

20. In Vivo Catalysis of Thrombin Inhibition by Antithrombin III or Heparin Cofactor II and Antithrombin effect: Differential Effects of Unfractionated Heparin and Dermantan Sulfate: Dermatan sulfate (inhibits thrombin by heparin cofactor II) inhibited prothrombin clearance by 70% and prothrombin accretion on to thrombi by 81% in rabbits with pre-existing thrombi. Results with hirudin (specific thrombin inhibitor) were comparable to that obtained with dermatan sulfate. On the other hand, 10 u/kg heparin (catalyzes the thrombin inhibition by antithrombin III) had no significant effect on prothrombin clearance and prothrombin accretion onto thrombi was only inhibited by 28% in rabbits with pre-existing thrombi. Thus indicating that dermatan sulfate and/or hirudin inhibited thrombus growth more effectively than heparin. It also suggests that thrombin in a thrombus is more accessible to inhibition by heparin cofactor II/dermatan sulfate and/or r-hirudin than antithrombin III/heparin.

21. Effect of Thrombin Inhibition in Porcine Platelet Interaction With Severe Damage Vessel Wall, Mildly Damage Vessel Wall, and Isolated Fibrillar Collagen Type I. Hirudin and R-Hirudin Versus Heparin in Arterial Thrombosis: Platelet deposition to mildly damage vessel wall and to isolated collagen type I fibrils was not reduced by hirudin or r-hirudin, and values did not differ from the platelet deposition in heparinized blood. However, rec-hirudin (100 u/ml) significantly reduced platelet deposition on severely damaged vessel wall (heparinized blood = $93 \pm 10 \times 10^6$ platelets/cm^2, rec-hirudin treated blood = $50 \pm 7 \times 10^{-6}$ platelets/cm^2). Thus, thrombus growth is dependent on local thrombin production at the site of severe wall damage and rec-hirudin is more effective than heparin in reducing platelet deposition at that site. Furthermore, natural hirudin was 5 times more potent than the rec-hirudin in the above experiments.

22. Comparison of R-Hirudin with Heparin as Adjunct to Streptokinase Thrombolysis in a Canine Model of Coronary Thrombosis: In anesthetized dogs, occlusive thrombus was induced in left anterior descending (LAD) coronary artery. Fifteen minutes before streptokinase (750,000 units in 60 min) administration dogs were given saline (control), rec-hirudin (0.3 mg/kg plus 0.3 mg/kg/hr, 1 mg/kg plus 1 mg/kg/hr or 2 mg/kg plus 2 mg/kg/hr) or heparin (60 units/kg plus 40 units/kg/hr or 100 units/kg plus 60 units/kg/hr) for unspecified duration). Vessel patency was

monitored for up to 180 min after the stoppage of streptokinase. In dogs which were not treated with any adjunctive agent (i.e. treated with saline), none of the vessels were recanalized with streptokinase. Both CGP 39393 and heparin dose dependently increased the incidence of reperfusion, total time of reperfusion and decreased the time to reperfusion and re-occlusion. In this experiment for a given APTT prolongation, rec-hirudin was about 5 times more effective than heparin in enhancing the effect of streptokinase.

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Table 1

Effects of CGP 39393, and heparin in conjunction with streptokinase on reperfusion and reocclusion parameters in anesthetized dogs

	Reperfusion				Reocclusion	
			Duration			
	Incidence	Time	Initial	Total	Incidence	Time
CGP 39393						
0.3	50% (4/8)	72 ± 31 (4)	46 ± 35 (4)	74 ± 29 (4)	50% (2/4)	9 (2)
1	75% (6/8)[a]	39 ± 5 (6)[a]	99 ± 23 (6)[a]	130 ± 7 (6)	33% (2/6)	28 (2)
2	100% (8/8)[a]	33 ± 6 (8)[b]	106 ± 21 (8)[b]	130 ± 13 (8)	38% (3/8)	43 ± 24 (3)
Heparin						
60	12% (1/8)	59 (1)	10 (1)	34 (1)	100% (1/1)	10 (1)
100	75% (6/8)	65 ± 16 (6)	46 ± 13 (6)	53 ± 11 (6)	83% (5/6)	40 ± 14 (5)

All time values (min) are mean ± SE. Incidence values indicate the percent (and fraction) of animals responding to a particular treatment. Numbers in parentheses reflect the number of animals corresponding to each data value. CGP 39393 (doses in mg/kg + mg/kg/hr) and heparin (doses in U/kg + 40 and 60 U/kg/hr, respectively) were administered 15 min before streptokinase (750,000 U over 1 hr).

[a]p <0.05, significantly different from heparin 60 value [b]p <0.05, significantly different from heparin 100 value

[Original data: Notebook reference - N90-386 pp. 63-118, Statistics Reports 91133 and 91162]

23. Effect of Thrombin Inhibitors on tPA induced Thrombolysis in Rat: In this experiment thrombus rich in red cells and fibrin was produced in distal aorta of rats by applying an external constriction after denuding the endothelium with a balloon catheter. Thrombolysis was induced with tPA (1 mg/kg i.v. bolus plus 1 mg/kg/hr for 30 min i.v. infusion). In addition, animals were also given saline (1 ml/kg bolus plus 2 ml/kg/hr for 80 min), heparin (40 units/kg bolus plus 40 units/kg/hr for 80 min), hirudin (1 mg/kg bolus plus 2 mg/kg/hr for 80 min), hirulog (low MW synthetic fragment of hirudin: 0.6 mg/kg bolus plus 2 mg/kg/hr for 80 min) or PPACK (D-Phe-Pro-ArgCH_2Cl [inhibits clot bound thrombin]: 0.5 mg/kg bolus plus 1.4 mg/kg/hr for 80 min). In this experiment the dosages of antithrombin used had comparable anticoagulant activity (as determined by APTT). Time to lysis was decreased in animals treated with thrombin inhibitor along with t-PA (saline 10 min, heparin 7 min, hirudin 7.5 min, Hirulog 4.4 min and PPACK 4.2 min) and the decrease was statistically significant in hirulog or PPACK treated rats. With respect to number re-occlusions, compared

to saline, heparin had no significant effect on this parameter. However, hirudin, hirulog, and PPACK significantly reduced the number of re-occlusions. The data suggests that heparin is less effective in inhibiting clot-bound thrombin and anti-thrombin III-independent inhibitors binds with clot-bound thrombin with variable potency.

24. Effects of Co-administration of R-Hirudin and Heparin (standard or low molecular weight) on Thrombus Formation in Rabbits (rabbit jugular vein thrombosis model): In this model rec-hirudin inhibited the thrombus growth by 34% and 41% at 0.3 and 1.0 mg/kg respectively. Standard heparin (80 IU) and low molecular weight heparin (LMWH: 80 IU) both gave similar results. Combine administration of r-hirudin and LMWH was more effective in inhibiting thrombus growth than the combined administration of rec-hirudin and standard heparin (approximately 51-53% versus 14-37%).

25. Effects of Hirudin on platelet Aggregation and Fibrin Deposition on Coronary Stents in Minipigs: Platelets and fibrin deposition on coronary tantalum stents having medial tears were significantly reduced when rec-hirudin (1 mg/kg) plus acetylsalicylic acid (ASA: 250 mg/animal) were given prior to stent implantation. Similar results but of lesser magnitude were observed when heparin (100 units/kg, i.v. bolus) plus ASA (250 mg/animal) or dextran (500 ml) plus ASA (250 mg/animal) plus heparin (100 units/kg, i.v. bolus plus 50 units/kg/hr) were given prior to stent implantation.

Treatment	Platelet Deposition (counts/stent)	Fibrin Deposition (mcg/stent)
Heparin+ASA	$234.7+/-145x10^6$	708+/-183
Heparin+Dextran+ASA	$103.2+/-18.9X10^6$	639+/-174
rec-Hirudin+ASA	$24.4+/-4.1x10^6$	197+/-36

Thus in this model, rec-hirudin was more effective than heparin in preventing the formation of platelet rich thrombi on coronary stents in the presence of deep arterial injury.

Secondary Pharmacology

1. Effects of CGP 39393 on the Cardiovascular System in Dogs: CGP 39393 (1-10 mg/kg I.V.) in dogs did not change heart rate, respiratory rate, left and right ventricular pressure, mean arterial pressure, platelet counts and fibrinogen levels.

2. Effects of CGP 39393 on the Cardiovascular System in Anesthetized Cats.

In the anesthetized cat, CGP 39393 (3-30 mg/kg; I.V.) induced only minor changes in hemodynamic parameters; ECG activity remained

unchanged. Short lasting increases in aortic dp/dt_{max} were seen at 20 and 30 mg/kg i.v.

3. Effects of CGP 39393 on the Rate and Force of Contraction in Isolated guinea pig Atria.

CGP 39393 (0.0043-0.43 u mole/l) had no significant effects on the rate of contraction in spontaneously beating right atria or on the force of contraction of electrically driven left atria of the guinea pig i.e. the voltage needed for electrical stimulation was not increased by the compound.

ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION (ADME):

Sponsor conducted in vitro and in vivo ADME studies with CGP 39393 in rats and dogs by using labeled (^{125}I-labeled) and non- labeled compound.

Absorption:

1. **Comparison of Two Analytical Methods, TCA and ELISA, for Measuring CCP 39393 in Plasma**
(Report # B 13/1991)

Both thrombin chromogenic assay (TCA) and ELISA methods were used to measure CGP 39393 in human plasma (study # RH/ET8). Both methods gave comparable results thus indicating that in human plasma biologically active metabolite(s) were not present or if present then they did not interfere with the TCA or ELISA assays. It should be noted that these two methods would not give comparable results if drug concentrations were to be measured in the urine because urinary metabolites might have varying biological activities.

2. **Pharmacokinetics of I.V. Administered CGP 39393 in Rats and Dogs:** (Study # B55/1989)

Sponsor has conducted pharmacokinetics studies after I.V. bolus injection of CGP 39393 in rats (n=3) and dogs (n-3) using a double antibody sandwich ELISA for the measurement of CGP 39393 in plasma and urine. The assay uses monoclonal mouse anti-hirudin antibodies and affinity purified anti-hirudin sheep anti-serum labeled with biotin. Following parameters were calculated from the plasma and urinary excretion data:

	Rat		Dog
Dose	1 mg/kg	10 mg/kg	1 mg/kg
t 1/2 alpha (min)	6.6	8.1	5.6
t 1/2 Beta (min)	26.4	30.7	42.3
AUC(0-inf.) (pmol-h/ml)	193	2435	760
Cl(tot.) ml/min Kg	12.39	9.88	3.17

[Limit of detection 2 pmole/ml for urine & 1 pmole/ml for plasma]

In rats, 11-18 % of the dose of 1 mg/kg and 42-47% of the dose of 10 mg/kg were excreted unchanged in the urine. In dogs, 62-70% of a dose of 1 mg/kg was excreted in the urine. The calculated $t_{1/2-\beta}$ for dogs had great variation (5.6 ± 2.1 min). It should also be noted that, when the given dose was increased from 1 to 10 mg/kg AUC (0-∞hr) increased over proportionally (12.6 times).

It is not possible to compare the above-mentioned data with published data of Markwardt et al (Pharmazie, 43: 202-207, 1988), due to the different analytical methods used. The method used by Markwardt et al is based on the biological activity of the analysate while the present method is based on the recognition of the analysate by two different antibodies. Nevertheless the pharmacokinetic parameters (e.g. AUC, clearance, and t1/2) of the previous method are incompatible with the result reported in this report. Furthermore Markwardt et al also reported that in dogs after i.v. bolus of CGP 39393, the renal clearance approximated total clearance. In the present report, only 62-70% of a dose of 1 mg/kg CGP 39393 was excreted in the urine in dogs, and the remaining 30-38% was not accounted for.

3. Half Life of Thrombin-Hirudin Complex in Rats

Methods: Anesthetized rats (n=6) were given mixture of thrombin-Hirudin complex (300 mcg/kg thrombin complexed with 39.6 mcg/kg CGP 39393) and free CGP 39393 (39.6 9/kg) via jugular cannula. Blood samples were collected from cannula after 0, 2, 10, 30, 60, 120 and 180 minutes of the e drug administration. The plasma concentration of the bound drug was measured by using [redacted] ELISA method and free drug levels were measured by TCA method.

Results: The free drug levels in plasma rapidly fell below the detection limit therefore no calculations could be made. Half life ($T_{1/2\alpha}$) for the bound drug was 19.5 min and $t_{(1/2\ \beta)}$ was 40.4 min. The volume of distribution and clearance values for the bound drug was 61 ml/kg and 0.48 ml/min respectively.

4. Bioavailability and Pharmacokinetics of CGP 39393 in Rats after I.V. or S.C Administration: (Report # B 19/1991)

Methods: Four male albino rats (Tif:RAIf[SPF]) were given a single i.v. or s.c. bolus dose of CGP 39393 (5 mg/kg). Blood samples were collected from retro-orbital site 24 hr before and 20, 40, 75, 150, 240 and 360 minutes after s.c. dosing and at 20, 40, 75, 150 and 240 minutes after i.v. dosing respectively. The plasma concentration of the drug was measured by using [redacted] ELISA method.

Results: The Tmax after s.c. dose was 40 minutes. The dose normalized AUC values after s.c. dose (5.5 mg/kg) was 253 (hr. nmol/l)/(mg/kg) and after i.v. dose was 306.5 (hr. nmol/l)/(mg/kg). The s.c. bioavailability was 83% (s.c. bioavailability in man is approximately 86-96%).

5. **Pharmacokinetic Study with Hirudin and Heparin in Nephrectomized and Normal Rats:**

The data was obtained from a study conducted by [redacted]

Methods: Four groups of male rats (6/group) were prepared by cannulation of right carotid artery and left jugular vein with PTFE cannula and in 2 of these groups, bilateral nephrectomy was performed. After 21 hr of recovery, a bolus dose of 1 mg/kg hirudin or 150 IU/kg heparin was administered. In 4 other groups of normal rats (4/group), hirudin was administered as a bolus dose of 1 mg/kg followed by 4-hr continuous perfusion at the rate of 0.00625, 0.0125, 0.0250 or 0.0500 mg/kg/min in hemoperfusion [redacted] activated charcoal apparatus to observe its effects on APTT and platelets. Blood samples were collected at 15, 30, 60, 120 and 240 min during infusion. In 2 additional groups, i.e., 1 normal and nephrectomized rats (6/group), 1 mg/kg hirudin or 150 IU/kg heparin was administered by bolus injection before dialysis. Hirudin or heparin was administered as a 4-hr infusion at the rate of 0.025 mg/kg/min. Blood samples at the intervals described above and 24 hr post dosing were collected for APTT and platelets count.

Results: APTT was prolonged in normal and nephrectomized rats and the effect was greater in nephrectomized rats than normal non-nephrectomized rats (>180 min in nephrectomized and 129.0 min in normal rats). In normal rats receiving hirudin during charcoal hemoperfusion, a dose related (from 0.05 to decrease of platelets was observed from 60 min post treatment and was of statistical importance after 120 min. The increase was due to enhanced degree of thrombocytopenia produced by hemoperfusion. APTT values were increased by 2 to 3 times in normal and nephrectomized rats after 15 min. Like normal rats treated with hirudin, the decrease in normal and nephrectomized animals treated with heparin, was gradual and delayed. In nephrectomized rat treated with hirudin, APTT

the remaining compound was not determined. The present study was undertaken to determine the excretion pattern of the compound in rat kidneys. Four male Wistar rats were administered intravenous bolus dose of 1 mg/kg and 8 rats were given a single i.v. dose of 15 mg/kg in saline. The kidneys of the animals treated with 15 mg/kg dose were separated after 30 min of dosing and the animals treated with 1 mg/kg were sacrificed 1 and 6 hr after the dosing. Their kidneys, liver, heart and lung separated. The tissues fixed and stained using sheep anti-hirudin in a ——— system.

After 30 min of the administration, CGP39393 was mostly detected in proximal tubules of all animals treated with 1 mg/kg and, only in few animals it was present in distal tubules. After high dose, the compound was seen in cortical (of all animals) and medullary tubules (a few of the animals). The compound concentrated in proximal tubule as discrete globules and probably lysosomes after 1 hr. The compound could not be detected in kidneys after 6 hr of the administration. CGP39393 was not detected in rat liver, lungs and heart. This study indicated that the compound was excreted in kidneys probably via lysosomal degradation.

9. **Placental Transfer in Rabbits**: (Research publication: Markwardt, F., Fink, G. et al, Pharmazie 43: 203-207, 1988)

The placental transfer of CGP 39393 was studied in pregnant rabbits by administering a single intravenous dose of 0.5 mg/kg ^{125}I-CGP 39393 in to the marginal vein on 27th day of gestation at 3 different times (20, 15 and 2 hr before sacrifice). The maternal blood and fetal heart blood, amniotic fluid and gastric juice were collected. The maternal and fetal blood contained hirudin and the concentration in the maternal blood was 60 times higher than fetal blood. Hirudin was present in 0.015 and 0.1% in amniotic and gastric juice, respectively showing that CGP 39393 passed the placental barrier in rabbit.

Metabolism & Excretion:

10. **Biotransformation of Single I.V. Dose of CGP 39393 in Rats**: DMET (EU) 2/1995

Methods: The renal and biliary (in bile) metabolites identification and excretion was studied after 30 mg/kg i.v. CGP 39393 (formulated with mannitol as used in clinical trials; vol=2 ml/kg) in Tif:RAIf(SPF) pedigree male 16 hr fasted rats (Ciba-Geigy). Three animals with bile duct cannulation were used to collect urine and bile for 24 hr for 24 hr. In addition, 2 rats (without bile cannulation) were treated with either 0 or 30 mg/kg CGP 39393 to exclude interfering effects of surgery. In the third experiment, 1 rat was treated with 30 mg/kg CGP 39393 and its liver

and kidneys were excised after 30 min and 1 untreated rat was a control. The urine and bile collected on ice for 8 hr or 8 to 24 hr during overnight treatment. In animals without bile duct cannulation, urine collected for 24 hr before, 8 hr after the dosing in 1 rat and up to 24 hr post dosing in the other animal. The free compound and its metabolites isolated from the urine and bile were quantified by a HPLC analysis. The free compound was estimated by ELISA procedure and the urinary and bile metabolites were estimated by

Results: Very low concentration of 0.2 to 1.2 nmol/l (0.01% of the dose) of free immunoreactive compound was seen in plasma of cannulated rats and most of the unchanged compound was present in bile samples. Two to 3 peaks of metabolites and no unchanged compound was reported in urine of these rats. By ELISA procedure, a biologically active metabolite hir (1-49 - lacking an amino acid) was detected in the bile. The HPLC analysis of rat urine samples gave 2 peaks of metabolites hir (1-49) and hir (1-52) and these were proteases induced hydrolyzed compounds. The ELISA analysis of the urine samples collected from cannulated rats showed no unchanged compound (sensitivity = 0.1 nmol/l) immediately to 8 hr of administration. The unchanged compound excreted was 45% and 12% in non-cannulated and bile cannulated rats, respectively. ELISA of liver and kidney homogenates showed no r-hirudin or immunoreactive metabolites 8 or 24 hr after treatment. But samples collected after 30 min of administration, hirudin and immunoreactive metabolites were reported in the kidneys. Sponsor did not determine the intracellular location of the metabolites. In rat liver only negligible amounts of free CGP 39393 was reported. MALDI-MS analysis of hirudin metabolites showed molecular mass of 4507, 5066, 5148 and 5396 for hir(1-44), hir(1-49), hir(1-50) and hir(1-52) respectively. Thus hirudin showed a variable metabolism in rats under the conditions of this study and it was hydrolyzed in at least metabolites in rat liver and 2 in kidneys.

11. **In Vitro Metabolism in Liver or Kidney of Rat, Dog, Baboon and Man**: (Study # DMET (EU) 3/1995)

Methods: In this study, the biotransformation of hirudin in homogenate fraction (S3) of liver and kidney of male (Tif:RAIf[SPF]) rat, dog (mongrel) and baboon (papio spec.) was estimated. Human liver homogenate was also used for studying the biotransformation of the compound. Rat liver and kidney supernatant S3 homogenate fractions were prepared and preserved in liquid nitrogen. The mixture of 180 ul of S3 liver or kidney homogenate fraction (10^7 cells - dil 1:5 v/v, pH 4.0) with 20 ul of 35.9 umol/l (250 mg/l) of CGP 39393 was incubated for 2 hr. The reaction was stopped with ice cold ethanol and the metabolites were detected by HPLC, ELISA or

[redacted] The change in metabolite pattern in kidney S3 fraction was observed at pH 4.0 and 7.4 by HPLC [redacted] and analyzed by [redacted]

Results:

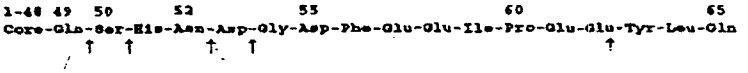
S3 Liver Fractions:

CGP 39393 was metabolized in to 6 metabolites (P2, P3, P6 to P9) by rat liver S3 homogenate fraction. The concentration of CGP 39393 was decreased by 39.6% within 2 hr and then decrease was slow thereafter. The metabolites P2/hir (1-49) and P3/hir (1-52) amounts were increased throughout the study period.

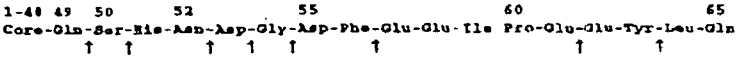
The proposed biotransformation of CGP 39393 in rat liver and kidney is shown in the following flow chart (taken from sponsor submission vol 1: 28, pp 5-15-109):

Figure 17: Biotransformation of CGP 39 393 by rat liver and kidney *in vitro*

Sites of hydrolysis of CGP 39 393 found upon incubations with rat liver S3 homogenate fractions:



Sites of hydrolysis of CGP 39 393 found upon incubations with rat kidney S3 homogenate fractions:



In dog liver, the recovery of CGP 39393 was 80 to 110%. Only one metabolite peak (P2) with retention time of 15 min was identified. No other metabolite was detected in liver homogenate.

In baboon liver fraction, the concentration of the compound was decreased at 8 and 24 hr observation periods. One peak of P2 (hir 1-49) was detected and recovery of CGP 39393 was 85 to 93%. In S3 liver fraction of man, 3 peaks corresponding to P2, P3 and P8 metabolites were seen. These were similar to hir (1-49), hir (1-52) and the parent compound, respectively.

S3 Kidney Fractions:

At pH 7.4, 6 metabolites (P3 to P6, P8 and P9) of CGP 39393 were seen in rat S3 kidney fraction after 30 min of incubation and 24 hr incubation resulted in disappearance of peaks P5, P6, P8 and P9 and peaks for P1 and P2 were seen. At pH 4.0, the parent compound was not seen and 2 metabolites, P3 and P5 were identified after 5 min and these disappeared and P2, P3 and P4 were seen from 120 min to 24 hr. Based on the similarity on chromatographic behavior, peak 3 and 5 were identified as hir(1-52) and hir(1-56). The recovery of these metabolites was 97 to 112% and 65-114% in CGP 39393 treated

fractions. Fractions P3, P5 and P6 were positive in ELISA immunogenic test whereas P8 and P9 did not show any response.

In dog kidney fraction, CGP 39393 contents were not decreased and no metabolites peaks were seen at pH 7.4. The recovery was from 75 to 92% in CGP 39393 treated fractions. At pH 4, four metabolites (P3, P5, P6 and P8) were reported and metabolites P2, P3, P4 and P5 were reported at 60 min and 3 peaks of P2, P3 and P4 were seen up to 24 hr of administration.

In baboon kidney S3 fraction, CGP 39393 concentration was not decreased and no metabolites peaks were seen at pH 7.4. The compound was degraded rapidly and main peaks of P5, P6 and P8 metabolites were seen after 5 min of administration. Thereafter, the main peaks were P2, P3 and P4. The parent compound was not detected and the recovery was up to 124.6%.

The molecular weight of the metabolites and their peaks in rat liver and kidney S3 are shown in the following table (table extracted from sponsors Table 14 and 15 (vol 1:28, pp 5-15-123 and 124):

Table 14: Molecular masses of metabolites of CGP 39 393 obtained *In vitro by* digestion with rat liver and Kidney S3 homogenate fractions at pH 7.4 determined by

Tissue	Metabolite/Peak	Mol. Weight [Dal]
Liver	hir(1-49)P2	5059.6
	hir(1-62)P6	6559.1
	hir(1-61)P6	6430.0
	hir(1-65)Hir	6963.6
Kidneys	hir(1-50)P2	5146.7
	hir(1-52)P3	5397.9
	hir(1-54)P3	5570.1
	hir(1-53)P3	5513.0
	hir(1-56)P5	5832.4
	hir(53-63)P5	1342.3
	hir(55-65)P5	1411.5
	hir(51-63)P5	1593.7
	hir(1-61)P6	6430.0
	hir(51-61)P6	1301.3
	hir(52-65)P6	1697.7
	hir(1-65)Hir	6963.6

This study demonstrated that CGP 39393 was extensively metabolized in rat, dog and baboon kidney S3 fractions and liver played a little role. The reduced/acidic pH of the medium (pH 4.0) increased the rate of metabolism of the compound. The biological activity of the metabolites was not studied.

12. **Elimination and Biotransformation in Situ in Perfused Rat Kidney**: (Study # DM(EU) 9/1994)

Methods: The study was conducted to investigate elimination and biotransformation in the in situ perfused rat renal perfusion method. The male rats (SD) were used as kidney donor and for collection of blood samples. The kidney was perfused in a perfusion apparatus and the animals were treated at the dose of either 10 or 40 mg/kg (n=2) CGP 39393 by the method of weighing the syringe before and after administration. Urine samples was collected every hour, volume measured and after the perfusion, the kidneys were cut, fixed in 0.4% formaldehyde solution and stored at -20°C. The metabolites (1:2 dil), urine (1:2 dil) and blood samples were tested at sponsor laboratory. The functions of the kidneys, perfusion pressure, perfusate flow, urine flow, urine appearance, organ appearance and ^{3}H-inulin clearance were determined. Glomerular filtration rate was determined by [redacted] concentration method, and plasma/renal clearance was calculated by a formula by [redacted] The perfusate clearance was calculated by 2 methods, i.e., [redacted]

Results:

CGP 39393 in the perfusate plasma decreased the perfusate flow in a monoexponential manner in perfused kidney with half life of 1.7 to 2.5 hr. The concentration of the compound was decreased slowly from 5 min to 2 hr after its administration and $t_{1/2}$ was 90 min to 145 min. The plasma inulin clearance (Cl_{pl}) was 0.28 to 0.43 ml/min (approx. 1/3rd of Cl_{pl} of ^{3}H-inulin). Using $AUC_{(0-120\ min)}$, the clearance for unchanged compound was 0.34 to 0.43 ml/min (^{3}H-inulin CL = 1.3 to 1.6 ml/min.kg). Only one peak for the unchanged compound (retention time = 20 to 45 min) was seen indicating that the drug in plasma perfusate was not metabolized in renal blood vessels.

Pattern and Identity of Metabolite excreted in the Urine of Perfused Kidney: No unchanged compound was detected, hir(1-50) and hir(1-52) in 16 to 22% and 33 to 41% respectively, were the main urinary metabolites over a period of 3 to 4 hr in kidneys treated with 10 and 40 mg/kg hirudin. The peak excretion of these metabolites in urine was in a dose proportion manner and was 45% and 56 to 68% at 10 and 40 mg/kg doses during 3 to 4 hr post dosing period. The unrecovered compound at these doses was 45% and 24 to 35%, respectively.

The [redacted] indicated that CGP 39393 and its metabolites were detected in uniform intensity in whole kidney samples but these were concentrated in epithelial and brush border of proximal and distal tubules of all the samples. Thus endocytosis at the brush border ends and degradation of lysosomes were the sites of accumulation.

Table 12

Semiquantitative balance of CGP 39 393, hir(1-50) and hir(1-52) in perfusate plasma and urine as % of dose.

compound	amounts of CGP 39 393, hir(1-50) and hir(1-52) in perfusate and urine [% of dose]							
	experiment 1 10 mg/kg a)		experiment 2 6 mg/kg		experiment 3 40 mg/kg a)		experiment 4 40 mg/kg	
	plasma 3 h	urine 0 - 3 h	plasma 4 h	urine 0 - 4 h	plasma 3 h	urine 0 - 3 h	plasma 4 h	urine 0 - 4 h
CGP 39 393 (A_t)	28.2	nd	23.2	nd	26.6	nd	23.0	nd
hir(1-50)	nd	12	nd	15	nd	25	nd	22
hir(1-52)	nd	3	nd	7	nd	15	nd	11
balance of CGP 39 393 and metabolites in plasma and urine at end of experiment ($A_t+A_{u(0-t)}$)	43		45		67		56	
proportion of dose removed from perfusion by sample collection ($A_{sc(0-t)}$)	14		9		9		9	
unrecovered proportion of dose ($A_{tu(0-t)}$) b)	43		46		24		35	

nd not detected

a) the balance was calculated for the time point 3 h since the last measured time point in plasma and/or urine was 3 h.

b) this dose proportion was assumed to have been taken up into the kidney by tubular reabsorption (endocytosis) and degraded in the endolysosomes of the tubular cells.

This study indicated that CGP 39393 was excreted mainly through kidneys in the rats. The results of the study could give only qualitative data because the viability of isolated kidney was good for about 2 hr.

13. **Renal Excretion and Biotransformation of an I.V. Dose in the Dog**: (Study # DMET(EU)1/1995, Protocol Test 94-7013)

Methods: The study was conducted to characterize the metabolism of the i.v. administered compound in the dogs. This study was conducted in 2 random-bred pedigree male beagle dogs by administering 1 mg/kg CGP 39393 in 3% aqueous mannitol as a slow i.v. injection in cephalic vein. Blood samples (4.5 or 9 ml) were collected at 0, 1, 5, 10, 20, 40, 70, 150 and 240 min after the administration of the compound and urine samples at 0-4, 4-8 and

8-24 hr of the administration of the compound. The compound was estimated by HPLC———————— in plasma and urine.

Results:

———— analysis method was used on urine CGP 39393 samples fractionated by HPLC technique. The compound having Mr 6969.3 (CGP 39393) was identified. The compound was excreted from 64 to 74% in urine within 4 to 8 hr. No plasma metabolites were detected in this study.

In conclusion, an intravenous bolus injection of CGP 39393 was seen to have a half life of 26.4 min in rats. Subcutaneous injection of CGP 39393 attained plasma peak levels within 50 min in rats. In dog, subcutaneously administered CGP 39393 produced rapid plasma peak but for a short time with half-life of about 42 min. CGP 3939 prolonged APTT by 2.2 to 3 times in rats but bilateral nephrectomy further increased APTT by >1.3 times. It was due to its impaired clearance. Intravenously or subcutaneously administered CGP 39393 was excreted in 4 hydrolyzed metabolites (not fully identified) in kidneys and only a small amount was excreted in rat bile. In dogs, it was excreted rapidly and mostly detected in cortical portion of proximal or distal tubules and it was not detected after 6 hr in kidneys. The bioavailability of subcuataneously administered CGP 39393, in comparison to intravenous route, was 83 and 86 to 90% in rat and man, respectively.

The comparison of PK parameters of Intravenously administered CGP 39393 in animals and man are shown below:

	Rat	Dog	Monkeys	Man
Dose	1 mg/kg	1 mg/kg	1 mg/kg	1 mg/kg
t 1/2 alpha (min)	6.6	5.6	--	--
t 1/2 Beta (min)	26.4	42.3	45	--
tmax (hr)	0.25-0.5	0.25	--	2.0
$AUC_{(0\text{-}Infin.)}$ (pmol-h/ml)	193	760	420	211
Cl(tot.) ml/min Kg	12.39	3.17	5	28-171

[Limit of detection 2 pmole/ml for urine & 1 pmole/ml for plasma]

TOXICOLOGY:

ACUTE TOXICITY

1. **Acute Toxicity Study in Rats and Dogs:**

GLP Requirement: A statement of compliance with Switzerland GLP regulation and quality assurance was included.

Methods: The acute toxicity of CGP 39393 was studied in Tif:RAIF rats (n=3/sex), and in a single male beagle dog. All animals were given a single i.v. injection of CGP 39393 in 5% dextrose. A dose of 100 mg/kg of CGP 39393 (10 ml/kg) was given to rats, and the dog (n=1) was given 30 mg/kg CGP 39393. All the animals were observed over a period of 14 days. Body weights of the rat were recorded predose and on days 3, 7, 10, and 15. At the end of the observation period, all rats were sacrificed and subjected to complete necropsy. In the dog PT, PTT and TT were evaluated predose, and 0.5, 1, 2, 3, 4, 5, 7 and 24 hr after dosing, food consumption were recorded daily and body weight weekly.

Results: None of the rats included in the study died, and no clinical signs were observed except for blue coloration of the tail and trace of blood at the injection site in six of six rats. A trace of blood was also observed in one ear in one out of six rats lasting up to 3 hrs post dose, sponsor claimed that body weight gain over the 14-day period was not affected, yet they failed to include "no drug" control group. No gross changes were observed at necropsy (data not shown). In dog, at all but the 24 hr blood sampling time points, a trickling of blood was evident at the site of puncture. Traces of the blood were also observed in the feces. PT was elevated for the first 4 hr as compared to predose values. PTT and TT were elevated for the first 7 hr as compared to predose values; with TT being affected the most. All these parameters returned to predose levels at the end of 24 hr after dosing. Body weights and food consumptions of the animals were not affected.

In the acute toxicity study, sponsor used a single dose level of 100 mg/kg in rats (n=6) and single dose level of 300 mg/kg in dogs (n=1). The studies did not yield useful information such as minimum lethal dose, maximum non-lethal dose and clinical signs of toxicity were not established. Hence sponsor has not conducted a valid acute toxicity study.

2. **Acute I.V. Toxicity Study in Mice and Rats**
(Report # 90063 and 90052)

Methods: The acute toxicity of CGP 39393 (Lot 810189) after a single dose administration was studied in mice and rats. Desired concentration of CGP 39393 was dissolved in 5% mannitol. No control animal group was included in the mice study, while control rats received i.v. injection of 5% mannitol. Highest dose tested in mice was 1600 mg/kg (volume of administration = 50 ml/kg), and in rats was 332 mg/kg (volume of administration = 10 ml/kg). Due to limitations of concentration and volume of administration, higher doses of CGP 39393 were not tried. All animals were observed for toxic signs and mortality for 14 days. At the end of observation period, all the animals were sacrificed and subjected to complete necropsy.

Results:

All animals (mice & rats) survived the treatment. No drug related toxic signs were evident in mice and rats and gross pathological examinations were normal. The LD_{50} value for mice was greater than 1600 mg/kg and the highest non-lethal dose for mice was 1600 mg/kg. In rats, the LD_{50} value was greater than 332 mg/kg and the highest non-lethal dose for rats was 332 mg/kg.

Acute Toxicity Study of CGP 39393 in Mice and Rats

Species (Strain)	No/sex group	Dosage Used (mg/kg)	LD_{50} (mg/kg) Males	Females	Highest Non-lethal Dose (mg/kg) Males	Females
Mice [Tac: (sw)fBR]	5	810 and 1600	--	--	1600	
Rats (Sprague-Dawley)	5 & 10	0, 85, 332	--	--	322	

3. **Acute I.V. Toxicity Study in Monkeys**: (Reports # 91-6016)

Methods: The acute toxicity of CGP 39393 (Lot 15/317/1) after a single i.v. (slow bolus, 10 ml/min) administration was studied in cynomolgus monkeys. Desired concentration of CGP 39393 dissolved in 4% mannitol. No control animal group was included in this study. One animal/sex were given 100 or 300 mg/kg of CGP 39393. All animals were observed for toxic signs and mortality for 14 days. At the end of observation period, all the animals were sacrificed and subjected to complete necropsy.

Results: All animals survived the treatment. No drug related toxic signs except dose related contusions on the hind limbs and or pelvic areas were seen in treated animals. In monkeys, the highest tested dose (300 mg/kg) was non-lethal.

SUBACUTE/SUBCHRONIC TOXICITY:

1. **1-Month Subcutaneous Tolerability Study in Rats**: (Study # 87-6256)

Testing Laboratory: Ciba-Geigy Ltd. Pharma Toxicology, Basel, Switzerland

Study Started: March 14, 1988 (initial group)/June 6, 1988 (Supplementary groups)

Study Completed: March 22, 1988 (initial group)/July 4, 1988 and August 2, 1988 (supplementary groups).

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

Animals: 6-8 weeks old Tif: RAIf [SPF] male (195-2769) and female (154-2349) rats.

Methods: In an exploratory 10-day subcutaneous tolerability study in rats, CGP 39393 was given a daily subcutaneous dose of 60 mg/kg (1 ml/Kg) for 10 consecutive days. Two of five rats died (days 8 & 10), cause of deaths was not determined. A marked hematoma at the injection site was seen in all animals from day 3 onwards. Sponsor concluded that there was no effect on body weight gain, yet they failed to, include "no drug" control group. The hematological parameters indicate anemia with reticulocytosis in one of three remaining rats along with increase in thrombin time, but due to the lack of proper control and sufficient number of animals tested it is difficult to ascertain the above mentioned findings. Upon microscopic examination, dilated blood vessels with thrombus formation and inflammation were seen at the injection sites. From this so called "dose range finding study"; the sponsor arbitrarily has chosen a single dose of 60 mg/kg for 1-month subacute toxicity study. Thus in the present study, a group of 15 male and 15 female rats (initial group) were given s.c. dose of 60 mg/kg (10 ml/Kg) for 7 days. The original treatment period was planned for 1 month, but the treatment was stopped after, 7th injection due to substantial blood loss at the injection sites and subsequent mortality. Thus in the initial group, nine of thirty (5 males and 4 females) died or had to be killed during the first week of treatment. Blood was taken from the remaining 21 rats at 24 hours post 7th dose, and all the rats were killed and necropsied. Another experiment was started (supplementary group). A second group of 15 male and 15 female rats were given s.c. dose of 20 mg/kg (1 ml/kg) for 28 days, a control group of rats (15 male and 15 female) was also evaluated. Control groups were injected with equivalent volume of the vehicle (5% dextrose). At the end of the treatment period all the animals were killed except 5/sex/group, which were observed for one additional month without treatment (recovery period). Clinical signs were observed twice daily, body weights daily during the administration period, 3 times per week during predose and recovery periods, food consumption two times per week. Blood chemistry, hematology and coagulation profile tests were performed on day 8 in the initial group and pretest and at the end of treatment and at the end of recovery period in the

Supplementary group. Urinalysis, eye examination, hearing test, and hemoccult test in feces were also performed at various time points. At the end of the treatment period or after the recovery period of 1 month (5/sex/dose), all the animals were sacrificed and subjected to complete necropsies.

Results:

1. **Observed Effects**: At 60 mg/kg and 20 mg/kg, clinical signs included s.c. hemorrhage in every rat, ventricumbency, paleness, hypothermia, irregular respiration, unkempt appearance, and reduced spontaneous activity. An improvement occurred during recovery period in 20 mg/kg dose group.

2. **Mortality**: In group receiving 60 mg/kg/day CGP 39393, there were 9 deaths (5 males & 4 females) out of 30 animals, during the first week of treatment, treatment was terminated after the 7th injection and the treated and the control rats were killed and necropsied on day 8. In the group receiving 20 mg/kg, four of thirty animals (2 males and 2 females) died.

3. **Body Weight/Food Consumption/Water Consumption**: There were no significant effects on body weight, food consumption, or water consumption.

4. **Hematology/Coagulation/Bone Marrow:** Marked anemia with severe reticulocytosis and thrombocytosis were noted in the 60 mg/kg dose group following 24 hr after the 7th injection. The same parameters were also altered but to a much lesser extent in the 20 mg/kg group after 28 days of treatment. All those parameters returned to baseline value at the end of the recovery period. Prothrombin time was not influenced by the treatments. Partial thromboplastin and thrombin times were erratic in rats. No occult blood was found in feces.

5. **Blood Chemistry/Urinalysis**: At a dose of 60 mg/kg, there were a significant elevation in bilirubin levels, 224% and 183% for male and female rats respectively compared to the corresponding control values. Also albumin levels were decreased only by 12% and 17% in male and female animals respectively, when compared to its corresponding control values. Urinary parameters were not affected by the treatment at either of the doses.

6. **Vital Signs/Physical Examination/Ophthalmic Examination**: Auditory activity and ocular changes were not affected by the treatment.

7. **Organ Weight Changes**: At 60 mg/kg, mean spleen weights were increased by 120% and 155% in male and female rats, respectively and the mean axillary lymph nodes weights were increased by 133% and 150% for male and female respectively. At 20 mg/kg no significant deviation in organ weights were found.

8. **Gross Pathology**. At the injection sites of both 60 and 20 mg/kg the treated rats reddening and hemorrhage were seen. At 60 mg/kg, thymus and axillary lymph nodes of some rats had reddened areas and spleen of some treated rats were increased in size.

9. **Histopathology**: The injection sites of all treated rats showed massive hemorrhage and inflammatory processes, thrombus formation, edema, dilation of the blood vessels, thrombus formation and fibrinoid necrosis of the vein wall and, extramedullary hematopoiesis in the spleen was reported. In addition at 60 mg/kg, slight dilated sinuses, numerous macrophages and a few erythrocytes were found in the axillary lymph nodes. Some of the microscopic findings at the injection site and spleen were still present in the recovery animals, but to a lesser extent. No internal hemorrhages were noted in any organ (including the brain) or tissue other than at the immediate sites of injection.

In summary, s.c. administration of CGP 39393 in rats at dose level of both 60 (7 days) or 20 (for 28 days) mg/kg/day CGP 39393 resulted in some mortality due to severe blood loss and anemia as a consequence of the strong anticoagulant potency of the compound. This potency was less expressed at 20 mg/kg. In the dose range finding study, sponsor had used a single dose of 60 mg/kg/day and did not include "no drug" control group, then arbitrarily selected a single dose of 60 mg/kg for 1-month toxicity study. Due to high mortality during the first week of treatment and in anticipation of substantially more death, this initial group treatment was terminated after 7th injection. In the main study (supplementary group), sponsor has reduced the daily dose to 20 mg/kg arbitrarily. The sponsor has not conducted a valid subacute toxicity study, the 'highest no effect dose' and maximum tolerated dose levels were not established.

2. 3-Month S.C. Tolerability Study in Rats

Testing Laboratories: Ciba-Geigy Ltd.
Basel, Switzerland

Study Started: May 22, 1989

Study Completed: September 21, 1989

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

Animals: Six to eight weeks old male (203-281 g) and female (154-236 g) Albino rats [Tif: RAIf (SPF)].

Drug Batch No.: 810388

Methods: Groups of 10 male and 10 female rats were given s.c. injection of CGP 39393 at daily doses of 0, 2.5, 5 and 10 mg/kg/day for 90 days. The control group received vehicle (glucose 45 mg/kg + 5 mg/kg PEG 4000 + 2.5 mg/g mannitol). Two additional groups (5 sex/group) received vehicle and 10 mg/kg/day CGP 39393 for 90 days and followed for 28 days of recovery period. An additional control group (7/sex) was also included and received 50 mg/kg of glucose for 90 days. All animals were observed for clinical sign(s) twice daily. Body weights were recorded 3 times/week during pretest period and recovery period, daily during the treatment phase. Food consumptions were recorded twice per week. Water intakes were recorded on week 1, 4, 8, 13 and 17. Hearing tests were monitored once prior to beginning of the study and at week 13 and 17, ophthalmic examinations were performed once prior to commencement of the study and at week 6, 13 and 17 of the study. At the end of the study period all animals were sacrificed except the animals in the recovery group, which were sacrificed at the end of 28 days of the recovery period. All animals were subjected to complete necropsy.

Results:

1. **Observed Effects**: Hematomas at the injection sites were seen in treated animals, and this was dose related. At high dose, one male had very large hematoma and one female had severe hemorrhage after blood sampling. No hematomas were observed in the control group animals.

2. **Mortality**: Three animals were killed in extremis (1 in 2.5 mg/kg group and 2 in 10 mg/kg group). The female animal at low dose had poor health, and at necropsy it was found that her esophagus was filled with sawdust. The two animals at high dose had large hematomas and severe hemorrhage at the injection site.

3. **Body Weight/Food Consumption/Water Consumption**: At the end of treatment period, the body weight gains were reduced by approximately 7% at high dose, compared to the control values. There were no significant effects on food and water consumptions.

4. **Hematology, Coagulation/Bone Marrow:** No significant effect(s) were observed.

5. **Blood Chemistry/Urinalysis**: No treatment related effects were seen.

6 **Vital Signs/Physical Examination/or Ophthalmic Examination/Hearing Test**: No treatment related changes were noted.

7. **Organ Weights**: No treatment related effects were observed.

8. **Gross Pathology**: Apart from the lesions at the injection sites, no other changes were observed at necropsy.

9. **Histopathology**: Hemorrhages were noted at the injection sites of the treated animals. Large hemorrhages were observed more often in female than male rats. These hemorrhages were accompanied with granulation and fibrous tissue and inflammatory cells. After the recovery period minimal to slight fibrosis with few inflammatory cells, and microscopic hemorrhages were noted in the treated rats. No other treatment related histopathological findings were observed in this study.

In conclusion, no clinical signs were observed except hematomas at the injection sites were seen in treated animals, and this was dose related. Hemorrhages were noted at the injection sites of the treated animals. Large hemorrhages were observed more often in females than male rats. These hemorrhages were accompanied with granulation and fibrous tissue and inflammatory cells. After the recovery period minimal to slight fibrosis with few inflammatory cells, and microscopic hemorrhages were noted in the treated rats. No other treatment related histopathlogical findings were observed in the study. No specific target organ of toxicity was identified except dysfunction of hemostasis mechanism and 'no effect dose' was established.

DOGS:

3. **7-Day I.V. Toxicity Study of CGP 39393 in Beagle Dogs**: (Report # 90077)

Testing Laboratories: [redacted]

Study Started: January 3, 1990

Study Completed: January 23, 1990

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

Animals: Seven months old male beagle dogs (9.5 - 10.8 kg)

Batch No.: 810189

Methods: Groups of 3 male beagle dogs each were given intravenous infusion (24 hour/day) of CGP 39393 at doses of 0 (vehicle), 1 and 4 mg/kg/hr for 7 days. The vehicle was 4% mannitol and 0.9% sodium chloride in sterile water for injection. The animals of 1 mg/kg/day group actually received 1.1 mg/kg/hr and the animal of 4 mg /kg/hr group received 4.1 - 4.2 mg/kg/hr. All animals were observed for clinical signs daily, body weights were recorded weekly and food consumption was recorded daily. Ophthalmic examinations were performed on all animals once prior to beginning of the study and on day of sacrifice. Blood samples were obtained from cephalic vein following an overnight fasting for hematological and blood chemistry tests from all animals 8 days prior to beginning of the study and on day 8 of the study. Overnight urine samples for urinalysis were also collected from each animal once on day -8 and again on day 8. At the end of the study period, all animals were sacrificed and subjected to complete necropsy.

Results:

1. **Observed Effects:** There was blood in the urine of treated animals (2 out of 3 at low dose on day 8, and 1 out of 3 animals at high dose). Minor bleeding from cuts or abrasions and contusions were seen in all animals at high dose. These effects were attributed to exaggerated pharmacology activity of the drug. There were no overt signs of toxicity.

2. **Mortality:** None.

3. **Body Weight/Food Consumption/Water Consumption:** No significant effects were observed.

4. **Hematology/Coagulation/Bone Marrow:** Slight anemia was seen in all animals belonging to high dose treatment group. This was accompanied by reticulocytosis and erythroblasts in 2 out of 3 high-dose treated animals. In addition, reticulocytosis was noted in 1 out of 3 low-dose animals. On day 8, Fibrinogen, activated partial thromboplastin time (APTT), prothrombin time (PT) were increased in all high-dose animals, and APTT and PT were also increased to a lesser magnitude in all low-dose animals. Only one animal in high dose group had significantly increased thrombin time (Animal #3011: pre treatment = 7.4 sec, post treatment 17.2 sec).

Effects on Hematological Parameters

Parameters	Control		Low Dose		High Dose	
	Pre	Post	Pre	Post	Pre	Post
Hemoglobin (g/L)	13.9	14.0	13.4	13.9	14.1	10.6
Red blood cells ($x 10^6$ cells/ml)	6.01	6.12	5.72	5.93	6.01	4.50
Hematocrit (Hct:%)	39.2	39.9	38.0	39.1	39.7	30.3
Reticulocytes (%)	0.2	0.4	0.2	0.9	0.2	2.1
Clotting Factor						
Fibrinogen(mg/dl)	275	214	309	314	348	699
APTT (Sec)	10.7	10.4	10.4	15.3	9.4	25.4
PT (Sec)	7.3	7.9	7.3	9.3	7.3	30.0
TT (Sec)	8.0	7.8	8.2	10.1	7.2	10.1

5. **Blood Chemistry/Urinalysis**: On day 8, the levels of total bilirubin were increased in treated animals than the corresponding control values (control = 0.17 mg/dl, mid dose = 0.26 mg/dl, and high dose = 0.41 mg/dl). This could be due to the increased red cell loss. Furthermore the total bilirubin values decreased in all animals including controls over the study period pre treatment = 0.65 - 0.73 mg/dl, post treatment = 0.17 - 0.41 mg/dl). No other treatment related findings were observed. Alpha 1 globulin levels were also decreased in treated animals at the end of 8 day (control = 0.51 g/dl; low dose = 0.27 g/dl and high dose = 0.33 g/dl). Again the alpha-1 globulin levels in all animals including controls were decreased over the study period (pretreatment = 0.70 - 0.76 g/dl, post treatment = 0.27 -0.51 g/dl). Few animals in low and high dose group had red blood cells in their urine. Due to small number of animals and the low incidence, no conclusion can be drawn at this time.

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

7. **Organ Weight Changes**: No treatment related effects were seen except liver weights were increased by 1.3 - 18.8% (relative weights: 13.6 - 26%) in treated animals compared to the control values, and this effect was not dose related.

8. **Gross Pathology**: Hemorrhages in the adventitia of the aorta, serosa of prostate, subcutaneous tissue, serosa or submucosae of the rectum, thymus, lung and fat around the infusion site were seen in animals treated at high dose (4 mg/kg/hr). Hemorrhages were also seen in low dose group animals but they were limited to the aortic adventitia of one animal and lung of a second dog. In the lung and trachea hemorrhages were often associated with inflammatory changes.

9. **Histopathology**: Increased erythropoiesis in the bone marrow and extremedullay hematopoiesis in the spleen were observed in 2 out of 3 dogs at high dose level. Congestion and erythrophagocytosis along with medullary plasmacytosis and histiocytosis in lymph node(s)

were found in 3 out of 3 dogs at high dose level and 1 out of 3 dogs at low dose level.

Thus the main findings at the end of 7 days of treatment were related to the pharmacological action of CGP 39393 (altered blood coagulation parameters, anemia, hemorrhage, and increased erythropoiesis). No specific target organ of toxicity was identified, and 'no effect dose' was not established.

4. 1-Month I.V. Tolerability Study in Beagle Dogs: (Study # 87-6257)

Testing Laboratories: Ciba-Geigy Ltd. Pharma Toxicology, Basel, Switzerland

Study Started: April 5, 1988

Study Completed: May 3, 1988

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

Animals: Three male and 3 female beagle dogs (8.6 - 12.1 Kg, 6-10 months old).

Methods: In the 10-day dose range finding study in beagle dogs, CGP 39393 (batch # R HHS-7014) was given intravenously to one male and one female dog at escalating doses of 10 (4 days), 20 (1 day), and 30 (5 days) mg/kg for 10 consecutive days. Both the dogs survived the dosing period. Increased incidences of defecation were observed after the first and second dose escalation and occasional traces of blood were seen at the injection site. Sponsor claimed that there were no treatment-related effects on body weight and food consumption and urinalysis (data not shown). Blood chemistry values for both animals for pre and post drug were within normal range. There were minor decreases in red cell parameters, and coagulation parameters were elevated after dosing, particularly the thrombin time, however all the values returned to normal levels by 24 hr post dose. No drug related pathological changes were observed in those tissues examined (organ weights data, gross/microscopic evaluation data are not shown). In the present study, three males and three female beagle dogs were given 20 mg/kg/day (1 ml/Kg) CGP 39393 (batch # 13/17511) as daily I.V. bolus injection for one month. A control group (3 males and 3 females) was given vehicle (5% glucose). Clinical signs, food consumption were recorded daily, body weight 3 times per week, blood chemistry and hematological measurements were performed pretest and during week 2 and 4 prior to injection. In addition

prothrombin time (PT), partial thromboplastin time (PTT), thrombin time (TT) and fibrinogen were determined at 2 and 4 weeks post 4 hr injection. Urinalysis, eye examination, neurological tests and cardiography were performed pretest and during week 4. At the end of the study all animals were killed and subjected to complete necropsy. It should be noted that the CGP 39393 used in this study has a different batch number than the one used in 10-day dose range finding study.

Results:

1. **Observed Effects**: Slight hemorrhage was noted at the injection site on day 7 and thereafter. Swelling and post traumatic hematoma was present at the injection sites.

2. **Mortality**: None.

3. **Body Weight/Food Consumption/Water Consumption**: There were no significant effects on body weight and food consumption of the animals.

4. **Hematology/Coagulation/Bone Marrow Changes**: Partial thrombin time (PTT) and thrombin times (TT) were significantly increased in samples collected from all dogs 4 hrs post dosing during weeks 2 and 4. Prothrombin time (PT) was only increased in two dogs during week 4 and post dose. A slight thrombocytopenia and elevated fibrinogen were also observed at week 4, along with severe changes in these three plasmatic coagulation parameters (PTT, PT, and TT) in 1 male dog (#180).

5. **Blood Chemistry/Urinalysis**: Blood chemistry parameters were not remarkable except only at week 4, one male dog (#180) had elevated alanine amino-transferase (222 %) and aspartate amino-transferase (267 %), and another female dog (#153) had elevated alanine-amino transferase (505 %). No treatment related changes in urinalysis were observed.

6. **Vital signs/Physical Examination/Cardiography/Ophthalmic Exam**: No treatment related effects were observed.

7. **Organ Weight:** Due to small number of animals and differences in age between control and treated dogs, no organ weight analysis was performed.

8. **Gross Pathology:** Hemorrhage was confirmed at the injection site(s) in all treated animals. Perivenous inflammatory infiltration was observed in three treated dogs.

9. **Histopathology:** Subacute to chronic perivascular inflammation in the heart, liver, lungs and cecum/colon were noted in the male dog (#180).

In summary, a dose of 20 mg/kg/day for 1-month in dogs caused hemorrhages, perivenous inflammatory reaction at the injection sites, increase plasmatic coagulation parameters, slight thrombocytopenia and elevation of fibronogen. Blood chemistry changes were limited to male dog (#180) and female dog (#153) and only at week 4. In this single dose group study no useful information could be found, the 'highest no effect dose' and 'maximum tolerated dose levels' were not established.

5. 3-Month I.V. Tolerability Study in Dogs
(Study #89-6052/VP 32/1989)

Testing Laboratories: Ciba-Geigy Ltd.,
Basel, Switzerland

Study Started: June 2, 1989

Study Completed: October 2, 1989

GLP Requirement: A statement of compliance with GLP Regulations and quality assurance unit was included.

Animals: 9-15 months old beagle dogs (9.6 - 13.9 kg)

Drug Batch No.: 810388

Methods: Groups of 3 male and 3 female dogs were given intravenous (bolus) injection of CGP 39393 at daily doses of 0, 10 and 25 mg/kg/day for 91 days. The control group received vehicle (glucose 50 mg/kg $\pm$ 12.5 mg/kg of PEG 4000 + 6.25 mg/kg of mannitol). An additional control group was also included which received 62.5 mg/kg of glucose. Additionally, 3 male and 3 female dogs were also treated at 25 mg/kg/day for 91 days, and were assigned for recovery studies and followed for 28 days. All animals were observed for clinical sign(s) daily, body weights were recorded once pretest and then 3 times a week, and food consumption were recorded daily. Ophthalmic and ECG examinations were performed once prior to beginning of the study, at week 6 (not performed in the glucose treated group) and at the end of the treatment period and also at the end of the recovery period. Neurological tests (observation of gait) were performed once pretest and at week 13 and 17 of the study. Blood samples were obtained from cephalic vein following an overnight fasting for hematological and blood chemistry tests from all animals once prior to the beginning of the study and at week 5, 9, 13 and 17 of the study. Overnight urine samples for urinalysis

were also collected at the above mentioned time intervals. At the end of study periods all animals were sacrificed except the animals in the recovery group, which were sacrificed at the end of 28 days of recovery period. All animals were subjected to complete necropsy.

Results:

1. **Observed Effects**: No clinical signs were observed except hemorrhages associated with the anticoagulant effect of the drug (exaggerated pharmacological effects). Clinical signs included swollen paws, blood in feces, decreased spontaneous activity, bleeding injuries and hematomas.

2. **Mortality**: There were 4 deaths (2 in 10 mg/kg/day treatment group and 2 at 25 mg/kg group) during the experimental period. All deaths were due to preterminal bleeding.

3. **Body Weight/Food Consumption/Water Consumption Changes**: No significant effects were observed in animals which died (or had to be killed).

4. **Hematology/Coagulation/Bone Marrow**: A moderate to marked anemia (reduced RBC-parameters, granulocytosis, polychromasia, roleau formation) was observed in animals which died or had to be killed (animal # 514 & 521 in low dose group and animal # 479 and 545 in high dose group). A moderate to marked anemia was also observed in all bleeding animals [animal # 439 (10 mg/kg), 520 and 530 (25 mg/kg)] at various time intervals.

Effects on-Coagulation Parameters (Post 4 hr Treatment

	Control		low Dose		High Dose	
Parameters	Male	Female	Male	Female	Male	Female
Fibrinogen (ng/dl)	162	113	191	178	238	186
APTT (Sec)	11.4	11.6	11.4	13.7	14.2	14.9
PT (sec)	6.4	6.3	7.1	7.2	7.2	7.9
TT (sec)	11.7	12.4	nmd	nmd	nmd	nmd

nmd= Incoagulable samples (longer than 167 sec)

Effects of CGP 39393 were measured 4 hours after injection during the whole administration period. At this time, thrombin time at both dose levels was greater than 167 sec, and APTT and PT values were prolonged dose dependently. However 24 hr after the injection all the values were within normal range.

5. **Blood Chemistry/Urinalysis**: All the animals which died and few animals with excessive bleeding showed impairment of the kidney functions. Some of these animals had marked increases in urea (up to 583%), creatinine (up to 77%), bilirubin (up to 274%) and cholesterol (up to 62%) levels. In the non-lethal case the changes

during treatment period were modest and were reversible. Urinalysis was normal except on week 5 there was marked protinuria in animal # 439 (female: 10 mg/kg).

6. **Vital Signs/Physical Examination/Ophthalmic Examination/ECG/Neurological Examination**: No abnormal effects were observed.

7. **Organ Weight**: No treatment related effects were seen.

8. **Gross Pathology**: Most of the findings were observed in 4 dogs (#514, 521, 479 and 545) which had to be killed in extremis or which died during the study period. All these animals had severe hemorrhages. The most affected organs/tissues were heart, urinary bladder, thymus, kidneys, G.I. tract, lymph nodes and injection sites. Furthermore, all the treated dogs had hemorrhages at the injection sites.

9. **Histopathology**: Vasculitis often accompanied by parivasculitis and fibrinoid necrosis of the vessel wall, inflammatory cell infiltration, edema, fibrin deposition and hemorrhage were observed in 4 dogs which had to be killed in extremis or which died during the study period. One dog of the low dose group (# 439) had vasculitis with intimal thickening, hemosiderosis, focal fibrosis in one heart auricle, perivasculitis accompanied by focal fibrosis in the thymus and slight inflammatory reaction in the kidneys. Fibrin deposition, edema, inflammatory cell infiltration (purulent or non-purulent), and granulation at the injection sites were much more severe in treated dogs than in the control dogs.

10. **Plasma Levels**: There was no accumulation of the drug after multiple daily administration of the drug. In few animals (2 at each dose levels) elevated plasma baseline concentration of CGP 39393 was observed, and resulted in uncontrolled bleeding and deaths.

11. **Immune Response**. In two series of experiments (which gave the same results), 14 out 18 dogs had elevated titers of antibodies against CGP 39393 on day 25. The vasculitis observed in same treated animals is considered to be due to the immunogenic potential of the drug in dogs. The titters of animals in the follow-up group all decreased to about the same level, irrespective of the titters on day 25.

In conclusion, no clinical signs were observed except hemorrhages associated with the anticoagulant effect of the drug (exaggerated pharmacological effects). There were 4 deaths (2 in 10 mg/kg group, and 2 in 25 mg/kg group) during the experimental period due to preterminal bleeding. Vasculitis was observed in 14/18 treated dogs and was considered to be due to the immunogenic potential of

the drug in dogs. No specific target organ of toxicity was identified except dysfunction of homeostasis mechanism, and 'no effect dose' was not established.

ADDENDUM TO STUDY BY THE REVIEWER:

1. In supervisory pharmacologist comments, Dr. Choudary stated that the 3-month i.v. toxicity study in dogs confirmed the immunogenicity of CGP 39393. Hemorrhage and inflammatory cell infiltration at the site of injection, increase in BUN, creatinine, bilirubin and cholesterol levels and proteinuria were reported in preterminally sacrificed dogs. Purulent myocarditis and fibrosis were seen 1/sex animals of 10 mg/kg/day treatment group and 1 female of 25 mg/kg/day treatment group. These changes suggested kidneys, liver and heart as the target organs of toxicity.

2. A partial report (#B33/1991: an amendment # 088 and its review dated 9/28/92) on the nature and quantity of the antibody complex indicated that concentration of antibody complex in plasma samples of dog # 404, 520 and 530 at 4 hr after the 44^{th} dose were 45.3, 31.8% and 14.1% of AUC values, i.e., mean of 30.4%. From 4 to 24 hr after the drug administration on day 44, the free drug plasma concentration decreased by about 50% in dog #404 while CGP 39393-antibody complex concentration expressed as % of AUC was increased and varied from 45.3% to 82.1%. A report on humoral immune response in animals (amendment dated December 6, 1993 and review date December 27, 1993) showed that 15 out of 18 dogs (5 out of 6 from low dose group and 10 out of 12 from high dose group) had significant increased levels of antibodies titers against CGP 39393. This report did not provide the information whether the circulating antibodies were confined in plasma or in other dog organs.

3. Sponsor submitted a report on the plasma levels of CGP 39393 (free and complex with antibodies) in 25 mg/kg/day CGP 39393 treatment group animals of the study. The blood samples were collected after 43, 44, 67 or 79 days of treatment. The calculated $t_{1/2}$ of antibody complex with CGP 39393 was 22.6 hrs, much longer than the $t_{1/2}$ of the native drug. The half lives of the compound on study day 44 in animals of 10 and 25 mg/kg/day treatment groups were 3.8 to 4.1 hr and, 4.5 and 4.5 hr for both groups of the animals on study day 89. The half life of a single dose was about 1.2 hour suggesting an accumulation of CGP 39393 in plasma after its prolonged treatment. The most of the compound was found in the form of antibody complex, a biologically active entity. From this report two conclusions were made: (1) The mean concentrations of CGP 39393 antibody complex in plasma samples of dogs increased after prolonged treatment (i.e., at 4 hr after 44^{th} dose about 30.4% which increased to from 45.3% to 82.1% after 89^{th} dose) and (2) From 4 to 24 hours after the 89^{th} dose of drug, the free drug plasma

concentration decreased by about 50%. Sponsor did not provide the information on the nature of compound antibody complex in other dog organs.

MONKEYS:

6. **14-Day I.V. Continuous Infusion Toxicity Study in Monkeys:** (Report # 52433)

Testing Laboratory: [redacted]

Study Started: May 29, 1991

Study Completed: July 24, 1991

Report Date: February 13, 1992

GLP Requirements: A statement of Compliance with GLP regulation was included.

Animals: Young adult Cynomolgus male and female monkeys (males: 2.8 - 4.9 kg and females: 2.5 - 3.5 kg)

Drug Batch #: 15/317/1

Methods: Groups of monkeys (3/sex) were given continuous I.V. infusion of 1.0 and 4.0 mg/kg/hr CGP 39393 for 14 days. The rate of infusion was 1 ml/kg/hr. The control group monkeys received the vehicle (4% mannitol in 0.9% saline) in similar fashion. All animals were observed twice daily for clinical signs and body weights were recorded twice weekly. Opthalmoscopic examinations were performed on all animals once pretest and once just before sacrifice. Blood samples were collected once during the treatment period and at the end of study period for hematological and serum chemistry tests. At necropsy, urine samples from bladder of few animals were collected for urinalysis. Additionally, blood samples (2 ml) were also collected from each animal immediately after the first, sixth and fourteenth day or preterminally for determining plasma levels of the drug. Furthermore, the results of plasma levels were reported as an addendum. All sacrificed animals were subjected to complete necropsy and histopathological examinations.

Results:

1. **Observed Effects:** Contusions on the hind limbs and/or abdominal regions were seen in treated monkeys. A rapid deterioration of the body conditions (cold to the touch, paleness, and weakness) was seen in high dose treated animals. Furthermore, due to severity of

the clinical signs, treatment of one low dose group male monkey (# 2021M) was stopped on day 8 of the study and the treatment of two monkeys belonging to high dose group (# 3122M & 3632F) were stopped on day 1 (21.62 hrs of infusion) of the study. These animals recovered and sacrificed at the end of the study period.

2. **Mortality**: A total of 6 animals died/sacrificed (control -1/6, low dose 1/6 and high dose = 4/6). The death of the control group animals was not treatment related, while the deaths in treatment groups were drug related.

3. **Body Weight/Food Consumption/Water Consumption Changes**: No treatment related effects were seen.

4. **Hematology/coagulation/Bone Marrow**: In all treated monkeys, the prothrombin (P.T.) and activated partial thromboplastin (A.P.T.T.) times were increased dose dependently. At 4 hr. after the start of infusion, the P.T. were 10.1 ± 0.21, 14.8 ± 1.5 and >30.0 sec. in control, low and high dose group animals, and corresponding A.P.T.T. values were 16.4 ± 0.7, 52.6 ± 8.5 and >60.0 sec. respectively. This finding was evident at all sampling time points. Additionally, fibrinogen levels were decreased significantly compared to control values in high dose treated monkeys. This exaggerated pharmacological effect adversely affected the red cells parameters (decreases in red blood cell counts, hemoglobin and hematocrit and increased reticulocytes).

5. **Blood Chemistry/Urinalysis Changes**: Two males and 2 females of high dose group which died after only 24+ hours of infusion, had abnormal serum chemistry results. Blood samples taken just before death revealed increased BUN, creatinine, glucose, creatinine phosphokinase and phosphorus and decreased cholesterol, total protein, albumin and globulin.

	High Dose Treated Animals			
Parameters	Males		Females	
	Pre (n=3)	Post (n=2)	Pre (n=3)	Post (n=2)
BUN	17.0±1.7	34.5±5.5	17.3±1.5	35.3±2.6
Creatinine	1.1±0.2	1.5±0.3	1.0±0.1	1.7±0.6
Glucose	91.3±21.7	324.0±7.1	77.3±22.2	353.0±7.1
Creatinine phosphokinase	94.0±3.4	2068±79	144.3±101	2168±2627
Phosphorus	5.7±0.9	8.3±0.6	5.7±0.7	12.4±3.3
Cholesterol	137±19	61±7.1	127±2.9	51±047
Total Protein	8.4±0.3	5.6±0.3	8.6±0.1	5.6±0.3
Albumin	4.2±0.3	3.0±0.1	4.1±0.2	2.8±0.3
Globulin	4.2±0.5	2.6±0.4	4.6±0.2	2.9±0.1

Similar results were seen in one of the female monkeys (# 2611) of low dose group that was killed on day 10 of the study.

It should be noted here that only one male and one female of high dose group were sacrificed at the end of the study period but these animals were treated only for one day. Therefore, no meaningful conclusion can be drawn from the results of serum chemistry test. Additionally, low dose treated monkeys terminal serum chemistry data represents the means of 3 males (one of them was treated for only 8 days) and 3 females. No serum chemistry abnormalities were evident in these animals. Urinalysis was normal.

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

7. **Organ Weight changes**: No treatment related effects were seen.

8. **Gross Pathology**: Pale discoloration of the carcass and/or multiple dark areas or foci in many different tissues and/or clot in the abdominal cavity and/or subcutaneous tissues were seen.

9. **Histopathological Changes**: Hemorrhages were seen in abdominal cavity, cecum, jejunum, lymph node (mandibular), salivary gland, seminal vesicle and subcutaneous tissue. Increased erythropoiesis in bone marrow was seen in treated animals (low dose: Males = 1/3 and females = 2/3; high dose: males = 2/3 and females = 3/3). Additionally, 1/3 males of low dose group had extramedullary hematopoiesis in the liver. Thrombosis, phlebitis, intimal proliferation, perivascular inflammation, and hemorrhages were seen at the infusion site in both control and treated monkeys.

The data indicate that two doses used in the study were lethal. Animals died/killed due to the exaggerated pharmacological effects. Sponsor should be asked to repeat the study with lower dose levels in order to define the toxicity profile and establish a no effect dose.

ADDENDUM TO THE STUDY:

In report #B36/1992, sponsor provided the plasma concentrations of CGP 39393 of monkeys including in 7-day continuous i.v. toxicity study. This report was partial and incomplete as the treatment of the high dose treatment group animals was stopped by study day 1 or 2. The data of plasma levels of animals of low dose treatment group could not be used for any toxicity information in monkeys. The study did not provide any useful conclusions for interpreting the compound toxicity in human.

REPRODUCTIVE TOXICOLOGY

1. S.C. Segment I. Fertility and General Reproductive Performance Study in Rats: (Report # 94055)

Testing Laboratories: Pharmaceuticals Division. Ciba-Geigy Corp., Summit, NJ.

Study Started: August 18, 1993

Study Completed: December 22, 1994 (report date)

GLP requirement: A statement of compliance with GLP requirements and quality assurance unit was included.

Animals: Crl: CD (SD)BR] rats (11-12 week old, males: 263-293 g and females: 202-270 g).

Drug Batch No.: 810192

Methods: The dose selection of the study was based on the results of repeat Segment II teratology study in Sprague Dawley rats (study #92099) and Segment III prenatal and post natal reproductive study in Sprague Dawley rats (study #93053). The segment II. Developmental toxicity study was conducted at subcutaneous doses of 0, 5 and 10 or 15 mg/kg/day CGP3939 and, segment III. Prenatal and postnatal toxicity study in rats at 0, 1, 5 and 10 mg/kg/day. In the present study, groups of 12 male and 24 female rats were given 0 (vehicle: 5% mannitol), 1, 5 and 10 mg/kg/day of CGP 39393 subcutaneously. The volume of administration was 1 ml/kg. The male rats were treated from 64 days prior to mating, during mating phase and until they were sacrificed (total duration of treatment: 97 days). Females were treated with 15 days prior to mating and throughout mating, gestation, lactation and till they were sacrificed (day 13 of gestation or day 21 of the lactation). Parents were observed daily for mortality and toxic signs. Body weights and food consumption were recorded weekly. The mating performance and fertility of both sexes were evaluated. About one-half pregnant rats were sacrificed on day 13 of gestation and examined for the number of corpora lutea, number of implants, the number dead or resorbed fetuses and number of live fetuses. The live fetuses were weighed, sexed and examined externally. About 12 dams/group were allowed to deliver spontaneously. The numbers of live/dead pups were recorded, and the live pups were weighed and sexed. The offspring were reared by the dams until weaning. Following delivery, pups were checked for clinical signs, mortality and body weight on days 0, 4, 7, 14, 21, 28 and 35 of lactation. On lactation day 4, litters with more than 8 pups were culled to 4

males and 4 females. During the nursing period, the growth and differential of the pups were observed, and development parameters were assessed (righting reflex, pinna detachment, tooth eruption, eyelid separation, visual and auditory function tests, testes descent, vaginal opening, learning ability test and open field test). On day 21 of post partum all dams were sacrificed and necropsied, and examined as mentioned above. On day 26 of post partum, 1 pup/sex/litter was selected for F_1/F_2 generation study. At 10 weeks of their age they were continuously, mated within each dose group and study was repeated as mentioned above except animals were not treated. F_2 generation rats were examined for abnormalities and then killed right after delivery.

Results: No mortality was seen during study period. Dose related hematomas at the injection sites were seen in all treated dams and in the mid and high dose treated males. Additionally, at necropsy, pale organs were seen in 1 high dose treated dam. No significant effect on body weight gains and food consumption were seen in males or females during the course of the study. Mating rates (control = 96%, low dose = 96%, mid dose = 92% and high dose = 96%) and pregnancy rates (control = 92%, low dose = 75%, mid dose = 83%, and high dose = 83%) were comparable in all study groups.

Dams-Sacrificed at Day 13

There were no significant changes in the pregnancy parameters (number of corpora lutea, pre- and post implantation loss, number of live fetuses).

HIRUDIN: A SUBCUTANEOUS FERTILITY AND REPRODUCTIVE TOXICITY (SEGMENT I) STUDY IN RATS (MIN 931090)

Table 7.15. Summary of the reproductive parameters derived from parental females sacrificed on gestational day 13 (Mean ± Standard Deviation)

Parameter	Dose level (mg/kg/day) control(0)	1	5	10
No. Pregnant females	12	11	9	12
No. Corpora lutea	18.00±2.80	16.09±2.81	18.33±4.27	17.83±2.21
No. Implants	16.83±1.90	14.64±2.66	16.56±2.88	16.50±2.47
No. Resorptions	0.92±1.00	0.18±0.40	0.11±0.33	0.42±0.79
No. Live fetuses	15.92±1.78	14.45±2.62	16.44±3.00	16.08±2.75
Preimplantation loss	1.17±1.70	1.45±1.63	1.78±1.64	1.33±1.30
%Preimplantation loss	5.71±7.74	8.64±9.09	8.63±6.77	7.49±7.51
Postimplantation loss	0.92±1.00	0.18±0.140	0.11±0.33	0.42± 0.79
%Postimplantation loss	5.29±5.44	1.17±2.61	0.79±2.38	2.68±5.02