CENTER FOR DRUG EVALUATION AND RESEARCH

APPROVAL PACKAGE FOR:

APPLICATION NUMBER
21-290

Pharmacology Review(s)

NDA 21,290

REVIEW AND EVALUATION OF TOXICOLOGY DATA

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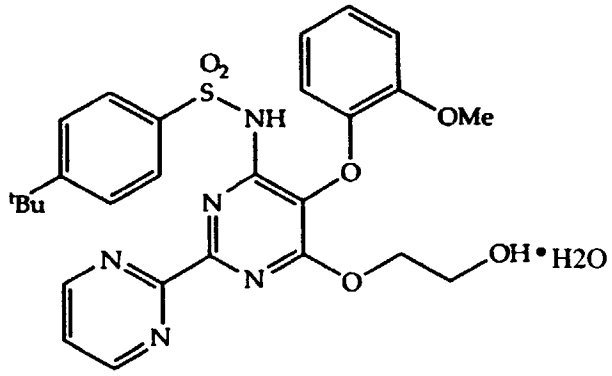
August 30, 2001

SUBMISSION DATE: 11/17/2000
CENTER RECEIPT DATE: 11/17/2000
REVIEWER RECEIPT DATE: 11/22/2000

SPONSOR: Actelion Pharmaceuticals US, Inc.

DRUG: Code Name: Ro 47-0203
Generic Name: Bosentan Trade Name: TRACLEER™
CAS Number: 157212-55-0

Chemical Structure:



Molecular Formula: $C_{27}H_{29}N_5O_6S \cdot H_2O$

Molecular Weight: 569.64 (anhydrate: 551.63)

FORMULATATION AND ROUTE OF ADMINISTRATION: Tablets for oral administration containing 62.5 mg or 125 mg bosentan and the following excipients: corn starch, pregelatinized starch, sodium starch glycolate, povidone , glyceryl behenate and magnesium stearate.

PHARMACOLOGICAL CLASS: Endothelin receptor antagonist

PROPOSED INDICATION: Treatment of pulmonary arterial hypertension

PROPOSED DOSAGE REGIME: The proposed maintenance dose is 125 mg, bid, following an initiation dose of 62.5 mg, bid, for 4 weeks.

IND UNDER WHICH CLINICAL TRIALS WERE CONDUCTED: IND[ ]

NDA 21,290

CHRONIC TOXICOLOGY STUDIES

6-Month Oral Toxicity Study of Ro 47-0203 in Rats

Location of Study Report: Vol 1.11, pg 1

Study Facilities:

Report No: B-159-691
Study Dates: 12/07/93-07/12/94

GLP Compliance: Statement indicates that this study was conducted in compliance with GLP regulations.

Animals: Male and female Wistar rats (Hanlbm strain) about 6 weeks of age at start of dosing; body weights: males, 131-166 g; females, 107-131 g. Rats were housed 2/same sex/cage, and allowed powdered rodent diet and tap water *ad libitum*.

Drug Administration: Ro 47-0203 (Batch GPM 0017) was administered in the diet as a powdered feed admixture. Information was not provided regarding whether the concentration of test agent in the diet was adjusted to maintain a constant intake on a mg/kg basis.

Dose Levels: 0, 40, 200 and 1000 mg/kg/day (18/sex/dose) for 26 weeks followed by a 4-week treatment free period (6 rats/sex/dose) to determine reversibility of any effects. Reversibility was assessed in rats form which plasma drug levels were determined. Doses were selected on the basis of 4-week oral toxicity studies in rats with administration via gavage at 20, 200 and 2000 mg/kg/day or dietary admix at 200, 600 and 1500 mg/kg/day. The control group received plain feed.

Observations/Measurements: Rats were observed daily for mortality and clinical symptoms. Body weight was determined weekly. Ophthalmoscopic examinations were performed during weeks 13 and 26 and at the end of the 4-week treatment free recovery period. Hematology and clinical chemistry values were determined (10 rats/sex/group) during treatment weeks 7, 14, and 26 and at the end of the 4-week recovery period. Urinalysis was performed (in 10 rats/sex/treatment group) on 18 hour urine samples collected during weeks 12, 25, and at the end of the recovery period. Thyroid hormone analyses (not GLP conditions) were performed on the serum of control and high dose rats assigned to toxicokinetic analysis and recovery groups. During week 7, serum T4 and rT3 (males and females) and T3 (females) were determined. Serum T3, T4, rT3 and TSH were also determined in males and females from control and high dose groups in study weeks 14, 24 and at the end of the recovery period. Bone marrow examinations using bone marrow smears collected within 5 minutes of sacrifice were performed on all rats. Rats were sacrificed at 26 weeks of treatment (12/sex/group) or after a 4-week treatment-free period (6/sex/dose) to assess reversibility of effects. Absolute and relative organ weights were determined for adrenal, pituitary gland, thyroid/parathyroid gland, brain, heart, kidneys, liver, ovaries and testes. The following tissues from control and high dose animals were examined histologically: adrenal, aorta, bone, bone marrow, brain, cecum, colon, duodenum, epididymides, eyes, heart, intestinal lymph nodes, jejunum, ileum, kidneys, liver, lungs, esophagus, ovaries, pancreas, pituitary gland, prostate, rectum, salivary glands, sciatic nerve, seminal vesicle, skeletal muscle, skin (from mammary area), spleen, stomach, testes, thymus, thyroid/parathyroid, trachea, urinary bladder,uterus and nose (4 transverse cuts including all the important structures of the nasal cavity).

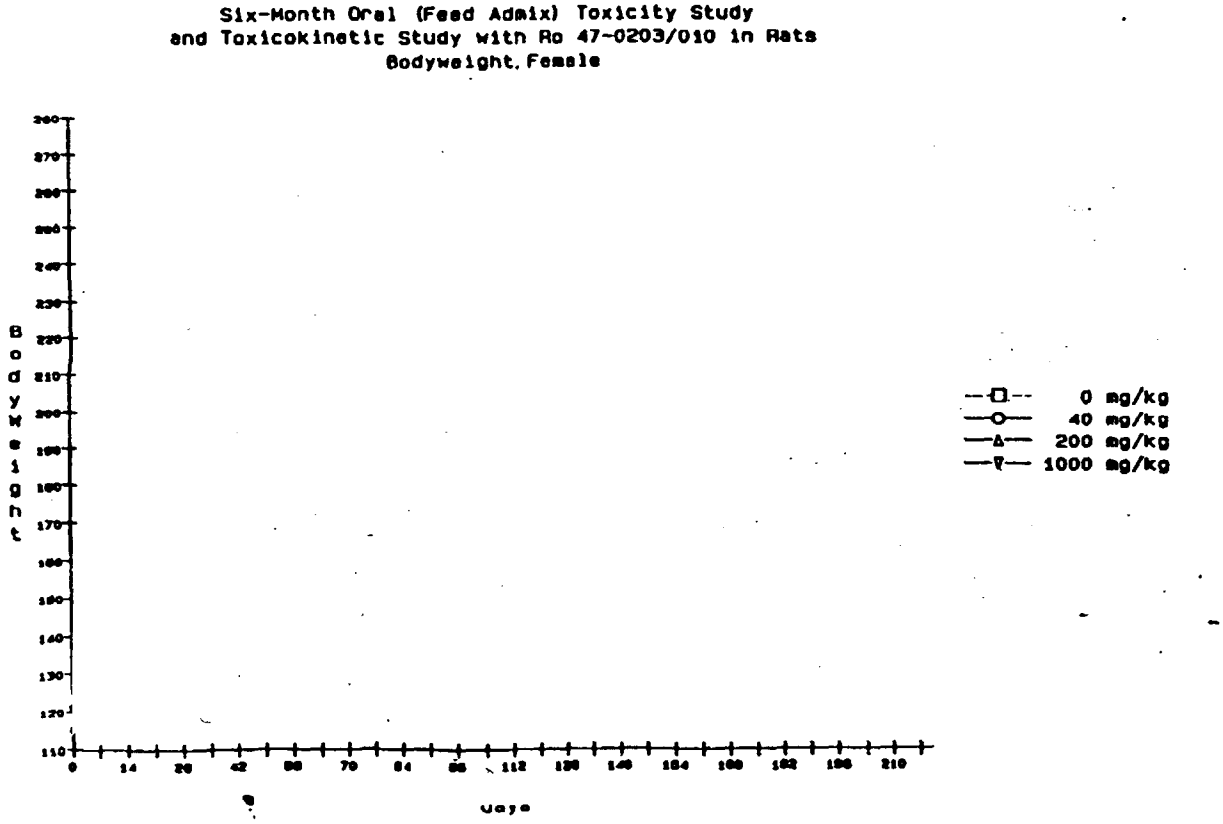
Interim Sacrifice: Two female rats (one control and one from the 1000 mg/kg/day group) were sacrificed in study week 20 to assess for respiratory infection.

Plasma Drug Levels: Determined in 6 rats/sex/group (1 rat/sex/group/time point) during weeks 1, 8, and 26 at 4:00, 7:00 and 11:00 a.m., and 3:00, 6:00 and 11:00 p.m., and during weeks 4, 13 and 19 at 8:00 a.m. Plasma levels of Ro 47-0203 were determined using an assay: limit of quantification in rat plasma of . ng/ml; average interassay precision of 7.7% in the concentration range of ng/ml; interassay accuracy from -4.6% to +5.1%.

Statistics: Dose related effects were evaluated using a rank test (Jonckheere test and Mann Whitney-U-test: Arch Toxicol (1985) 58: 57-58). Adjusted organ weights were determined using an empirically derived constant, b. Sponsor indicates that this correction allows a differentiation between effects on body weight and effects on specific organs.

[Adjusted organ weight (g) = absolute organ weight (g) x (100/body weight)b].

Test agent related findings: There was no test agent related mortality; one control rat died on day 26. Female body weight gain was reduced by 8% at 1000 mg/kg/day compared to concurrent control. Male body weights and body weight gains were not affected by any dose of bosentan.



At week 7, mean hematocrits for male rats receiving 200 and 1000 mg bosentan/kg /day were 6% and 8% lower, respectively, than concurrent control, and mean hematocrit in female rats receiving 1000 mg bosentan/kg/day was 9% lower than concurrent control. Hematocrit was not affected by bosentan at other time point. Reticulocytes in male and female rats given 1000 mg/kg/day were not greater than concurrent control at any time point.

Serum T4 and rT3 levels in female rats given 1000 mg/kg/day were ~37% greater than concurrent control at week 7. Serum T3 level was also increased by this dose of bosentan compared to concurrent control during week 29 (at end of 4-week recovery period). Lower doses of bosentan were not evaluated for effects on thyroid hormones. Serum TSH levels in male and female rats given 1000 mg/kg/day were not greater than concurrent control at any time point.

Bone marrow lymphocytes in female rats at 40, 200 and 1000 mg/kg/day were, respectively, 30%, 20% and 30% lower than concurrent control. After 4-weeks of recovery following 1000 mg/kg/day, lymphocytes were still (36%) lower than concurrent control. These differences in bone marrow lymphocytes were not considered test agent related since they were not dose-related. Bone marrow eosinophils were increased in male and female rats at 200 and 1000 mg/kg in a dose-related way.

Bone Marrow Eosinophils (% Increase from Concurrent Control)

Gender	Dose (mg/kg/day		
	40	200	1000
Male	37	42[a]	80[a]
Female	36	51[a]	118[a]

[a] Indicates significant difference from concurrent control at $P<0.05$

Liver weights relative to body weight at 1000 mg/kg/day were 11% and 12% higher than concurrent control in male and female rats, respectively; absolute liver weights at this dose were 9% and 10% higher in male and females, respectively. Adrenal weight relative to body weight at 1000 mg/kg/day was 23% higher than concurrent control in female rats; absolute adrenal weight at 1000 mg/kg/day was 20% higher. Thyroid weight/body weight ratio was not affected by any dose of bosentan . (Absolute organ weights were not provided in the study report submitted in the NDA. Absolute organ weights were provided in the study report submitted in the IND.)

Bosentan produced a dose related increase in stertorous breathing, particularly in female rats. Severity score was increased at 1000 mg/kg/day compared to concurrent control. No bacteria or virus specific for the respiratory tract was detected in two female rats sacrificed on day 131 to determine if stertorous breathing was related to respiratory tract infection.

Male and Female Rats Combined

Stertorous Breathing	Dose (mg/kg/day)			
	0	40	200	1000
Incidence (%)	5/36[a] (13%)	12/36 (33%)	14/36 (38%)	29/36 (80%)
Mean Severity Score	1.0[b]	1.0	1.0	2.0
Mean Onset (Days)	126	121	122	121

[a] Number of rats exhibiting this finding/total number of rats in treatment group.

[b] Grade 1, trace; grade 2, slight; grade 3, moderate; grade 4, marked; grade 5, severe.

Male and Female Rats, by Severity

Gender	Stertorous Breathing	Dose (mg/kg/day)			
		0	40	200	1000
Male	Total Incidence	4/18[a]	8/18	6/18	11/18
	Severity	all trace	all trace	all trace	8/18[a], trace 3/18, slight
Female	Total Incidence	1/18	4/18	8/18	18/18
	Severity	all trace	all trace	6/12, trace 2/12, slight	5/18, trace 4/18, slight 3/18, moderate 1/18, marked 5/18, severe

[a] Number of rats exhibiting this finding/total number of rats in treatment group.

There were histopathological findings in the respiratory epithelium of the anterior part of the nasal cavity of male and female rats. Nasal goblet cell hypertrophy and hyperplasia were observed in both control and 1000 mg/kg/day treatment males and all female treatment groups. Goblet cell hyperplasia was characterized by the formation of intra epithelial pseudoglandular formation resembling a cryptlike invagination of the pseudoduct. Bosentan at 1000 mg/kg/day increased the severity of nasal goblet cell hypertrophy/hyperplasia in female rats compared to concurrent control.

Inflammation of the respiratory and squamous epithelium of the nasal cavity was noted in one male at 1000 mg/kg/day and in both control and 1000 mg/kg/day treated females. Bosentan at 1000 mg/kg/day increased the incidence and severity of inflammation in female rats compared to concurrent control.

Gender	Dose (mg/kg/day)	Goblet Cell Histopathology: Incidence[a] (Severity Grade)[b]		
		Hypertrophy	Hyperplasia	Inflammation
Male	0	4/12 (1.8)	3/12 (2.3)	0
	40	0	0	0
	200	0	0	0
	1000	4/12 (1.8)	3/12 (1.7)	1/12 (1.0)
Female	0	11/12 (1.8)	6/12 (1.2)	2/12 (1.0)
	40	9/12 (1.3)	4/12 (1.0)	0
	200	10/12 (1.8)	6/12 (1.3)	0
	1000	11/12 (3.1)	7/12 (3.4)	4/12 (1.3)

[a] Number of rats exhibiting this finding/total number of rats in treatment group.

[b] Grade 1, trace; grade 2, slight; grade 3, moderate; grade 4, marked; grade 5, severe.

Four weeks following cessation of treatment at 1000 mg/kg/day, female rats still exhibited goblet cell hypertrophy and hyperplasia compared to concurrent control. However, the severity was similar to that seen in concurrent control, suggesting at least partial reversal of findings.

Female Rats	Dose (mg/kg/day)	Goblet Cell Histopathology During Recovery Incidence (Severity Grade)	
		Hypertrophy	Hyperplasia
	0	2/6 (1.0)	0
	1000	4/6 (2.0)	4/6 (1.5)

[a] Number of rats exhibiting this finding/total number of rats in treatment group.

[b] Grade 1, trace; grade 2, slight; grade 3, moderate; grade 4, marked; grade 5, severe.

Severe (grade 5) tubular atrophy of the testes was observed in rats given 200 mg/kg/day (2/2 rats evaluated), but no atrophy was observed in rats receiving a 5-fold higher dose (0/12 rats evaluated).

Testicular atrophy	Dose (mg/kg/day)			
	0	40	200	1000
Number evaluated	12	0	2	12
Number observed (Severity grade)*	0	-	2 (5, 5)	0

* Severity grade 5 is the highest severity grade.

Thyroid follicular cell hypertrophy and hyperplasia were not observed in male and female rats given bosentan (all doses), or vehicle.

Test agent doses calculated on basis of feed intake approximated target doses.

Dose (mg/kg/day)	Calculated Dose (mg/kg/day)	
	Male	Female
40	41±2	40±3
200	203±11	202±8
1000	1017±57	1012±51

Values shown are means±SD.

Toxicokinetic analysis demonstrated that exposure (AUC) to test agent was dose-related but not dose-proportional, particularly at the highest dose given (1000 mg/kg/day). Exposure in female rats was 2-4 fold greater than in male rats. Exposure remained relatively constant throughout the 26 week treatment period, with the exception of male rats at 1000 mg/kg/day where a decrease in exposure with treatment duration was noted.

Dose (mg/kg/day)	$AUC_{0-24\ hr}$ (ug.h/ml)					
	Male			Female		
	Week 1	Week 8	Week 26	Week 1	Week 8	Week 26
40	13.7	12.9	12.1	43.5	26.4	29.8
200	69.6	61.7	50.0	128	139	173
1000	209	144	98.5	445	542	371

The no observed adverse effect level (NOAEL) was 40 mg/kg/day in male and female rats. In male rats receiving 200 mg/kg/day, elevations in bone marrow eosinophils were noted; at 1000 mg/kg/day, increased liver weight and stertorous breathing were observed. In female rats receiving 200 mg/kg/day, elevations in eosinophils and stertorous breathing were observed; at 1000 mg/kg/day, decreased body weight gain, increased liver and adrenal weights, and nasal histopathology were observed. Although there was evidence of alterations of thyroid hormone levels at 1000 mg/kg/day, this effect was not consistent across time and is therefore considered inconsequential.

Similarly, although hematocrit was 6-8% lower than concurrent control in male rats given 200 and 1000 mg/kg/day, and 9% lower in female rats given 1000 mg/kg/day, this effect was considered inconsequential, and likely unrelated to drug, because of its slight magnitude and the fact that it was only noted at one time point (7 weeks of treatment).

NDA 21,290

12-Month Oral Toxicity Study of Ro 47-0203 in Dogs

Location of Study Report: 1.13, pg 1

Study Facilities:

Report No: RRB-167375
Study Dates: 11/14/95 to 11/15/96

GLP Compliance: Statement indicates that this study was conducted in compliance with GLP regulations.

Animals: Male and female Beagle dogs (8-9 months of age; body weights at start of study: males, 7.5-12.8 kg and females, 6.7-10.7 kg,). Dogs were housed up to 4 per double spaced indoor kennel, and allowed feed and tap water *ad libitum*. The sponsor did not indicate whether dogs were segregated by sex.

Drug Administration: Ro 47-0203 (micronized; Batch 410003A40) was administered orally in gelatine capsules, daily, 7 days/week for 52 weeks. Concurrent control dogs received empty gelatine capsules.

Dose Levels: 0, 60, 180 and 500 mg/kg/day (4/sex/dose)

Observations/Measurements: Dogs were observed daily for mortality and clinical symptoms. Body weight was determined weekly. Ophthalmoscopic examinations and ECGs (conscious dogs) were performed prior to study and at 6 and 12 months of treatment. Hematology and clinical chemistry were determined on venous blood samples taken from overnight fasted dogs. Blood samples were collected prior to study and at 3, 6 and 12 months of treatment.

Erythropoietin levels were determined (non-GLP) at 3 months of treatment. Serum IgG levels in dogs given 500 mg/kg/day and concurrent control dogs were determined (non-GLP) prior to treatment and at 3, 6 and 12 months of treatment. Bile collected at sacrifice was analyzed (non-GLP) for cholesterol, calcium, phosphate, bile acids, sodium, potassium, chloride, phospholipids and glutathione. Urine was collected by catheterization prior to treatment and at 3, 6 and 12 months of study. Dogs were sacrificed at the end of the study and subjected to necropsy. Absolute and relative organ weights were determined for adrenal, brain, heart, kidneys, liver, ovaries, testes, thymus and thyroids. Serum antibody specific to bosentan was measured using serum collected at 6 months of treatment. In this assay, Ro 47-0203 is conjugated with guinea pig albumin, and the ability of serum to bind to this conjugate is monitored using an ELISA assay.

The following tissues were examined histologically from all dogs from all dose groups: adrenal, aorta, bone, bone marrow (sternum), brain, epididymides, esophagus, eyes, gall bladder, kidneys, large intestine (cecum, colon, rectum), liver, lungs with bronchi, mesenteric lymph nodes, ovaries, pancreas, pituitary gland, prostate, salivary glands, sciatic nerve, skeletal muscle, skin (from mammary area), small intestine (duodenum, jejunum, ileum), spleen, stomach, testes, thymus, thyroid/parathyroid, tonsils, trachea, urinary bladder and uterus (corpus).

Four sections of the heart were sampled at necropsy and examined histologically:

right atrium, including valves, coronary vessels and coronary fat;
left ventricle, including papillary muscle and coronary vessels;
right ventricle, including papillary muscle;
septum, including coronary vessels of *facies auricularis*.

Bone marrow smears from the sternum of all dogs were prepared but not examined.

ECGs were monitored in conscious dogs given 500 mg/kg/day and conscious concurrent control dogs prior to treatment and at 6 and 12 months of treatment (leads I, II, III, aVR, aVL, aVF, CV5RL, CV6LL, CV6LU and CV10). It was not stated when ECGs were taken relative to dosing.

Plasma levels of bosentan were determined on day 1 and during weeks 13, 26 and 52 of treatment (the specific day of blood sampling was not provided). Blood samples were taken from all dogs predose and at 1, 3, 7 and 24 hours post dosing. Clearance of bosentan *in vitro* from primary hepatocytes taken at sacrifice after 52 weeks of treatment was determined in one male dog given 500 mg/kg/day and one concurrent control (non-GLP).

Levels of unchanged bosentan and its metabolites Ro 48-5033, Ro 47-8634 and Ro 64-1056 were determined in bile and liver from dogs given 500 mg/kg/day. Samples were taken 24 hours after the last dose of bosentan was administered (Study Report B-167176).

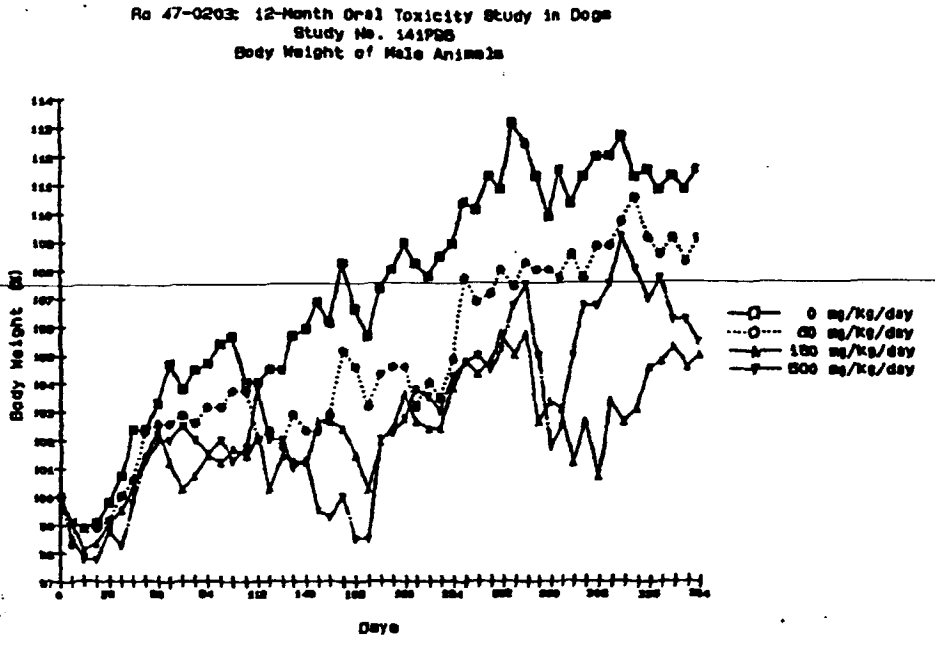
Test Agent Related Findings

One male dog (No. 4611) given 500 mg/kg/day was sacrificed prior to scheduled sacrifice on day 315 (38 days after treatment was stopped) after exhibiting clinical symptoms while on treatment. On day 247, this dog was found recumbent in the kennel 1.5 hours after dosing, was apathetic, sedated and the nictating membrane was prolapsed. The dog appeared dehydrated with enophthalmia and exsiccosis, delayed capillary refilling and pale mucus membranes. Heart rate was 60 beats per minute and arrhythmic, and breathing rate was 20 respirations per minute. An ECG showed sinus bradycardia, (which is normal for conscious dogs). Symptoms were absent the following morning, but test agent was discontinued for 3 days. Treatment was reinstated, and symptoms reappeared twenty five days later (day 276) prior to the morning dose. Clinical chemistries and serum liver enzymes were not monitored on this day. Dog 4611 was again recumbent, appeared anesthetized, showed paddling movements, and exhibited a heart rate of 60 beats per minute. Treatment was again discontinued and the dog recovered, after losing 2 kg body weight. The dog was sacrificed on day 315 after a symptom-free, treatment-free period of 38 days. On day 176 (prior to clinical symptoms) dog 4611 showed marked elevations in several liver enzymes compared to concurrent control and baseline: ALT (25X), ALP (14X), GGT (12X), GLD (30X). Several other clinical chemistry markers of liver function were elevated slightly compared to concurrent control and baseline: AST (2X), total bilirubin (1.4X), triglycerides (3X). On day 247, the day of first appearance of clinical symptoms, liver enzymes were elevated but not to the same extent as on day 92: ALT (3X), ALP (4X), GGT (12X), GLDH (7X), total bilirubin (1.3X), total protein (1.2X). Plasma bosentan level was not determined at this time. Temperature was recorded, and found to be normal (37.8° C). On day 276, the day of reappearance of clinical symptoms, liver enzyme levels and plasma bosentan levels were not determined. Temperature was found to be normal (38°C). Hematology examination of dog 4611 on the day of symptoms (day 247) showed slightly low red blood cell parameters, increased relative count of segmented neutrophils (90%) and monocytes (12%) and complete absence of lymphocytes. These findings were not noted on the scheduled blood sampling days 92 and 176.

One female dog (No. 4454) given 500 mg/kg/day exhibited pale mucus membranes, weak behavior and body weight loss on study day 69. Treatment was discontinued for 7 days. RBC, hemoglobin, PCV and platelets were markedly decreased, and fibrinogen was increased compared to baseline. Minimally increased alkaline phosphatase, GGT and total cholesterol, and decreased bilirubin were noted. Fecal samples taken on day 71 were free of salmonella, shigella or parasites. Dosing was reinstated on day 76 at 180 mg/kg/day. Additional hematology examinations taken on days 73, 80, 87 and 90 showed recovery towards normal. Reticulocytes increased 17 fold within this period. On day 92, the day of the scheduled examination, reticulocytes were still elevated, but other hematology parameters had returned to normal. Bone marrow smears prepared from a biopsy of the *os ilium* on day 78 showed complete erythro- and myelopoiesis and a few megacaryocytes (non-GLP). Serum taken on day 80 was negative for ehrlichiosis. X-ray of the abdomen on day 83 was normal. Treatment at 500 mg/kg/day was reinstated on day 84, and clinical findings did not reoccur.

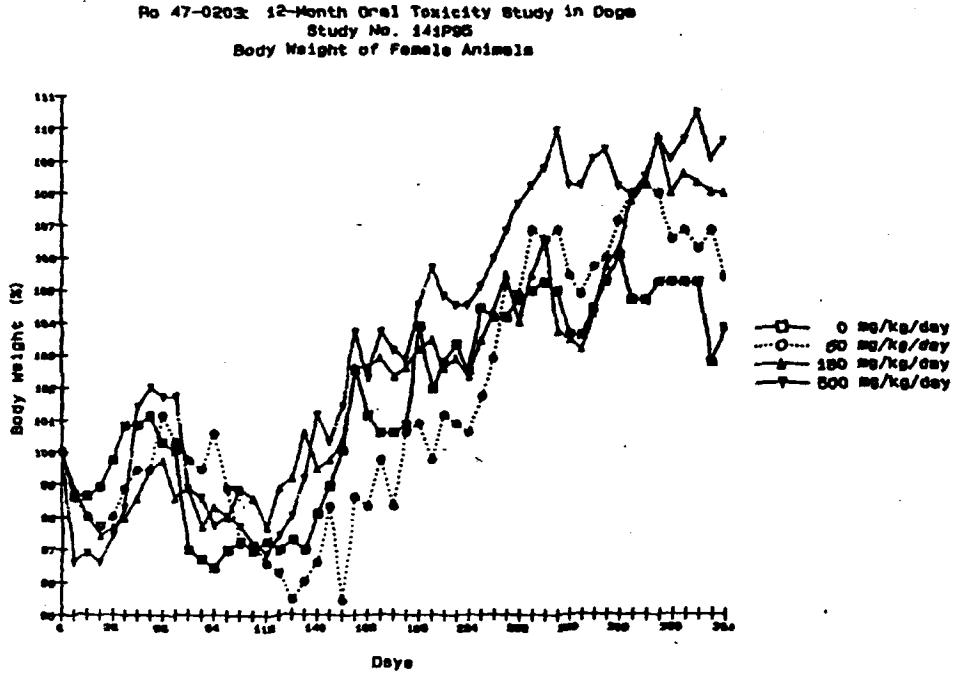
All dogs survived to scheduled sacrifice at 12 months of treatment, with the exception of male dog 4611, which was sacrificed on day 315, 38 days after stopping drug treatment.

Mean body weights normalized to baseline are shown for all treatment groups. Body weights and body weight gain were not significantly affected by test agent.



Male No. [redacted] was killed on day 315.

4611



On day 92, erythrocytes and hemoglobin were lower than concurrent control in males given 500 mg/kg/day. Activated partial thromboplastin time (APTT) was greater than concurrent control in males given 180 and 500 mg/kg/day. Mean reticulocyte counts (and their standard deviations) tended to be greater than concurrent control in males and females given 500 mg/kg/day. Large increases in reticulocytes were seen in one male and female dog given 500 mg/kg/day; male dog 4611 and female dog 4454 showed reticulocyte counts of 18 and 29, respectively. Female dog 4454 also showed lower hemoglobin (7.0 mM) and erythrocyte counts (4.86) than concurrent control.

On day 176, hemoglobin was lower than concurrent control in females given 500 mg/kg/day; APTT was lower than concurrent control in males at 180 and 500 mg/kg/day and in females at 500 mg/kg/day.

On day 328, there were no significant treatment related differences from concurrent control.

Treatment Day	Parameter	Sex	Dose (mg/kg/day)			
			0	60	180	500
92	RBC (10^{12}/L)	M	6.35±0.59	6.56±0.53	6.05±0.43	5.36±0.47*
		F	6.99±0.65	6.50±0.63	6.84±0.82	5.86±0.75
	Hemoglobin (mM)	M	9.20±0.96	9.57±0.81	8.88±0.48	7.97±0.49*
		F	10.55±0.93	9.40±0.87	10.02±1.32	8.48±1.21
	Reticulocyte (0.001)	M	3.25±1.26	3.75±2.99	2.25±1.50	7.75±7.59
		F	7.00±3.46	2.50±0.58	3.50±1.73	9.75±12.84
	APTT (sec)	M	10.25±0.50	9.75±0.50	9.00±0.82*	8.75±0.50*
		F	9.50±0.58	9.50±0.58	10.00±0.00	9.25±0.50
176	RBC (10^{12}/L)	M	6.18±0.42	6.83±0.62	6.27±0.67	5.96±0.18
		F	6.84±0.34	6.26±1.05	6.25±0.49	6.36±0.54
	Hemoglobin (mM)	M	9.38±0.65	10.57±1.02	9.95±1.07	9.00±0.64
		F	10.7±0.55	9.73±1.55	9.77±0.83	9.45±0.87*
	Reticulocyte (0.001)	M	2.00±1.15	3.00±2.16	1.25±0.50	1.75±0.50
		F	2.00±2.00	1.00±0.00	2.75±1.26	2.75±0.96
	APTT (sec)	M	10.50±0.58	10.00±0.00	9.25±0.50*	9.50±0.58*
		F	10.25±0.50	10.25±0.50	9.75±0.50	9.50±0.58*

* $P < 0.05$ compared to concurrent control

Individual hematology values for female dog 4454, which was given 500 mg/kg/day and showed clinical signs of toxicity on day 69, are provided below. Baseline values for this dog were similar to concurrent control. However, on day 69, erythroid parameters and platelets were markedly lower than concurrent control. By day 176, erythroid parameters had recovered spontaneously to values similar to concurrent control. Reticulocytes in dog 4454 were higher than concurrent control on days 73, 90, and 92, and returned to concurrent control value day 176.

Treatment Day*	RBCs (10^{12}/L)	Hemoglobin (mM)	PVC (l)	APTT (sec)	Fibrinogen (g/l)	Platelets (10^9/l)	Reticulocytes (0.001)
0							
69							
73							
80				nd	nd		
87				nd	nd		nd
90							
92							
176							
358							

*Treatment discontinued on day 69, reinstated at 180 mg/kg/day on day 76, and increased to 500 mg/kg/day on day 84.

^ Values for other female dogs *(in italics)* given bosentan at 500 mg/kg/day are provided for comparison since concurrent control values are not available for day 69.

nd, not determined

Individual hematology values for male dog 4611, which was given 500 mg/kg/day and showed clinical signs of toxicity on days 247 and 276, are shown below. RBCs, hemoglobin, PVC and APTT were slightly lower than baseline on days 92, 176 and 247. Reticulocytes were greater than concurrent control on day 92. No data was provided for day 276.

Treatment Day*	RBCs (10^{12}/L)	Hemoglobin (mM)	PVC (l)	APTT (sec)	Fibrinogen (g/l)	Platelets (10^9/l)	Reticulocytes (0.001)
0							
92							
176							
247^							
276^	nd	nd	nd	nd	nd	nd	nd
315^^							

*Treatment discontinued on day 247, reinstated on day 250, and terminated permanently on day 276.
nd, not determined
^Clinical symptoms were observed on days 247 and 276.
^^Sacrificed on day 315 after 38 days off-drug treatment

Erythropoietin levels in dogs 4454 and 4611 at three months of treatment were similar to concurrent control.

Determination of erythropoietin

Dose	Animal No.	Males		Females	
mg/kg/day		predose	3-month	predose	3-month
Control	4637				
	4645				
	4609				
	4631				
500	4649				
	4611				
	4641				
	4627				
Control	4498				
	4458				
	4472				
	4450				
500	4456				
	4448				
	4464				
	4454				

Serum IgG levels in dogs given 500 mg/kg/day were similar to those observed in concurrent controls. Antibody specific for bosentan was not observed in serum from dogs (all dose groups) at 6 months of study.

At 180 and 500 mg/kg/day, mean serum liver enzymes were increased in male and female dogs compared to concurrent control. Alkaline phosphatase was greater than concurrent control at 180 and 500 mg/kg/day at 92, 176 and 358 days of treatment. GGT was greater than concurrent control on days 176 and 358. AST was greater than concurrent control in males given 180 and 500 mg/kg/day, but only on day 358. Variation in ALT, ALP, GGT and GLD in males given 500 mg/kg/day was large on day 176 compared to concurrent control; this large variation is due to large increases in serum liver enzyme levels in dog 4611.

Treatment Day	Parameter	Dose (mg/kg/day)			
		0	60	180	500
92 (n=4)	Bilirubin (Total, μM) M	1.85±0.15	1.59±0.27	1.72±0.46	1.66±0.16
	F	2.14±0.29	1.85±0.20	1.75±0.34	1.81±0.19
	Bile Acid (μM) M	nd	nd	nd	nd
	F	nd	nd	nd	nd
	AST (μkat/l) M	0.55±0.07	0.49±0.04	0.52±0.04	0.48±0.11
	F	0.60±0.09	0.42±0.03	0.43±0.09	0.48±0.03
	ALT (μkat/l) M	0.67±0.14	0.67±0.27	0.56±0.07	0.97±0.68
	F	0.75±0.20	0.47±0.08	0.55±0.20	0.42±0.09*
	ALP (μkat/l) M	1.38+0.49	1.30±0.31	2.43±0.44*	2.63±1.56*
	F	1.27±0.36	1.62±0.71	2.56±0.73*	2.30±0.94*
	GGT (μkat/l) M	0.05±0.00	0.07±0.01	0.07±0.01	0.07±0.01
	F	0.10±0.03	0.07±0.01	0.08±0.02	0.13±0.03
	GLD (μkat/l) M	nd	nd	nd	nd
	F	nd	nd	nd	nd
176 (n=4)	Bilirubin (Total, μM) M	2.13±0.38	2.06±0.46	1.78±0.26	2.11±0.74
	F	2.44±0.33	2.24±0.49	1.88±0.38	1.72±0.32*
	Bile Acid (μM) M	2.92±2.23	3.17±0.63	2.65±1.35	4.78±5.09
	F	4.70±1.44	3.63±2.22	4.72±0.98	4.35±0.30
	AST (μkat/l) M	0.56±0.09	0.51±0.07	0.53±0.11	0.71±0.61
	F	0.50±0.11	0.46±0.08	0.40±0.09	0.46±0.06
	ALT (μkat/l) M	0.69±0.12	0.75±0.34	0.57±0.09	**6.75±12.17**
	F	0.73±0.36	0.50±0.12	0.49±0.21	0.59±0.23
	ALP (μkat/l) M	1.23±0.39	1.33±0.27	2.99±1.21*	**8.65±11.04***
	F	1.37±0.44	2.15±0.88	2.81±1.03*	3.00±1.26*
	GGT (μkat/l) M	0.05±0.01	0.06±0.01	0.07±0.01*	**0.24±0.32***
	F	0.12±0.02	0.13±0.09	0.10±0.00	0.14±0.04
	GLD (μkat/l) M	0.08±0.01	0.09±0.01	0.09±0.05	**0.84±1.41**
	F	0.09±0.02	0.09±0.01	0.08±0.01	0.17±0.14

* P< 0.05 compared to concurrent control.
nd, not determined
Bold indicates large variation due to large increases in serum enzymes in a single dog (#4611) on day 176.
AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GGT, gamma-glutamyl transferase; GLD, glutamate dehydrogenase.

Table: continued.

Treatment Day	Parameter		Dose (mg/kg/day)			
			0	60	180	500
358 (n=3)	Bilirubin (Total, μM)	M	1.89±0.48	1.96±0.35	1.79±0.27	1.67±0.12
		F	1.98±0.57	2.15±0.31	1.76±0.33	1.52±0.46
	Bile Acid (μM)	M	2.92±0.92	2.35±0.42	4.53±1.37	1.50±0.72
		F	2.80±1.40	1.65±0.89	**10.18±9.81^**	**10.82±11.63^^**
	AST (μkat/l)	M	0.53±0.07	0.46±0.06	0.43±0.07*	0.39±0.05*
		F	0.58±0.17	0.50±0.21	0.43±0.12	0.43±0.08
	ALT (μkat/l)	M	0.60±0.13	1.29±0.50	0.74±0.44	0.69±0.21
		F	0.76±0.46	0.48±0.07	0.52±0.25	0.40±0.10*
	ALP (μkat/l)	M	0.99±0.32	1.24±0.28	2.10±0.46*	1.66±0.55*
		F	0.88±0.34	1.13±0.22	1.83±0.50*	1.94±0.71*
	GGT (μkat/l)	M	0.06±0.01	0.07±0.01	0.08±0.02*	0.08±0.00*
		F	0.09±0.01	0.15±0.03	0.15±0.05	0.11±0.08
	GLD (μkat/l)	M	0.06±0.02	0.15±0.11	0.11±0.07	0.10±0.03
		F	0.10±0.06	0.08±0.01	0.08±0.03	0.06±0.02

*P< 0.05 compared to concurrent control.
Bold indicates large variation due to large increases in bile acids in one or two dogs.
^ Individual bile acid values (. 1)
^^ Individual bile acid values [dog 4454])

Serum levels of bone alkaline phosphatase levels were not treatment related at any time point evaluated (data not shown), indicating that the ALP increases noted were due to elevated liver ALP.

Individual values for serum liver enzymes for male dog 4611 are compared to those for other male dogs given 500 mg/kg/day in the following table. Pretreatment serum liver enzyme levels were similar in all dogs. On day 92, ALP for dog 4611 was twice concurrent control. On day 176, dog 4611 exhibited extremely high increases in ALT, ALP, GGT and GLD compared to concurrent control. Enzyme levels returned towards control on day 247, but still were elevated compared to control values seen on day 176. On day 315, (38 days following termination of treatment with bosentan), AST and ALP were only 2 to 4 times greater than control values seen on treatment day 358.

Treatment Day	Liver Parameter	500 mg/kg/day		Concurrent Control	
		Dog 4611	Remaining three dogs at 500 mg/kg/day	Mean ±SD	Highest Concurrent Control Value
Day -6 (Pretreatment)	Bilirubin (Total, μM)		1.35, 1.92, 1.65	1.83±0.29	2.22
	AST (μkat/l)		0.53, 0.65, 0.64	0.53±0.04	0.58
	ALT (μkat/l)		0.56, 0.71, 0.61	0.61±0.11	0.77
	ALP (μkat/l)		1.38, 1.10, 1.88	2.27±0.64	3.02
	GGT (μkat/l)		0.06, 0.09, 0.07	0.06±0.00	0.06
	GLD (μkat/l)		nd	nd	nd
Day 92	Bilirubin (Total, μM)		1.75, 1.66, 1.43	1.85±0.15	2.06
	AST (μkat/l)		0.34, 0.50, 0.48	0.55±0.07	0.57
	ALT (μkat/l)		0.60, 0.61, 0.61	0.67±0.14	0.86
	ALP (μkat/l)		1.44, 1.34, 3.15	1.38±0.49	2.06
	GGT (μkat/l)		0.05, 0.07, 0.08	0.05±0.00	0.05
	GLD (μkat/l)		nd	nd	nd
Day 176	Bilirubin (Total, μM)		1.92, 1.83, 1.50	2.13±0.38	2.45
	AST (μkat/l)		0.36, 0.38, 0.48	0.56±0.09	0.67
	ALT (μkat/l)		0.88, 0.48, 0.65	0.69±0.12	0.83
	ALP (μkat/l)		2.10, 2.50, 4.99	1.23±0.39	1.74
	GGT (μkat/l)		0.08, 0.08, 0.07	0.05±0.01	0.06
	GLD (μkat/l)		0.21, 0.07, 0.12	0.09±0.06	0.17
	Bile Acids (μM)		2.5, 2.1, 2.1	2.7±1.3	4.6
Day 247 (First Occurrence of Dog 4611's clinical signs)	Bilirubin (Total, μM)		nd	nd	nd
	AST (μkat/l)		nd	nd	nd
	ALT (μkat/l)		nd	nd	nd
	ALP (μkat/l)		nd	nd	nd
	GGT (μkat/l)		nd	nd	nd
	GLD (μkat/l)		nd	nd	nd

Treatment Day	Liver Parameter	500 mg/kg/day		Concurrent Control	
		Dog 4611	Remaining three dogs at 500 mg/kg/day	Mean ±SD	Highest Concurrent Control Value
Day 315[a] (Dog 4611 Sacrificed)	Bilirubin (Total, μM)		nd	nd	nd
	AST (μkat/l)		nd	nd	nd
	ALT (μkat/l)		nd	nd	nd
	ALP (μkat/l)		nd	nd	nd
	GGT (μkat/l)		nd	nd	nd
	GLD (μkat/l)		nd	nd	nd
Day 358	Bilirubin (Total, μM)		1.78, 1.69, 1.54	1.89±0.49	nd
	AST (μkat/l)		0.38, 0.35, 0.45	0.53±0.07	0.60
	ALT (μkat/l)		0.92, 0.51, 0.65	0.60±0.14	0.78
	ALP (μkat/l)		1.55, 1.18, 2.26	0.99±0.33	1.36
	GGT (μkat/l)		0.08, 0.08, 0.08	0.06±0.01	0.07
	GLD (μkat/l)		0.11, 0.06, 0.12	0.06±0.02	0.09

[a] Dog 4611 sacrificed on day 315, 38 days after termination of bosentan treatment in this dog.
nd, not determined

Triglyceride levels were greater than concurrent control in dog 4611 on day 176.

Treatment Day	Triglycerides (mM)			
	Dog 4611[a]	Remaining three dogs at 500 mg/kg/day	Concurrent Control (Mean±SD)	Highest Concurrent Control Value
-6		0.26, 0.30, 0.38	0.30±0.07	0.40
92		0.29, 0.45, 0.52	0.40±0.15	0.52
176		0.33, 0.35, 0.87	0.40±0.08	0.46
247		nd	nd	nd
358		0.21, 0.19, 0.26	0.32±0.09	0.42

Bold indicates 2-fold increase in dog 4611 compared to the highest concurrent control value.
[a] Dog 4611 sacrificed on day 315.
nd, not determined

Blood urea nitrogen, creatinine and plasma electrolytes for dog 4611 were not treatment related at any time point evaluated. Mean treatment group values for these parameters were also not treatment related at any time point evaluated (data not shown).

Individual values for serum liver enzymes for female dog 4454 were compared to those seen in other female dogs given 500 mg/kg/day. Pretreatment values were similar for all dogs. On all treatment days, ALP levels for several dogs given 500 mg/kg/day, including dog 4454, were greater than concurrent control. Liver enzymes were evaluated on day 69 since this dog exhibited clinical symptoms on this day.

Treatment Day	Liver Parameter	500 mg/kg/day		Concurrent Control	
		Dog 4454	Remaining three dogs at 500 mg/kg/day	Mean ±SD	Highest Concurrent Control Value
Day -6 (Pretreatment)	Bilirubin (Total, μM)		1.44, 2.61, 2.46	1.84±0.43	2.40
	AST (μkat/l)		0.55, 0.51, 0.79	0.56±0.09	0.68
	ALT (μkat/l)		0.56, 0.59, 0.70	0.64±0.13	0.80
	ALP (μkat/l)		1.67, 1.30, 0.88	1.53±0.36	1.92
	GGT (μkat/l)		0.08, 0.08, 0.09	0.07±0.02	0.08
	GLD (μkat/l)		nd	nd	nd
Day 69 (Unscheduled testing on day of symptoms)	Bilirubin (Total, μM)		1.88, 2.05, 2.25	nd	nd
	AST (μkat/l)		0.48, 0.43, 0.41	nd	nd
	ALT (μkat/l)		0.40, 0.68, 0.43	nd	nd
	ALP (μkat/l)		2.95, 2.64, 1.20	nd	nd
	GGT (μkat/l)		0.08, 0.09, 0.09	nd	nd
	GLD (μkat/l)			nd	nd
Day 92	Bilirubin (Total, μM)		1.63, 2.06, 1.83	2.14±0.30	2.40
	AST (μkat/l)		0.45, 0.49, 0.45	0.60±0.09	0.68
	ALT (μkat/l)		0.45, 0.46, 0.48	0.75±0.20	1.03
	ALP (μkat/l)		3.08, 2.58, 0.93	1.27±0.36	1.62
	GGT (μkat/l)		0.10, 0.10, 0.16	0.10±0.03	0.11
	GLD (μkat/l)		nd	nd	nd
Day 176	Bilirubin (Total, μM)		1.36, 2.03, 1.94	2.44±0.33	2.80
	AST (μkat/l)		0.46, 0.39, 0.54	0.50±0.11	0.60
	ALT (μkat/l)		0.44, 0.89, 0.65	0.73±0.36	1.22
	ALP (μkat/l)		3.77, **4.01**, 3.00	1.37±0.44	0.44
	GGT (μkat/l)		0.11, 0.17, 0.11	0.12±0.02	0.14
	GLD (μkat/l)		0.08, 0.17, 0.38	0.08±0.01	0.09
	Bile Acids (μM)		4.5, 4.1, 4.1	4.7±0.10	6.0

Bold indicates ≥2-fold increase compared to the highest control value.
nd, not determined

Table: continued

Treatment Day	Liver Parameter	500 mg/kg/day		Concurrent Control	
		Dog 4454	Remaining three dogs at 500 mg/kg/day	Mean ±SD	Highest Concurrent Control Value
Day 358	Bilirubin (Total, μM)		1.33, 2.20, 1.27	1.97±0.57	2.38
	AST (μkat/l)		0.45, 0.33, 0.52	0.58±0.17	0.79
	ALT (μkat/l)		0.40, 0.31, 0.55	0.76±0.46	1.41
	ALP (μkat/l)		2.23, 1.73, 1.07	0.89±0.35	1.40
	GGT (μkat/l)		0.14, 0.12, 0.19	0.09±0.01	0.11
	GLD (μkat/l)		0.06, 0.07, 0.09	0.10±0.06	0.19
	Bile Acids (μM)		10.4, 2.2, 2.3	2.8±1.4	4.8

Bile analyzed at necropsy on day 365 showed dose-related decreases in cholesterol in males given 180 and 500 mg/kg/day, and in females given 500 mg/kg/day compared to concurrent control. Bile acids and phospholipids were lower, and inorganic phosphate was higher than concurrent control in males and females given 500 mg/kg/day. Biliary sodium and glutathione levels were lower than concurrent control in females given 500 mg/kg/day, but liver glutathione was not treatment related. Biliary calcium and potassium were also not treatment related (data not shown).

Sex	Parameter	Dose (mg/kg/day)			
		0	60	180	500
Male	Biliary Cholesterol (mM)	0.96±0.45	0.63±0.17	0.32±0.04*	0.19±0.08*
	Biliary Phospholipids (mM)	47.15±4.61	44.12±0.69	39.38±3.08	39.07±6.02
	Biliary Bile Acids (mM)	138.45±18.43	140.75±8.61	115.03±19.79	110.8±19.75*
	Biliary Inorganic Phosphate (mM)	0.12±0.03	0.13±0.09	0.45±0.21	1.02±0.59*
	Biliary Sodium (mM)	227.00±10.42	233.35±27.26	230.25±17.29	224.00±26.21
	Biliary Glutathione (μM)	475.76±84.02	412.33±72.45	396.26±10.00	415.97±33.45
	Liver Glutathione (μM)	4156±464	4102±219	4498±657	4086±421
Female	Biliary Cholesterol (mM)	0.60±0.22	0.67±0.21	0.30±0.08	0.09±0.03*
	Biliary Phospholipids (mM)	46.77±4.96	45.25±3.52	39.77±2.94	28.46±6.24*
	Biliary Bile Acids (mM)	136.77±24.38	122.22±21.00	108.88±20.06	80.60±24.37*
	Biliary Inorganic Phosphate (mM)	0.03±0.03	0.04±0.04	0.11±0.08	0.51±0.39*
	Biliary Sodium (mM)	251.25±8.77	230.75±23.99	225.50±22.123	193.50±15.55*
	Bilrary Glutathione (μM)	532.08±63.36	473.47±99.16	410.13±82.59	393.70±102.07*
	Liver Glutathione (μM)	4425±734	3751±1051	4349±1283	5013±533

*P<0.05 compared to concurrent control

In female dogs given bosentan at 500 mg/kg/day, serum albumin was lower than concurrent control on treatment days 92 and 358. Globulins were greater than concurrent control in female dogs given 500 mg/kg/day on days 176 and 358. There were no consistent effects of bosentan on serum proteins in male dogs.

Serum Proteins in Female Dogs

Treatment Day	Parameter	Dose (mg/kg/day)			
		0	60	180	500
92 (n=4)	Total Protein (g/l)	55.1±1.8	50.3±1.0	53.1±1.0	51.7±2.0*
	Albumin (g/l)	34.9±2.6	30.6±1.7	31.3±2.3	30.7±2.2*
	α-Globulin (g/l)	6.2±0.6	6.4±0.6	6.9±0.7	6.9±1.0
	β-Globulin (g/l)	10.4±0.9	9.9±1.0	10.8±0.9	10.4±1.7
	γ-Globulin (g/l)	3.6±0.4	3.5±0.2	4.2±0.6	4.0±0.9
176 (n=4)	Total Protein (g/l)	55.1±2.6	51.4±1.7	53.8±1.4	55.8±1.2
	Albumin (g/l)	35.1±2.1	31.0±1.3	32.8±1.8	32.7±0.6
	α-Globulin (g/l)	7.2±0.3	8.2±0.5	8.0±1.1	8.7±1.0*
	β-Globulin (g/l)	9.1±0.3	10.4±1.3	9.7±0.4	10.4±1.1
	γ-Globulin (g/l)	3.7±0.3	4.6±0.8	3.3±0.5	4.0±0.4
358 (n=4)	Total Protein (g/l)	55.8±3.2	52.9±1.1	54.2±2.3	57.0±1.0
	Albumin (g/l)	37.4±2.4	34.9±1.1	32.8±3.3*	33.1±1.6*
	α-Globulin (g/l)	6.0±0.5	6.6±0.8	7.6±1.5*	8.3±0.8*
	β-Globulin (g/l)	8.8±1.1	8.2±0.6	10.1±1.1	11.1±1.2*
	γ-Globulin (g/l)	3.6±0.6	3.2±0.5	3.7±1.0	4.5±0.8

*P<0.05 compared to concurrent control

Serum Proteins in Male Dogs

Treatment Day	Parameter	Dose (mg/kg/day)			
		0	60	180	500
92 (n=4)	Total Protein (g/l)	53.7±3.1	53.8±2.2	53.4±1.5	51.6±2.2
	Albumin (g/l)	32.7±1.8	32.5±2.4	31.2±0.9	30.2±1.9*
	α-Globulin (g/l)	6.6±0.6	6.9±0.8	7.5±0.9	6.8±0.3
	β-Globulin (g/l)	10.1±0.7	10.4±0.7	11.0±0.4	11.1±0.8*
	γ-Globulin (g/l)	4.3±0.9	3.9±0.3	3.7±0.2	3.5±0.9
176 (n=4)	Total Protein (g/l)	53.9±2.7	54.2±1.9	52.0±0.9	57.1±6.0
	Albumin (g/l)	32.6±1.0	31.2±1.5	28.7±1.7	32.5±3.3
	α-Globulin (g/l)	7.6±1.2	7.8±0.5	8.8±0.6	9.4±1.4*
	β-Globulin (g/l)	9.3±1.1	10.3±0.5	10.4±0.7	10.9±1.3
	γ-Globulin (g/l)	4.5±0.9	4.8±0.4	4.2±0.2	4.3±0.5
358 (n=3)	Total Protein (g/l)	55.6±3.0	56.7±1.5	54.8±1.1	53.3±1.5
	Albumin (g/l)	34.6±2.1	36.4±1.3	32.9±1.7	32.5±2.4
	α-Globulin (g/l)	7.2±1.4	6.9±0.7	7.8±0.7	7.4±0.5
	β-Globulin (g/l)	9.3±1.2	9.6±0.7	10.1±0.9	9.5±0.6
	γ-Globulin (g/l)	4.5±0.8	3.9±0.5	4.0±0.2	4.0±0.3

*P<0.05 compared to concurrent control

Liver weights relative to body weight were greater than concurrent control in males given 180 and 500 mg/kg/day and in females given 500 mg/kg/day. Relative kidney and brain weights were greater than concurrent control in males given 180 and 500 mg/kg/day. Relative adrenal weights were greater than concurrent control in males given 500 mg/kg/day. Sponsor did not statistically analyze absolute organ weights for differences from concurrent control.

Sex	Organ		Dose (mg/kg/day)			
			0	60	180	500
Male	Liver	Weight (g)	335±46	289±37	393±46	346±48
		Relative Wt (g/kg)	28.3±2.2	30.6±3.2	36.3±5.3*	36.1±2.1*
	Kidney	Weight (g)	53.8±8.0	44.9±4.7	60.3±3.0	54.3±13.2
		Relative Wt (g/kg)	4.5±0.2	4.8±0.6	5.5±0.4*	5.7±1.2*
	Adrenal	Weight (g)	1.13±0.24	1.11±0.18	1.18±0.11	1.27±0.08
		Relative Wt (g/kg)	0.096±0.012	0.119±0.027	0.109±0.010	0.134±0.009*
	Brain	Weight (g)	75.5±7.4	75.4±5.3	80.7±7.5	76.6±2.9
		Relative Wt (g/kg)	6.4±0.3	8.0±0.7	7.4±0.6*	8.0±0.5*
Female	Liver	Weight (g)	288±46	304±38	376±165	437±75
		Relative Wt (g/kg)	31.0±4.2	33.7±4.2	38.7±9.9	45.3±1.23*
	Kidney	Weight (g)	43.8±4.3	42.1±3.2	44.9±13.2	51.5±9.8
		Relative Wt (g/kg)	4.7±0.6	4.7±0.2	4.7±0.5	5.3±0.6
	Adrenal	Weight (g)	1.30±0.15	1.34±0.18	1.25±0.18	1.41±0.16
		Relative Wt (g/kg)	0.141±0.017	0.147±0.017	0.134±0.009	0.149±0.033
	Brain	Weight (g)	71.4±2.5	74.0±7.7	74.2±2.7	74.0±5.9
		Relative Wt (g/kg)	7.7±0.9	8.2±1.4	8.1±1.6	7.8±1.2

*P<0.05 compared to concurrent control
Relative Wt, organ weight/body weight ratio

Absolute and relative organ weights for dog 4611 were similar to organ weights of other male dogs given 500 mg/kg/day (data not shown).

Gross pathology showed depressed areas in the kidneys of one male and one female given 500 mg/kg/day, and discoloration of the kidney in one male at 180 mg/kg/day and two females at 500 mg/kg/day. Renal cortical interstitial fibrosis (consistent with old infarcts) and renal tubular atrophy were seen in one male (dog 4611) and one female (dog 4448) given 500 mg/kg/day and corresponded with the grossly depressed areas of the kidney. The sponsor speculated on an infectious basis for these renal findings since renal vascular lesions were not observed in these dogs.[1] Renal pigmentation was more severe than concurrent control in dogs given 500 mg/kg/day. The uterus was enlarged in one female at 180 mg/kg/day and one female at 500 mg/kg/day. Several gallstones were seen in one female given 180 mg/kg/day. These stones were about 0.2 cm in diameter, black-brown in color and friable.

Pigmented canaliculi were seen microscopically in the livers of males and females given 500 mg/kg/day and hepatocyte degeneration was seen in one female given 500 mg/kg/day. Fibrosis of the liver was not observed in any dog, including dog number 4611, which showed markedly high serum liver enzymes (bosentan was discontinued in this dog for 38 days prior to sacrifice). Gallbladder pigment deposits were more frequent in males given 500 mg/kg/day and in females given 180 and 500 mg/kg/day than in concurrent controls. Gallbladder vacuolation was more common in males and females at 180 and 500 mg/kg/day than in concurrent controls. Moderate (grade 3) retinal atrophy of the eyes (outer nuclear layer, photoreceptor layer) was observed in one dog given 500 mg/kg/day. The incidences of testicular atrophy and germ cell degeneration were not drug-related.

Organ	Finding	Sex	Incidence (Severity)			
			0 mg/kg/day	60 mg/kg/day	180 mg/kg/day	500 mg/kg/day
Liver	Pigmented Canaliculi	M F	0 0	0 0	0 0	2 (2.0) 3 (1.0)
	Hepatocyte Degeneration	M F	0 0	0 0	0 0	0 1
	Cytoplasmic Rarefaction	M F	1 (1.0) 3 (1.0)	0 1 (1.0)	0 1 (2.0)	3 (1.75) 4 (1.5)
Gallbladder	Pigment Deposits	M F	0 1 (1.0)	1 (1.0) 1 (1.0)	3 (1.0) 1 (1.0)	4 (1.5) 4 (1.5)
	Vacuolation	M F	0 0	1 (1.0) 1 (2.0)	3 (1.3) 2 (1.5)	4 (1.5) 2 (1.0)
Spleen	Hemopoiesis	M F	0 0	0 0	0 1	0 1
Kidney	Pigmentation	M F	4 (1.0) 1 (1.0)	4 (1.5) 1 (1.0)	4 (1.5) 2 (1.0)	4 (2.8) 4 (1.8)
	Interstitial Fibrosis and Tubular Atrophy	M F	0 0	0 0	0 0	1 (3.0) 1 (2.0)
Eye	Retinal Atrophy (Bilateral)	M F	0 0	0 0	0 0	1 (3.0) 0

Severity grade: 1, minimal; 2 slight; 3 moderate; 4, marked

[1] Sponsor submitted information on June 10, 2001.

Plasma Drug Levels

Toxicokinetic evaluation showed that exposure (AUC) was dose-related, but not dose-proportional on all days evaluated (table and figure). AUCs decreased markedly with repeated administration at all doses evaluated (figure). AUCs declined by 60-75% from day 1 to week 13, but were similar at weeks 13, 26 and 52, indicating that steady state was reached by week 13.

Sex	Dose (mg/kg/day)	AUC (μg.hr/ml)			
		Day 1	Week 13	Week 25	Week 52
Male	60	556±283	134±18	195±28	207±62
	180	805±376	324±137	392±192	393±192
	500	2160±1490	756±586	791±510	466±393*
Female	60	770±258	228±111	162±63	237±188
	180	2090±464	554±226	539±66	415±225
	500	2390±837	599±200	757±255	510±160

*(n=3)

C_{max} was dose-related but not dose-proportional. C_{max} was also time related, and decreased by 60-70% from day 1 to week 13, but was similar at weeks 13, 26 and 52, indicating that steady state had been reached by week 13.

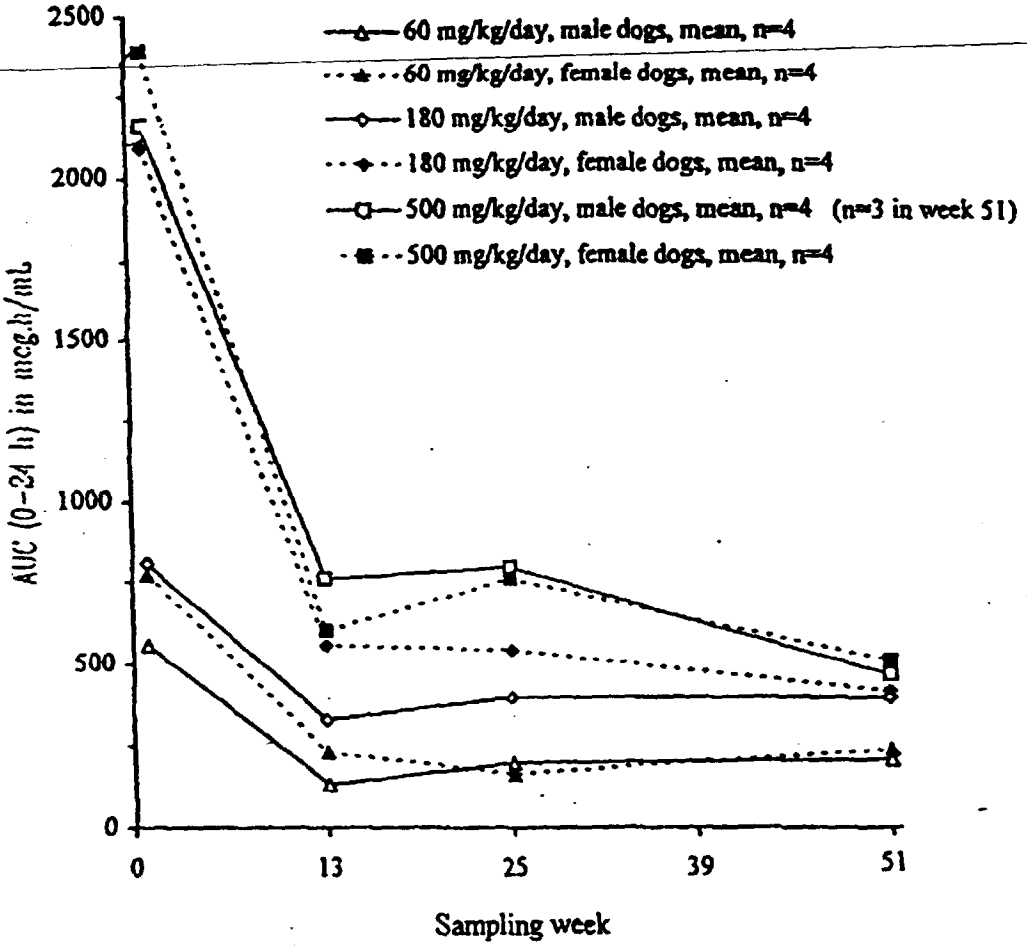
Sex	Dose (mg/kg/day)	C_{max} (μg/ml)			
		Day 1	Week 13	Week 25	Week 52
Male	60	53±12.1	19±4.1	28±4.0	21±3.5
	180	84±25.9	47±16.3	62±31	45±23
	500	183±99	65±44	73±44.2	44±28.0*
Female	60	65±17.6	29±9.6	21±5.3	22±12.5
	180	163±39.7	53±17.9	69±19.3	40±17.5
	500	198±36.2	56±11.1	84±31.6	43±10.3

*(n=3)

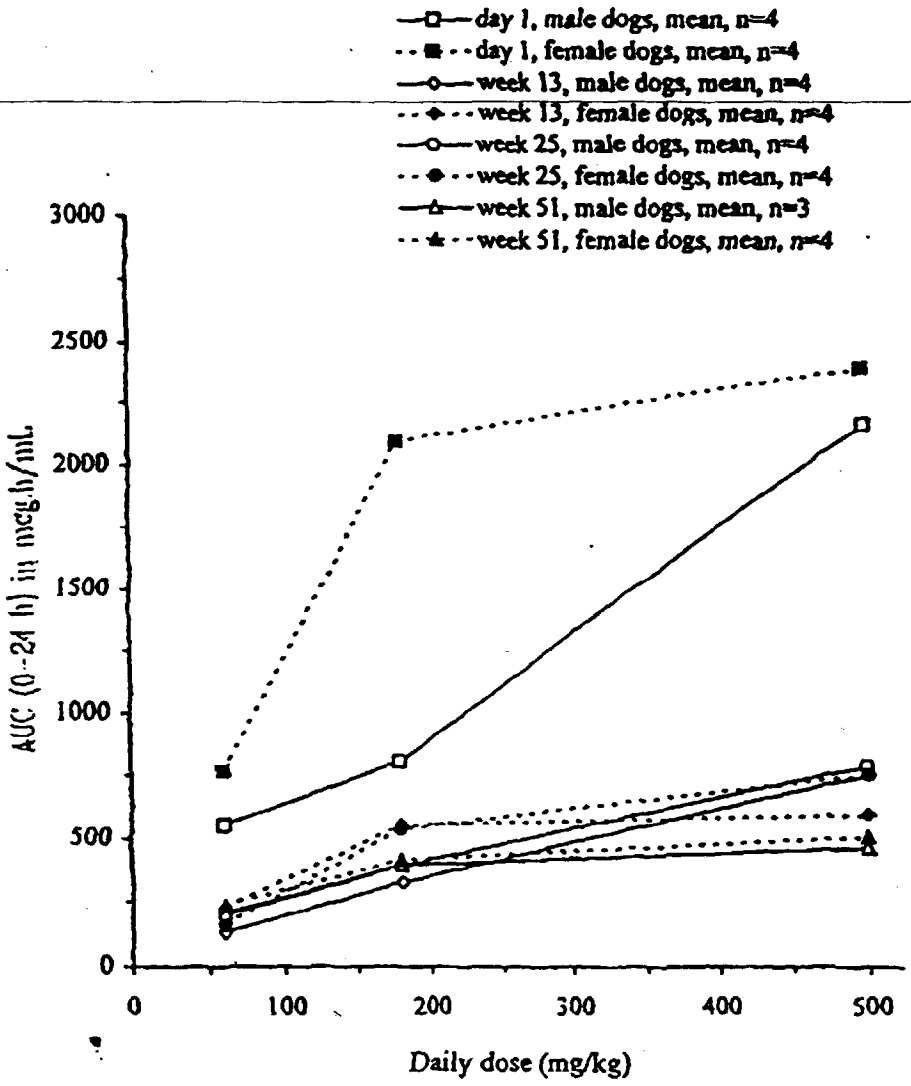
The decrease in AUC with repeated administration suggested that elimination half life was decreased by bosentan. The sponsor tested this hypothesis *in vitro* in a non-GLP study. Metabolic clearance of bosentan by primary hepatocyte culture from one male dog given 500 mg/kg/day for 12 months was 3-4 times higher than that for a male concurrent control dog, consistent with this hypothesis.

Dose (mg/kg/day for 52 weeks)	Male Dog No.	Hepatic Metabolic Clearance (μl/hr/10^6 cells)
0	4637	
500	4641	

12-month oral toxicokinetics of Ro 47-0203 in dogs (Protocol No. 141P95):
Changes in systemic exposure with time



12-month oral toxicokinetics of Ro 47-0203 in dogs (Protocol No. 141P95):
Changes in systemic exposure with dose



Individual AUCs are shown for all dogs given 500 mg/kg/day. Male dog 4611 showed the highest AUC and C_{max} on day 1, but by day 176, AUC and C_{max} were similar to those of other male dogs given this dose. C_{min} for dog 4454 was higher than for other female dogs at weeks 13, 25 and 51.

Parameter	Sex	Dog No.	Day 1	Week 13	Week 25	Week 51
AUC (μg.hr/ml)	Male	4649				
		4611				**nd**
		4641				
		4627				
	Female	4456				
		4448				
		4464				
		4454				
C_{max} (μg/ml)	Male	4649				
		4611				nd
		4641				
		4627				
	Female	4456				
		4448				
		4464				
		4454				
C_{min} (μg/ml)	Male	4649				
		4611				nd
		4641				blq
		4627				
	Female	4456				
		4448				
		4464				
		4454				

nd, not determined
blq, below limit of quantification of 0.050 μg/ml

Levels of unchanged bosentan in bile and liver in individual dogs given 500 mg/kg/day are shown below. Levels in liver were much lower than those in bile.

Individual concentrations of unchanged Ro 47-0203 and its metabolites Ro 48-5033, Ro 47-8634 and Ro 64-1056 in bile samples collected at the end of a 12 month chronic oral treatment (24 h after the last administration) with bosentan at 500 mg/kg/day (Protocol 141P95)

Dog identification	Ro 47-0203 (µg/mL)	Ro 48-5033 (µg/mL)	Ro 47-8634 (µg/mL)	Ro 64-1056 (µg/mL)
F4448				
F4454				
F4456				
F4464				
M4627				
M4641				
M4649				
mean (µg/mL)	268	1830	752	208
S.D. (µg/mL)	146	715	257	120

Individual concentrations of unchanged Ro 47-0203 and its metabolites Ro 48-5033, Ro 47-8634 and Ro 64-1056 in liver samples collected at the end of a 12 month chronic oral treatment (24 h after the last administration) with bosentan at 500 mg/kg/day (Protocol 141P95)

Dog identification	Ro 47-0203 liver (µg/g)	Ro 47-0203 plasma (µg/mL) (1)	Ro 47-0203 liver/plasma	Ro 48-5033 (µg/g)	Ro 47-8634 (µg/g)	Ro 64-1056 (µg/g)
F4448						
F4454						
F4456						
F4464						
M4627						
M4641						
M4649						
mean (µg/g or µg/mL)	4.97	1.49	-	3.07	0.61	0.17
S.D. (µg/g or µg/mL)	4.03	1.56	-	1.59	0.52	0.10
total amount in the liver (µg)*	2500	-	-	1500	300	100
total amount in the liver (% of dose)**	0.05	-	-	0.03	<0.01	<0.01

(1) data from reference 2
*assuming 500 g liver weight
**assuming 10 kg body weight

REPRODUCTIVE TOXICOLOGY STUDIES

Study of Fertility and Early Embryonic Development to Implantation in Rats with Oral Administration of Ro 47-0203

Location of Study Report: Vol 37, pg 277

Study Facility:

Study No.: 219R93
Report No.: 153691
Study Dates: 11/09/1993 – 03/1994

GLP Compliance: Yes

Animals: albino rats (group median weight on the day the dosing was started: 320-334 g, males; 201-208 g, females) were housed 2/same sex per cage (except for mating and parturition) and allowed feed and water *ad libitum*.

Drug Administration: Ro 47-0203 (Lot No. GFR 0038) was suspended in aqueous caroxymethyl cellulose and given orally by gavage. Males were given test agent daily for 28 days prior to mating and until mating was completed (maximum of 11 days). Females were given test agent for 14 days prior to mating and continued through gestation day 7 (implantation).

Dose Levels: 0, 60, 300, 1500 mg/kg/day[2] (26 rats/sex/treatment group)

Observations/Measurements: Rats from respective treatment groups were mated overnight by placing one male and one female in a breeding cage. Females without signs of copulation were paired for a maximum of 11 consecutive days. Day 0 of gestation was based on evidence of mating (copulation plug).

Male and female rats were observed daily for mortality and clinical signs of toxicity. Male rats were sacrificed following breeding and necropsied. Testes were weighed and the sperm was collected from the left caudal epididymidis for determination of sperm concentration and motility.

Female rats were sacrificed on Day 20 of gestation. Uteri were excised and examined for numbers of corpora lutea and implantions. Fetuses were weighed, examined externally for abnormalities, and fixed in Wilson's solution. Fetuses were not examined for soft tissue or skeletal abnormalities.

Plasma Drug Levels: Not determined.

[2] Doses were chosen based on a pilot study showing saturation of exposure in pregnant rats given bosentan at doses ≥600 mg/kg/day. In this pilot study (study number not provided), plasma AUCs for bosentan were determined in 10 pregnant rats/dose. Body weights and days of gestation on which sampling was conducted were not provided. Bosentan was given orally at 200, 600 and 2000 mg/kg/day for 10 days. Blood samples were taken on days 1, 5 and 10 at 1, 2 and 7 hours (1 rat/dose/time point). AUCs were 87, 209 and 230 μg.hr/ml on day 1; 39, 79 and 82 μg.hr/ml on day 5; and 44, 82 and 132 μg.hr/ml on day 10 in pregnant rats given 200, 600 or 2000 mg/kg/day, respectively.

Drug Associated Findings

Several male rats died on study or were sacrificed moribund (2, 1, 1, 4 deaths at 0, 60, 300, 1500 mg/kg/day, respectively). All male deaths appeared to be related to dosing error. One female (1500 mg/kg/day) died on study; the cause of death was not determined.

Reproductive performance of male and female rats was not drug related, as shown in the following tables.

219R93

STUDY OF FERTILITY AND EARLY EMBRYONIC DEVELOPMENT TO IMPLANTATION IN RATS WITH ORAL ADMINISTRATION OF RO 47-0203/015
MATING PERFORMANCE AND MATING SUCCESS

		CONTROL PLACEBO		60 MG/KG	300 MG/KG	1500 MG/KG
Females placed with males	N	26		26	26	26
Total number inseminated [d]	N	24	f	25	26	24
female mating index	%	92.3		96.2	100.0	92.3
pregnant	N	22	f	21	19	24
female fertility index	%	91.7		84.0	73.1	100.0
Males placed with females	N	22		23	24	20
mated	N	22	f	23	24	20
male mating index	%	100.0		100.0	100.0	100.0
with females pregnant	N	21	f	20	18	20
male fertility index	%	95.5		87.0	75.0	100.0
Females with defined day 0 pc	N	24		24	26	24
Mating days until day 0 pc	MEDIAN	2.0	d	2.0	3.0	2.5
	Q1	1.0		1.3	1.0	2.0
	Q3	3.0		4.0	4.0	4.0
Day 1 to 4	N	24	f	23	24	23
	%	100.0		95.8	92.3	95.8
Day 5 to 8	N	0	f	1	2	0
	%	0.0		4.2	7.7	0.0
Day 9 to 14	N	0	f	0	0	1
	%	0.0		0.0	0.0	4.2
Day 15 to 21	N	0	f	0	0	0
	%	0.0		0.0	0.0	0.0

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test

219R93

STUDY OF FERTILITY AND EARLY EMBIYONIC DEVELOPMENT TO IMPLANTATION IN RATS WITH ORAL ADMINISTRATION OF RO 47-0203/015
SUMMARY OF REPRODUCTION DATA (C-SECTION)

		CONTROL PLACEBO		60 MG/KG	300 MG/KG	1500 MG/KG
Pregnant, used for calculation	N	22		20	18	22
Resorptions: Total	N	46		29	35	25
No. per animal	MEDIAN	2.0	d	1.0	1.0	1.0
	Q1	1.0		0.3	0.0	0.0
	Q3	2.5		2.0	3.0	2.0
% of impl. per group	%	18.8		11.3	14.8	8.6
% of impl. per animal	MEDIAN	14.8	u	8.0	7.4	7.7
	Q1	7.1		1.6	0.0	0.0
	Q3	28.5		16.1	21.3	15.7
Resorptions: Early	N	46		29	32	25
% of resorp. per group	%	100.0		100.0	91.4	100.0
Resorptions: Late	N	0		0	3	0
% of resorp. per group	%	0.0		0.0	8.6	0.0
Postimplantation Loss	N	46		29	35	25
No. per animal	MEDIAN	2.0	d	1.0	1.0	1.0
	Q1	1.0		0.3	0.0	0.0
	Q3	2.5		2.0	3.0	2.0
% of impl. per group	%	18.8		11.3	14.8	8.6
% impl. per animal	MEDIAN	14.8	u	8.0	7.4	7.7
	Q1	7.1		1.6	0.0	0.0
	Q3	28.5		16.1	21.3	15.7
Viable Male Fetuses	N	116	f	106	96	137
	%	50.9		46.7	47.8	51.7
Female Fetuses	N	112	f	121	105	128
	%	49.1		53.3	52.2	48.3
Fetal Body Weight (g)	MEDIAN	3.4	d	3.3	3.3	3.3
	Q1	3.2		3.1	3.2	3.2
	Q3	3.5		3.6	3.5	3.6
	N LITTERS	21		19	18	22

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test u=Kruskal-Wallis + Mann-Whitney U

21283)

STUDY OF FERTILITY AND EARLY EMBRYONIC DEVELOPMENT TO IMPLANTATION IN RATS WITH ORAL ADMINISTRATION OF RO 47-0203/015
SUMMARY OF REPRODUCTION DATA (C-SECTION)

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Pregnant, used for calculation	N	22	20	18	22
Corpora Lutea	N	306	288	260	317
No. per animal	MEDIAN	14.5 d	15.0	15.0	14.0
	Q1	13.0	13.3	13.8	13.0
	Q3	16.0	16.0	16.3	16.0
Preimplantation Loss	N	32	32	24	27
% per group	%	10.5	11.1	9.2	8.5
% per animal	MEDIAN	7.7 u	6.3	7.0	6.5
	Q1	4.4	0.0	0.0	0.0
	Q3	14.7	13.5	16.6	14.3
Implantation Sites	N	274	256	236	290
No. per animal	MEDIAN	13.0 d	14.0	14.0	13.0
	Q1	12.0	12.0	12.0	12.8
	Q3	14.0	15.0	15.0	15.0
Fetuses	N	228	227	201	265
No. per animal	MEDIAN	11.5 d	13.0	12.0	13.0
	Q1	8.5	10.3	10.5	11.0
	Q3	13.0	14.0	14.0	13.0
Alive	%	100.0	100.0	100.0	100.0
Dead	%	0.0	0.0	0.0	0.0
Live Fetuses	N	228	227	201	265
No. per animal	MEDIAN	11.5 d	13.0	12.0	13.0
	Q1	8.5	10.3	10.5	11.0
	Q3	13.0	14.0	14.0	13.0
Dead Fetuses	N	0	0	0	0
No. per animal	MEDIAN	0.0	0.0	0.0	0.0
	Q1	0.0	0.0	0.0	0.0
	Q3	0.0	0.0	0.0	0.0
% of impl. per group	%	0.0	0.0	0.0	0.0
% of impl. per animal	MEDIAN	0.0 u	0.0	0.0	0.0
	Q1	0.0	0.0	0.0	0.0
	Q3	0.0	0.0	0.0	0.0

Statistical key: d=ANOVA + Dunnett-test u=Kruskal-Wallis + Mann-Whitney U

Testes weights, sperm counts and sperm motility were not drug-related.

STUDY OF FERTILITY AND EARLY EMBRIONIC DEVELOPMENT TO IMPLANTATION IN RATS WITH ORAL ADMINISTRATION OF BO 47-0203/015

SUMMARY OF TESTES WEIGHTS AND CAUDA WEIGHTS in gram

GROUP		TESTES WEIGHT
A	N	24
	MEAN	3.29
	SD	0.32
	SIGN	N.S.
B	N	25
	MEAN	3.24
	SD	0.59
	SIGN	N.S.
C	N	25
	MEAN	3.12
	SD	0.65
	SIGN	N.S.
D	N	23
	MEAN	3.37
	SD	0.39
	SIGN	N.S.

GROUP		CAUDA WEIGHT
A	N	24
	MEAN	0.15
	SD	0.03
	SIGN	N.S.
B	N	25
	MEAN	0.15
	SD	0.03
	SIGN	N.S.
C	N	25
	MEAN	0.16
	SD	0.04
	SIGN	N.S.
D	N	23
	MEAN	0.17
	SD	0.03
	SIGN	N.S.

A = Control
B = 60 mg/kg
C = 300 mg/kg
D = 1500 mg/kg

Jonckheere-Test : **: P <= 1% ; *: 1% < P <= 5%
U-Test : ##: P <= 1% ; #: 1% < P <= 5%

N.S. statistically not significant

STUDY OF FERTILITY AND EARLY EMBRYONIC DEVELOPMENT TO IMPLANTATION IN RATS WITH ORAL ADMINISTRATION OF BO 47-0203/015

SUMMARY OF SPERM MOTILITY AND SPERM COUNT

GROUP		SPERM MOTILITY
A	N	24
	MEAN [a]	33.67
	SD	10.26
	SIGN	N.S.
B	N	25
	MEAN	30.60
	SD	12.10
	SIGN	N.S.
C	N	25
	MEAN	29.80
	SD	12.18
	SIGN	N.S.
D	N	23
	MEAN	36.35
	SD	14.41
	SIGN	N.S.

GROUP		SPERM COUNT
A	N	24
	MEAN [b]	74.13
	SD	32.28
	SIGN	N.S.
B	N	25
	MEAN	73.04
	SD	53.56
	SIGN	N.S.
C	N	25
	MEAN	90.56
	SD	74.79
	SIGN	N.S.
D	N	23
	MEAN	80.70
	SD	40.66
	SIGN	N.S.

A = Control
B = 60 mg/kg
C = 300 mg/kg
D = 1500 mg/kg

a: immotile sperms/200 sperms counted : b Mean in 10^6/ml base suspension

Jonckheere-Test : **: P <= 1% ; *: 1% < P <= 5%
U-Test : ##: P <= 1% ; #: 1% < P <= 5%

N.S. statistically not significant

Study of Embryo-Fetal Development and Postnatal Development in the Rat Following Oral Administration of Ro 47-0203

Location of Study Report: Vol 38, pg 1

Study Facility:

Study No.: 051R94
Report No.: 153693
Study Dates: 04/04/1994 – 10/1994

GLP Compliance: Yes

Animals: Female albino rats weighing from 198-201 g on gestation day 0, were housed individually and allowed feed and water *ad libitum*.

Drug Administration: Ro 47-0203 (Lot No. GFR 0038) was suspended in aqueous carboxymethyl cellulose and given orally by gavage to female rats. The Caesarean section group was given bosentan from gestation day 6 through gestation day 15. The spontaneously delivery group was given bosentan from gestation day 6 through lactation day 22.

Dose Levels: 0, 60, 300, 1500 mg/kg/day (42 female rats per treatment group. Approximately half of the dams in each group underwent cesarean section on day 20 of gestation. The remaining dams were permitted to deliver spontaneously.)

Mating Procedure: Each female was placed with an untreated male, overnight, in a breeding cage. Mating was determined by the presence of an ejected copulatory plug. The day evidence of mating was detected was designated day 0 of gestation.

Observations Measurements

F0-Generation:

All dams were weighed and observed daily for mortality and clinical signs of toxicity. Dam weights were not corrected for gravid uterine weights.

Cesarean section dams were sacrificed on Day 20 of gestation. Kidneys, lungs, liver and other organs (not specified) were macroscopically inspected. Uteri and ovaries were removed and inspected for reproductive parameters. Corpora lutea, implantations and fetuses were counted. Live fetuses were sexed, weighed, and examined for external abnormalities. Half of the live fetuses were examined viscerally; the remaining fetuses were examined for skeletal anomalies. Dead fetuses were examined macroscopically and excluded from the study.

Gestation lengths were recorded for dams allowed to deliver spontaneously and rear their young. The number of live and dead pups were recorded. Pups found dead on day 1 of lactation were considered stillborn; stillbirth was not confirmed by a lung test for air content since heart vessels were to be preserved for examination. The sponsor stated that the lung test interfers with examination of heart blood vessels. Dams were sacrificed for necropsy after weaning their pups. The number of corpora lutea and implantations were determined. Dams that did not deliver were sacrificed one week after the expected date of parturition and the uteri were inspected for implantations.

F1 generation:

Preweaning period- Pups were inspected for milk intake (the sponsor did not indicate how milk intake was determined) on lactation days 2-4, and weighed on lactation days 1, 4, 12 and 23. Pups were evaluated for auditory startle on lactation days 16, 17 and 19, and pupil contraction on day 21. All pups that died on study were weighed, sexed and examined for soft tissue abnormalities.

Postweaning Period- Learning and memory (swimming test, single trial) were assessed in male and female pups (20 /sex/dose). Sexual maturation was assessed in one male and one female per litter. Female sexual maturation was evaluated as the time to vaginal opening between days 38-49 post partum. Male sexual maturation was evaluated as the time to balano-preputial skinfold cleavage between days 42-55 post partum. Reproductive performance was assessed by cohabitating one male and one female (same dose group but not of the same litter) for 14 days. Mating index, male fertility index, female fertility index, gestation index, pre and post implantation loss, live birth index, viability index and lactation index were determined.

Plasma Drug Levels: Not determined.

Drug Associated Findings

For cesarean section dams, maternal body weight gains (uncorrected for gravid uterine weight) on gestation days 16 and 20 were 11-12% lower at 1500 mg/kg/day than concurrent control. Two cesarean section dams given 1500 mg/kg/day died due to dosing errors. Body weight gains at lower doses were not different than concurrent control. In spontaneous delivery dams, maternal body weights during gestation and lactation were unrelated to drug. Mammary glands at necropsy were poorly developed in spontanously delivery dams given 300 and 1500 mg/kg/day. This finding was associated with and attributed to complete litter loss and loss of suckling in these dams (0, 0, 24 and 58% of litters from dams given 0, 60, 300 and 1500 mg/kg/day, respectively).

The numbers of post-implantation losses were unrelated to drug treatment for cesarean delivery. Fetal weights and sex ratios were comparable across dose groups.

For spontaneous delivery dams, the number of dams delivering and the number of pups delivered were unrelated to drug. The median gestation duration was slightly increased above concurrent control duration at 300 and 1500 mg/kg/day. The number of dams with stillborn pups was increased in all Ro 47-0203 treatment groups. Pup weights at delivery were lower than concurrent control at 1500 mg/kg/day. Pup survival was markedly reduced at 300 and 1500 mg/kg/day. At 1500 mg/kg/day, only 14% of pups survived until lactation day 23, whereas in the concurrent control group, 83% of pups survived until lactation day 23.

Teratogenic effects observed in fetuses delivered via cesarean delivery included agenesis of the soft palate in the litter of one dam given 300 mg/kg/day and in 14 litters from dams given 1500 mg/kg/day, and shortened tongues in 5 litters of dams given 1500 mg/kg/day. Abnormal origin of the right subclavian artery was observed in one litter given 1500 mg/kg/day; this finding was absent in other treatment groups.

Abnormalities of the skull were common at 1500 mg/kg/day, and included shortened and misshapen mandibles (6 and 5 litters, respectively), abnormally shaped palatine (3 litters), abnormally shaped tympanic annulus (11 litters) and hyoid bone (18 litters), fusion of the pterygoid process with the tympanic annulus (16 litters) and bent internal pterygoid process (9 litters).

Teratogenic effects observed in spontaneous delivery fetuses that died on study included agenesis of the soft palate, anophthalmia and microphthalmia at 300 and 1500 mg/kg/day. Abnormal origin of the right subclavian artery was observed at 1500 mg/kg/day. These findings were absent in dead fetuses from other treatment groups.

REPRODUCTION TOXICITY Embryotoxicity (caesarian section)						
Ref. to document.: Volume: Page: to Addendum No.: Report date: May 5, 1995 Number: Study period (years): 1994						
Species/Strain: Rat/ Albino (						
Number of animals: 86 females						
Generation of parental animals: <0>						
Administration route: p.o.						
Treatment of controls: placebo						
Evidence of mating - Day 0 of gestation	Treatment of the females from day:6 to day: 15	Section of females on day: 20				
Methods of examination of the young: (litter reduction: yes < > no <x>) <x> Skeleton < > Histology	<x> Soft tissue < > Biochemistry	Others: yes < > no <x>				
Study group			(1)Contr	(2)	(3)	(4)	(5)
Dosage <mg/kg/day>			0	60	300	1500	
P A R.	Females with evidence of mating		21	21	21	23	
	Pregnant females		18	21	18	23	
	Eval. pregn. females		18	21	18	21	
LITTER	median per litter	Corpora lutea	15.0	14.0	14.5	14.0	
		Implantations	13.5	14.0	13.0	12.0	
		Live foetuses	12.0	13.0	12.5	10.0	
		Dead foetuses	0	0	0	0	
		Postimplantation loss	1.5	1.0	1.5	2.0	
		Weight of foetuses (g)	3.5	3.3	3.4	3.3	
S	Sex ratio of foetuses(m/f,%)		46/54	50/50	53/47	51/49	
Study conducted by the applicant: yes <x> no < > Study in compliance with GLP: yes <x> no < >							

Only 21 of 23 pregnant females given 1500 mg/kg/day were evaluated at cesarean section as 2 had died due to dosing errors.

REPRODUCTION TOXICITY Pre- and postnatal development (spont. delivery)

Ref. to document.: Volume: Page: to Addendum No.:
Report date: May 5, 1995 Number: Study period (years): 1994

Species/Strain: rat/ Albino

Number of animals: 82 females

Generation of parental animals: <0>

Administration route: p.o.

Treatment of controls: placebo

Treatment of the females	Evidence of mating =	
DG 6 through DL 22	day 0 of gestation	

Methods of examination of the young: (litter reduction: yes < > no <x>)
<x> Observation | <x> Development | <x> Behaviour
< > Histology | < > Biochemistry | Others: yes <x> no < >

	Study group	(1)Contr	(2)	(3)	(4)	(5)
	Dosage <mg/kg/day >	0	60	300	1500	
PARENTS	Females with evidence of mating	21	21	21	19	
	Pregnant females	20	20	19	17	
	Females with delivery	19	20	18	16	
	Median duration of gestation	22	22	23	23	
LITTER MEDIAN	Implantations	14.0	14.0	14.0	14.0	
	Pups delivered (total)	13.0	12.0	11.0	12.0	
	Live pups day 1 p.p.	13.0	12.0	11.0	7.0	
	Survivors day 4 p.p.	12.0	12.0	6.0	1.0	
	Survivors at weaning	10.0	10.0	5.0	0.0(mean:1.1)	
	Weight at birth (g)	5.4	5.6	5.7	5.1	
	Weight at weaning (g)	35.1	36.6	40.4	46.6	
	Sex ratio at weaning in % (m/f)	46/54	46/54	50/50	35/65	

Study conducted by the applicant: yes <x> no < >
Study in compliance with GLP: yes <x> no < >

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (REARING SUB-GROUP)
PREGNANCY AND LITTER DATA (REARING)

		CONTROL PLACEBO		60 MG/KG	300 MG/KG	1500 MG/KG
Females on study	N	21		21	21	19
Females Mated	N	21	f	21	21	19
Mating Index	%	100.0		100.0	100.0	100.0
Females Pregnant	N	20	f	20	19	17
Female Fertility Index	%	95.2		95.2	90.5	89.5
Females with Liveborn	N	19	f	20	18	16
Gestation Index	%	95.0		100.0	94.7	94.1
Females Surviving Delivery	N	19	f	20	18	16
	%	90.5		95.2	85.7	84.2
Duration of Gestation	MEDIAN	22.0	d	22.0	23.0*	23.0#
	Q1	22.0		22.0	22.0	23.0
	Q3	22.0		23.0	23.0	23.0
with Stillborn Pups	N	2	f	4	6	11#
	%	10.5		20.0	33.3	68.8
with all Stillborn	N	0	f	0	0	0
	%	0.0		0.0	0.0	0.0
Females with all Resorptions	N	1	f	0	1	1
	%	4.8		0.0	4.8	5.3
Females Pregnant surviving assumed delivery date	N	20	f	20	19	17
	%	95.2		95.2	90.5	89.5
Pups Delivered (total)	N	237		235	191	184
	MEDIAN	13.0	d	12.0	11.0	12.0
	Q1	12.0		11.0	8.8	11.0
	Q3	14.0		13.8	13.0	13.0
Liveborn	N	234	f	224*	170#	140#
Live Birth Index	%	98.7		95.3	89.0	76.1
Stillborn	N	3	f	11*	21#	44#
	%	1.3		4.7	11.0	23.9

Statistical Key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test * = p<0.05 # = p<0.001

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (REARING SUB-GROUP)
PREGNANCY AND LITTER DATA (REARING)

		CONTROL PLACEBO		60 MG/KG	300 MG/KG	1500 MG/KG
Females with Entire Liveborn Litter Dying and/or Missing, Cannibalized sacrificed moribund						
days 1-4	N	0	f	0	5*	8#
	%	0.0		0.0	27.8	50.0
days 1-23	N	0	f	1	5*	11#
	%	0.0		5.0	27.8	68.8
Pups Dying, Missing, Cannibalized, Sacrificed moribund						
day 1	N	0	f	0	7**	22#
	%	0.0		0.0	4.1	15.7
days 2-4	N	32	f	27	55#	86#
	%	13.7		12.1	32.4	61.4
days 5-12	N	5	f	6	9	10*
	%	2.1		2.7	5.3	7.1
days 13-23	N	3	f	0	1	2
	%	1.3		0.0	0.6	1.4
days 1-23	N	40	f	33	72#	120#
	%	17.1		14.7	42.4	85.7
Pups surviving 4 days Viability Index	N	202	f	197	108#	32#
	%	86.3		87.9	63.5	22.9
Pups surviving 21 days Lactation Index	N	195	f	191	98#	20#
	%	83.3		85.3	57.6	14.3
Implantation sites per Litter	N	264		274	241	206
	MEDIAN	14.0	d	14.0	14.0	14.0
	Q1	13.0		12.3	11.8	12.0
	Q3	15.0		15.0	15.0	14.0
Resorptions	N	27	f	39	50**	22
	%	10.2		14.2	20.7	10.7

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test * = p<0.05 ** = p<0.01 # = p<0.001
Resorptions = difference between the number of implantation sites and the number of pups delivered

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (C-SECTION SUB-GROUP)
SUMMARY OF FETAL VISCERAL OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	18	21	18	18
Fetuses Evaluated	N	101	123	91	95
PALATE/MOUTH					
Litter Incidence	N	0	0	1	14
Fetal Incidence	N	0	0	1	54
A AGENESIS OF SOFT PALATE					
Fetal Incidence	N	0 f	0	1	54
	%	0.0	0.0	1.1	56.8
Litter Incidence	N	0 f	0	1	14
	%	0.0	0.0	5.6	77.8
BRAIN/SKULL					
Litter Incidence	N	0	1	1	0
Fetal Incidence	N	0	1	2	0
A HYDROCEPHALUS INTERNUS					
Fetal Incidence	N	0 f	1	2	0
	%	0.0	0.8	2.2	0.0
Litter Incidence	N	0 f	1	1	0
	%	0.0	4.8	5.6	0.0
BLOOD VESSELS					
Litter Incidence	N	2	5	6	4
Fetal Incidence	N	2	5	7	7
V BRACHIOCEPHALIC TRUNK SHORTENED					
Fetal Incidence	N	1 f	4	6	2
	%	1.0	3.3	6.6	2.1
Litter Incidence	N	1 f	4	5	1
	%	5.6	19.0	27.8	5.6

Statistical key: f-Chi-square + Fishers exact test ‡ = p<0.001
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (C-SECTION SUB-GROUP)
SUMMARY OF FETAL SKELETAL OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	18	21	18	21
Fetuses Evaluated	N	112	135	103	108
MISCELLANEOUS					
Litter Incidence	N	1	1	1	2
Fetal Incidence	N	1	5	1	4
V GENERALIZED RETARDATION OF OSSIFICATION					
Fetal Incidence	N	1 f	0	0	1
	%	0.9	0.0	0.0	0.9
Litter Incidence	N	1 f	0	0	1
	%	5.6	0.0	0.0	4.8
A MULTIPLE MALFORMATIONS					
Fetal Incidence	N	0 f	5	1	3
	%	0.0	3.7	1.0	2.8
Litter Incidence	N	0 f	1	1	1
	%	0.0	4.8	5.6	4.8
SKULL					
Litter Incidence	N	5	6	15	21
Fetal Incidence	N	7	11	34	99
V INTERNAL PTERYGOID PROCESS SLIGHTLY BENT					
Fetal Incidence	N	0 f	3	23#	16#
	%	0.0	2.2	22.3	14.8
Litter Incidence	N	0 f	2	13#	7**
	%	0.0	9.5	72.2	33.3
V TYMPANIC ANNULUS ABNORMAL SHAPE					
Fetal Incidence	N	0 f	0	6*	21#
	%	0.0	0.0	5.8	19.4
Litter Incidence	N	0 f	0	6*	11#
	%	0.0	0.0	33.3	52.4
V HYOID BONE ABNORMAL SHAPE					
Fetal Incidence	N	0 f	3	7**	74#
	%	0.0	2.2	6.8	68.5
Litter Incidence	N	0 f	2	4	18#
	%	0.0	9.5	22.2	85.7

Statistical key: f=Chi-square + Fishers exact test * = p<0.05 ** = p<0.01 # = p<0.001
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (C-SECTION SUB-GROUP)
SUMMARY OF FETAL SKELETAL OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	18	21	18	21
Fetuses Evaluated	N	112	135	103	108
A MANDIBLE SHORTENED					
Fetal Incidence	N	0 f	0	0	17#
	%	0.0	0.0	0.0	15.7
Litter Incidence	N	0 f	0	0	6*
	%	0.0	0.0	0.0	28.6
V PART OF PALATINE ABNORMAL SHAPE					
Fetal Incidence	N	0 f	0	0	5*
	%	0.0	0.0	0.0	4.6
Litter Incidence	N	0 f	0	0	3
	%	0.0	0.0	0.0	14.3
A MANDIBLE MISSHAPED					
Fetal Incidence	N	0 f	0	0	11#
	%	0.0	0.0	0.0	10.2
Litter Incidence	N	0 f	0	0	5
	%	0.0	0.0	0.0	23.8
V ZYGOMATIC ARCH ABNORMAL SHAPE					
Fetal Incidence	N	0 f	0	0	14#
	%	0.0	0.0	0.0	13.0
Litter Incidence	N	0 f	0	0	7**
	%	0.0	0.0	0.0	33.3
A TONGUE SHORTENED					
Fetal Incidence	N	0 f	0	0	8**
	%	0.0	0.0	0.0	7.4
Litter Incidence	N	0 f	0	0	5
	%	0.0	0.0	0.0	23.8
A FUSION OF PTERYGOID PROCESS WITH TYMPANIC ANNULUS					
Fetal Incidence	N	0 f	0	1	62#
	%	0.0	0.0	1.0	57.4
Litter Incidence	N	0 f	0	1	16#
	%	0.0	0.0	5.6	76.2
CRANIAL BONES INCOMPL.OSSIF.					
Fetal Incidence	N	7 f	6	5	0*
	%	6.3	4.4	4.9	0.0
Litter Incidence	N	5 f	4	2	0*
	%	27.8	19.0	11.1	0.0

Statistical key: f=Chi-square + Fishers exact test * = p<0.05 ** = p<0.01 # = p<0.001
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (C-SECTION SUB-GROUP)
SUMMARY OF FETAL SKELETAL OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	16	21	18	21
Fetuses Evaluated	N	112	135	103	108
R SUPRAOCCIPITAL INCOMPL.OSSIF.					
Fetal Incidence	N	0 f	0	1	4
	%	0.0	0.0	1.0	3.7
Litter Incidence	N	0 f	0	1	4
	%	0.0	0.0	5.6	19.0
V ADDITIONAL BONE ELEMENT					
Fetal Incidence	N	0 f	0	1	4
	%	0.0	0.0	1.0	3.7
Litter Incidence	N	0 f	0	1	3
	%	0.0	0.0	5.6	14.3
V TYMPANIC ANNULUS NOT APPARENT					
Fetal Incidence	N	0 f	0	0	1
	%	0.0	0.0	0.0	0.9
Litter Incidence	N	0 f	0	0	1
	%	0.0	0.0	0.0	4.8
A INTERNAL PTERYGOID PROCESS BENT					
Fetal Incidence	N	0 f	0	1	19
	%	0.0	0.0	1.0	17.6
Litter Incidence	N	0 f	0	1	9**
	%	0.0	0.0	5.6	42.9
CERVICAL VERT.					
Litter Incidence	N	5	8	1	8
Fetal Incidence	N	8	9	6	12
R ARCH INCOMPLETELY OSSIFIED					
Fetal Incidence	N	7 f	8	1	12
	%	6.3	5.9	2.9	11.1
Litter Incidence	N	5 f	7	2	8
	%	27.8	33.3	11.1	38.1

Statistical key: f=Chi-square + Fishers exact test ** = p<0.01 # = p<0.001
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (REARING SUB-GROUP)
SUMMARY OF PUPS DIED OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	10	7	15	15
Pups Evaluated	N	23	23	43	66
PALATE/MOUTH					
Litter Incidence	N	0	0	6	13
Pup Incidence	N	0	0	16	47
A AGENESIS OF SOFT PALATE					
Pup Incidence	N	0 f	0	8*	29#
	%	0.0	0.0	18.6	43.9
Litter Incidence	N	0 f	0	5	12#
	%	0.0	0.0	33.3	80.0
A PARTLY AGENESIS OF SOFT PALATE					
Pup Incidence	N	0 f	0	8*	18**
	%	0.0	0.0	18.6	27.3
Litter Incidence	N	0 f	0	4	5
	%	0.0	0.0	26.7	33.3
EYES					
Litter Incidence	N	0	0	1	1
Pup Incidence	N	0	0	1	1
A MICROPHTHALMIA					
Pup Incidence	N	0 f	0	1	0
	%	0.0	0.0	2.3	0.0
Litter Incidence	N	0 f	0	1	0
	%	0.0	0.0	6.7	0.0
A ANOPHTHALMIA					
Pup Incidence	N	0 f	0	1	1
	%	0.0	0.0	2.3	1.5
Litter Incidence	N	0 f	0	1	1
	%	0.0	0.0	6.7	6.7
BRAIN					
Litter Incidence	N	1	0	0	0
Pup Incidence	N	1	0	0	0

Statistical key: f-chi-square + Fishers exact test * = p<0.05 ** = p<0.01 # = p<0.001
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (REARING SUB-GROUP)
SUMMARY OF PUPS DIED OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	10	7	15	15
Pups Evaluated	N	23	23	43	66
A DILATATION OF LATERALS VENTRICLES					
Pup Incidence	N	1 f	0	0	0
	%	4.3	0.0	0.0	0.0
Litter Incidence	N	1 f	0	0	0
	%	10.0	0.0	0.0	0.0
BLOOD VESSELS					
Litter Incidence	N	0	0	0	1
Pup Incidence	N	0	0	0	1
A ARTERY ABNORMAL ORIGIN/ROUTE					
Pup Incidence	N	0 f	0	0	1
	%	0.0	0.0	0.0	1.5
Litter Incidence	N	0 f	0	0	1
	%	0.0	0.0	0.0	6.7
KIDNEY					
Litter Incidence	N	1	0	1	1
Pup Incidence	N	1	0	1	1
R RENAL PAPILLA REDUCED					
Pup Incidence	N	1 f	0	1	1
	%	4.3	0.0	2.3	1.5
Litter Incidence	N	1 f	0	1	1
	%	10.0	0.0	6.7	6.7
URETERS					
Litter Incidence	N	1	0	1	1
Pup Incidence	N	1	0	1	1

Statistical key: f=Chi-square + Fishers exact test
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

STUDY OF EMBRYO-FETAL AND PRE- AND POSTNATAL DEVELOPMENT IN THE RAT WITH ORAL GAVAGE OF RO 47-0203/015 (REARING SUB-GROUP)
SUMMARY OF PUPS DIED OBSERVATIONS

		CONTROL PLACEBO	60 MG/KG	300 MG/KG	1500 MG/KG
Litters Evaluated	N	10	7	15	15
Pups Evaluated	N	23	23	43	66
V CONVOLUTED URETER					
Pup Incidence	N	1 f	0	0	0
	%	4.3	0.0	0.0	0.0
Litter Incidence	N	1 f	0	0	0
	%	10.0	0.0	0.0	0.0
A CONVOLUTED URETER					
Pup Incidence	N	0 f	0	1	1
	%	0.0	0.0	2.3	1.5
Litter Incidence	N	0 f	0	1	1
	%	0.0	0.0	6.7	6.7

Statistical Key: f-Chi-square + Fishers exact test
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

Median body weights of surviving pups on lactation day 23 were higher than concurrent control at 300 and 1500 mg/kg/day (35, 36, 40 and 47g at 0, 60, 300 and 1500 mg/kg/day, respectively); this finding is likely due to the smaller surviving litter sizes in higher dose dams. Development of surviving pups (auditory startle, pupil contraction, memory and learning) was unrelated to drug. Reproductive development and performance of surviving pups were unrelated to drug. Female mating indexes were 94.7%, 94.7%, 100% and 80% (18/19, 18/19, 13/13 and 4/5) at doses of 0, 60, 300 and 1500 mg/kg/day. The lower mating index of 80% was not attributed to drug since only 5 females were mated.

REPRODUCTION TOXICITY Breeding a F2-generation
Ref. to document.: Volume: Page: to Addendum No.:
Report date: May 5, 1995 Number: RRB'153'693 Study period (year) :1994

Species/Strain: Rat/ Albino

Number of animals: 56 females, 53 males

Generation of parental animals: <0>

Administration route:no treatment, except during lactation via mother's milk

Treatment of controls: no treatment

Treatment of the females DL 1 through DL 22 (via mother's milk)	Evidence of mating = day 0 of gestation	F1-generation breeding a F2-generation

Methods of examination of the fetuses

< > Skeleton	< > Soft tissue	Others: yes < > no <x>
< > Histology	< > Biochemistry	External examination

			(1)Contr	(2)	(3)	(4)	(5)
Study group							
Dosage <mg/kg/day >			0	60	300	1500	
P	Females with evidence of mating		18	18	13	4	
A	Pregnant females		17	18	12	4	
R.	Eval. pregn. females		17	18	12	4	
LITTER	median per dam or per litter	Corpora lutea	14.0	16.0	14.0	13.0	
		Implantations	14.0	14.0	13.5	13.5	
		Fetuses	13.0	12.5	13.0	10.5	
		Dead fetuses	0.0	0.0	0.0	0.0	
		Postimplantation loss	1.0	1.0	1.0	2.0	
		Weight of fetuses (g)	not determined				
S	Sex ratio of fetuses (m/f,%)		not determined				

Study conducted by the applicant: yes <x> no < >
Study in compliance with GLP: yes <x> no < >

Study of Embryo-Fetal Development and Postnatal Development in the Rat Following Oral Administration of Ro 47-0203: Litter Exchange

Location of Study Report: Vol 39, pg 1

Study Facility:

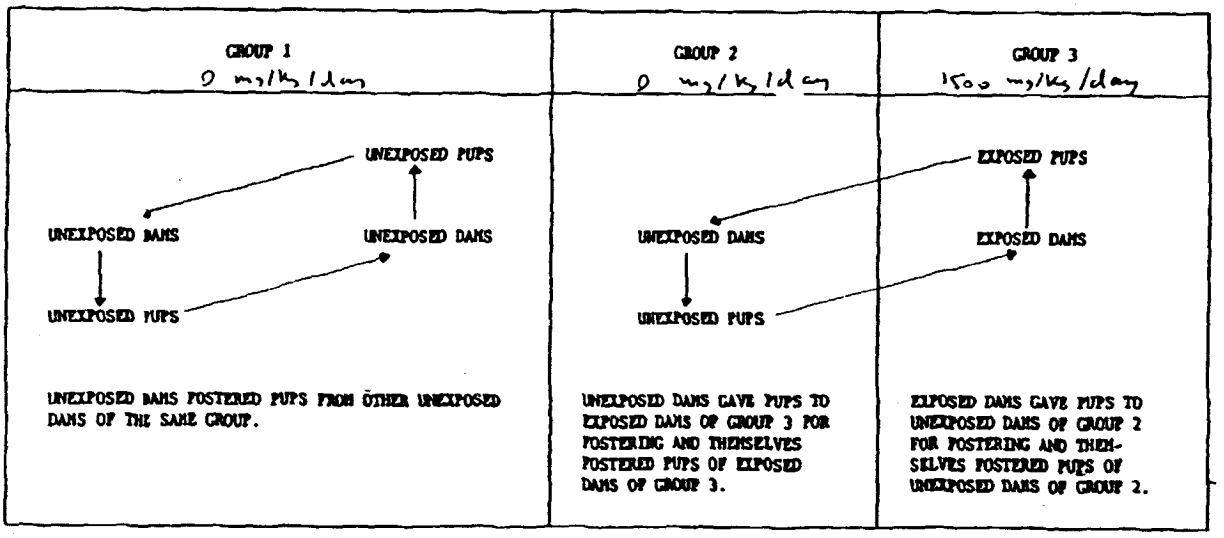
Study No.: 114R94
Report No.: 163303
Study Dates: 06/06/1994 – 07/12/1994

GLP Compliance: Yes

Animals: Female Wistar rats weighing from 181-219g (70-84 days of age) on gestation day 0 were housed individually and allowed feed and water *ad libitum*.

Drug Administration: Ro 47-0203 (Lot No. GFR 0038) was suspended in aqueous carboxymethyl cellulose and given orally by gavage to pregnant female rats from gestation day 6 through lactation day 12.

Dose Levels: 0, 0, 1500 mg/kg/day (groups 1, 2 and 3); 20 female rats per treatment group. Groups 1 and 2 served as concurrent control and unexposed litter exchange females, respectively.



Mating: Each female was placed with one untreated male in a breeding cage, overnight. Mating was determined by the presence of an ejected copulatory plug. The day evidence of mating was detected was designated day 0 of gestation.

Litter exchange: Litters were exchanged between untreated dams and dams given 1500 mg/kg/day before the first milk intake on the day of parturition. Litters from untreated dams that were raised by drug-treated dams were designated group 2 litters. Litters from drug-treated dams that were raised by untreated dams were designated group 3 litters.

Observations/Measurements: All dams were weighed on gestation days 0, 6-18 and 20; and lactation days 1, 4 and 12. Average food consumption was monitored on gestation days 0-6, 6-13, 13-20 and lactation days 1-4, and 4-12.

Dams were observed daily for mortality and clinical signs of toxicity. Dams were allowed to deliver spontaneously. Dams were sacrificed on lactation days 13-15 and necropsied. Uteri were removed and examined.

Pups were sacrificed on lactation days 13-15, sexed, and subjected to external macroscopic examination. Internal organs were examined macroscopically. Heads were fixed in Wilson's solution and examied. Pups which were found dead on study were fixed in Wilson's solution and examined for soft tissue abnormalities.

Plasma Drug Levels: Not determined.

Drug Associated Findings

Duration of gestation was similar across treatment groups, as were numbers of corpora lutea, implantations and pre- and postimplantation losses. Absence of milk in mammary glands was observed at necropsy in 26% of untreated dams raising litters that were treated in utero at 1500 mg/kg/day; this finding was absent in concurrent control dams and drug-treated dams raising untreated litters. Absence of milk in mammary glands of group 2 untreated females likely resulted from fetal deaths and lack of suckling.

Pup survival was markedly reduced in litters treated *in utero* at 1500 mg/kg/day but raised by untreated dams (group 3). In comparison, pup survival was not lower than concurrent control in litters from untreated dams, but raised by drug-treated dams (group 2).

Agenesis of the soft palate and shortening of the innominate artery were observed in litters treated *in utero* at 1500 mg/kg/day but raised by untreated dams (group 3). These findings were absent in litters from untreated dams that were raised by drug-treated dams (group 2).

REPRODUCTION TOXICITY Pre- and Postnatal Development

Ref. to document.: Volume: Page: to Addendum No.:
Report date: October 20, 1997 Number: RRB-163'303 Study period (years): 1994

Species/Strain: Rat /Wistar

Number of animals: 60 f

Generation of parental animals: <0>

Administration route: oral (gavage)

Treatment of controls: Vehicle (Ro 47-0203/022)

Sperm in vag. smear = day 0 of gestation	Treatment of females from gestation day 6 to lactation day 12	

Methods of examination of the pups: (litter reduction: yes < > no < x >)

< > Skeleton	< x > Soft tissue	Others: yes < x > no < > litter exchange
< > Histology	< > Biochemistry	External examination

Study group			(1) Contr	(2)	(3)		
Dosage <mg/kg/day>			0	0	1500		
P	Females with sperm		21	19	20		
A	Pregnant females		19	19	20		
R.	Females with delivery		19	19	20		
	Median duration of gestation		22	21	22		
L	median per dam	Implantations	13.0	14.0	14.0		
I		Live births	197	221	209		
T		Stillbirths	12	0	7		
T		Survivors day 4 pp	182	214	87		
E		Survivors at DG 12	178	197	61		
		Weight at birth (g; day life 1)	5.5	5.4	5.7		
R		Weight at weaning (g; day life 12)	17.1	17.1	17.1		
S	Sex ratio of live newborns (m/f)		48.9 51.1	52.3 47.7	50.8 49.2		

Study conducted by the applicant: yes < x > no < >
Study in compliance with GLP: yes < x> no < >

DG, day of gestation

LITTER FOSTERING STUDY IN RATS WITH ORAL ADMINISTRATION OF RO 47-0203/015
PREGNANCY AND LITTER DATA (REARING)

		GROUP 1	GROUP 2	GROUP 3
Females with Entire Liveborn Litter Dying and/or Missing, Cannibalized^ Sacrificed moribund				
days 1-4	N	0 f	0	4
	%	0.0	0.0	20.0
days 1-12	N	0 f	0	5*
	%	0.0	0.0	25.0
Pups Dying, Missing, Cannibalized, Sacrificed moribund				
day 1	N	0 f	0	0
days 2-4	N	15 f	7*	122#
	%	7.6	3.2	50.4
days 5-12	N	4 f	17*	26#
	%	2.0	7.7	12.4
days 1-12	N	19 f	24	150#
	%	9.6	10.9	71.9
Pups Surviving 4 days Viability Index	N	182 f	214*	87#
	%	92.4	96.8	41.6
Pups Surviving 12 days Lactation Index	N	178 f	197	61#
	%	90.4	89.1	29.2
Implantation Sites per Litter	N	234	245	232
	MEDIAN	13.0 d	14.0	14.0
	Q1	12.0	13.0	12.0
	Q3	14.3	15.0	15.0
Resorbed Implants	N	25 f	24	16
	%	10.7	9.8	6.9

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test * = p<0.05 # = p<0.001
Resorbed Implants = difference between the number of implantation sites and the number of pups delivered

LITTER FOSTERING STUDY IN RATS WITH ORAL ADMINISTRATION OF RO 47-0203/015
SUMMARY OF PUP VISCERAL OBSERVATIONS (PUPS FOUND DEAD)

		GROUP 1	GROUP 2	GROUP 3
Litters Evaluated	N	8	4	17
Pups Evaluated	N	24	16	111
PALATE/MOUTH				
Litter Incidence	N	0	0	15
Pup Incidence	N	0	0	90
A AGENESIS OF SOFT PALATE				
Pup Incidence	N	0 f	0	79#
	%	0.0	0.0	71.2
Litter Incidence	N	0 f	0	11**
	%	0.0	0.0	64.7
A PARTIAL AGENESIS OF SOFT PALATE				
Pup Incidence	N	0 f	0	11
	%	0.0	0.0	9.9
Litter Incidence	N	0 f	0	6
	%	0.0	0.0	35.3
BLOOD VESSELS				
Litter Incidence	N	0	0	3
Pup Incidence	N	0	0	3
V INNOMINATE ARTERY SHORTENED				
Pup Incidence	N	0 f	0	3
	%	0.0	0.0	2.7
Litter Incidence	N	0 f	0	3
	%	0.0	0.0	17.6

Statistical key: f=Chi-square + Fishers exact test ** = p<0.01 # = p<0.001
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION

Study of Embryo-Fetal Development and Postnatal Development in the Rat Following Oral Administration of Ro 47-0203 (batch containing higher levels of impurities Ro 47-0005, Ro 47-4056 and Ro 47-9931)

Location of Study Report: Vol 38, pg 321

Study Facility:

Study No.: 0218R94
Report No.: 163300
Study Dates: 01/05/95-02/20/95

GLP Compliance: Yes

Animals: Mated female Wistar rats weighing from 162-212 g on gestation day 0 were housed individually and allowed feed and water *ad libitum*.

Drug Administration: Ro 47-0203 [Lot No. GFR 0071; containing bosentan plus the impurities Ro 47-0005 (0.1%), Ro 47-4056 (0.6%) and Ro 47-9931 (0.3%)] was suspended in aqueous carboxymethyl cellulose and given orally by gavage to female rats on gestation days 6 through 20. The day evidence of mating was detected was designated day 0 of gestation.

Dose Levels: 0, 30, 60, 120, 300 mg/kg/day (20 female rats per treatment group)

Observations/Measurements

Dams were observed daily for clinical signs of toxicity. Gestation lengths were recorded. Maternal body weights were determined on gestation days 0, 6, 16 and 20, and lactation days 1 and 4. Maternal food intakes were monitored during gestation days 0-20.

Dams were allowed to deliver spontaneously. The number of live and dead pups were recorded. Pups found dead on day 1 of lactation were considered stillborn. Dams were sacrificed for necropsy after weaning their pups. Dam's internal organs were observed macroscopically. The number of corpora lutea and implantations were determined.

All drug-treated pups and some of the concurrent control pups were sacrificed on day 4 of lactation and necropsied. (two pups/litter from the concurrent control treatment group were sacrificed on lactation days 1, 2 and 3 and necropsied). Following external examination, all pups were examined for deviation of the soft palate. The heart and thymus were examined in situ. Approximately half of the pups were prepared for skeletal examination of the upper part of the body, including the first four ribs, shoulder girdle, neck and skull. The remaining pups were fixed in Wilson's solution and examined for soft tissue anomalies. Pups found dead during the study were examined as described above.

Plasma Drug Levels: Not determined.

Drug Associated Findings : The numbers of corpora lutea, implantations, pre- and post-implantation losses were unrelated to drug treatment. Pup weights at delivery and sex ratios were comparable across dose groups. The number of bosentan-treated dams with stillborn was slightly higher than concurrent control. This effect was observed at doses as low as 30 mg/kg/day but was not dose-related.

The percentage of pups surviving through 4 days of lactation was lower in the 300 mg/kg/day treatment group than in the lowest dose group (30 mg/kg/day). Concurrent control pup survival was not available since some concurrent control pups were sacrificed on lactation days 1, 2 and 3.

Agenesis of the soft palate was observed in pups from three dams given 120 mg/kg/day and eight dams given 300 mg/kg/day. Dose-related skeletal and blood vessel variations were observed in pups from dams given bosentan at doses as low as 30 mg/kg/day.

REPRODUCTION TOXICITY Embryo-fetal toxicity (spontaneous delivery)							
Ref. to document.: Volume: Page: to Addendum No.:							
Report date: October 30, 1997 Number: B-163'300 Study period (years): 1994/95							
Species/Strain: Rat							
Number of animals: f 100							
Generation of parental animals:<0>							
Administration route:oral (gavage)							
Treatment of controls:Vehicle (Ro 47-0203/022)							
Sperms of vag. smear = Day 0 of gestation		Treatment of the females from day: 6 to day: 20 of gestation			Section of females on day:4-23 of lacation		
Methods of examination of the young: (litter reduction: yes < x controls only > no < x>)							
< x > Skeleton		< x > Soft tissue			Others: yes < > no < >		
< > Histology		< > Biochemistry					
Study group			(1) Contr	(2)	(3)	(4)	(5)
Dosage mg/kg/day (*spiked batch)			0	30	60	120	300
P	Females with sperm		20	20	20	20	20
A	Pregnant females		18	19	16	19	18
R	Dams delivering		16	16	16	18	16
L	median per pregn. female	Corpora lutea	13.5	14.0	14.0	14.0	
I		Implantations	13.5	12	13	14	12
T		Live foetuses	not determined				
T		Dead foetuses	not determined				
E		Resorptions (%)	8.0	14.4	7.7	9.2	8.1
R	group median	Weight of pups DL1 (g)	5.7	6.0	5.8	5.8	5.8
S	Sex ratio of foetuses (m/f)		47.1/52.9	51.9/48.1	43.5/56.5	47.8/52.2	54.7/45.3
Study conducted by the applicant: yes < x> no < >							
Study in compliance with GLP: yes < x > no < >							

RO 47-0203/015 SEGMENT II: ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL AND ON PRE- AND POSTNATAL DEVELOPMENT IN THE RAT
PREGNANCY AND LITTER DATA (REARING)

		CONTROL PLACEBO		30 MG/KG	60 MG/KG	120 MG/KG	300 MG/KG
Females on Study	N	20		20	20	20	20
Females Pregnant	N	18	f	19	16	19	18
Female Fertility Index	%	90.0		95.0	80.0	95.0	90.0
Females with Liveborn	N	16	f	16	16	18	16
Gestation Index	%	88.9		84.2	100.0	94.7	88.9
Females Surviving Delivery	N	16	f	16	16	18	16
	%	80.0		80.0	80.0	90.0	80.0
Duration of Gestation	MEDIAN	22.0	d	22.0	22.0	22.0	22.0
	Q1	22.0		22.0	22.0	22.0	22.0
	Q3	22.0		22.0	22.8	22.0	22.0
with Stillborn Pups	N	0	f	1	0	2	2
	%	0.0		6.3	0.0	11.1	12.5
with all Stillborn	N	0	f	0	0	0	0
Females with all Resorptions	N	1	f	0	0	0	1
	%	5.0		0.0	0.0	0.0	5.0
Females Pregnant surviving assumed delivery date	N	17	f	16	16	18	17
	%	85.0		80.0	80.0	90.0	85.0
Pups Delivered (total)	N	173		161	180	218	181
	MEDIAN	12.5	d	10.5	12.0	13.0	12.0
	Q1	8.5		7.5	11.0	11.5	10.3
	Q3	14.0		13.0	13.0	14.0	12.8
Liveborn	N	173	f	160	180	216	179
Live Birth Index	%	100.0		99.4	100.0	99.1	98.9
Stillborn	N	0	f	1	0	2	2
	%	0.0		0.6	0.0	0.9	1.1

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test

RO 47-0203/015 SEGMENT II: ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL AND ON PRE- AND POSTNATAL DEVELOPMENT IN THE RAT
PREGNANCY AND LITTER DATA (REARING)

		30 MG/KG	60 MG/KG	120 MG/KG	300 MG/KG
Females with Entire Liveborn Litter Dying and/or Missing, Cannibalized Sacrificed moribund days 1-4	N	0 f	1	0	0
	%	0.0	6.3	0.0	0.0
Pups Dying, Missing, Cannibalized, Sacrificed moribund day 1	N	1 f	5	1	4
	%	0.6	2.8	0.5	2.2
days 2-4	N	5 f	5	12	14
	%	3.1	2.8	5.6	7.8
days 1- 4	N	6 f	10	13	18*
	%	3.8	5.6	6.0	10.1
Pups Surviving 4 days Viability Index	N	154 f	170	203	161*
	%	96.3	94.4	94.0	89.9
Implantation Sites per Litter	N	188	195	240	197
	MEDIAN	12.0 d	13.0	14.0	12.0
	Q1	10.3	12.0	12.8	12.0
	Q3	13.0	14.0	15.3	14.0
Resorbed Implants	N	27 f	15*	22	16
	%	14.4	7.7	9.2	8.1

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test * = $p<0.05$
Resorbed Implants = difference between the number of implantation sites and the number of pups delivered

Comment: On lactation days 1, 2 and 3, two pups of each control litter were sacrificed in order to evaluate the normal development of the anatomical strurcures which are sensitive to drug influence. Therefore, the data of the control litters were not comparable with that of the dose groups and were omitted from the table. Statistical evaluations were performed using the lowest dose group instead of the controls. This is justifiable, because our historical controls (e.g. pups dying between days of lactation 1-4, pup weights day 1 and 4 or the viability index), were comparable to the values of this lowest dose group.

RO 47-0203/015 SEGMENT II: ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL AND ON PRE- AND POSTNATAL DEVELOPMENT IN THE RAT
SUMMARY OF PUP VISCERAL OBSERVATIONS

		CONTROL PLACEBO	30 MG/KG	60 MG/KG	120 MG/KG	300 MG/KG
Litters Evaluated	N	13	16	15	18	16
Pups Evaluated	N	35	80	87	103	90
PALATE/MOUTH						
Litter Incidence	N	0	0	0	3	8
Pup Incidence	N	0	0	0	4	14
A PARTIAL AGENESIS OF SOFT PALATE						
Pup Incidence	N	0 f	0	0	4	14*
	%	0.0	0.0	0.0	3.9	15.6
Litter Incidence	N	0 f	0	0	3	8**
	%	0.0	0.0	0.0	16.7	50.0
BLOOD VESSELS						
Litter Incidence	N	0	2	0	0	1
Pup Incidence	N	0	2	0	0	1
V INNOMINATE ARTERY SHORTENED						
Pup Incidence	N	0 f	1	0	0	0
	%	0.0	1.3	0.0	0.0	0.0
Litter Incidence	N	0 f	1	0	0	0
	%	0.0	6.3	0.0	0.0	0.0
A ARTERY ABNORMAL ORIGIN/ROUTE						
Pup Incidence	N	0 f	1	0	0	0
	%	0.0	1.3	0.0	0.0	0.0
Litter Incidence	N	0 f	1	0	0	0
	%	0.0	6.3	0.0	0.0	0.0
A ACCESSORY ARTERY						
Pup Incidence	N	0 f	0	0	0	1
	%	0.0	0.0	0.0	0.0	1.1
Litter Incidence	N	0 f	0	0	0	1
	%	0.0	0.0	0.0	0.0	6.3
URETERS						
Litter Incidence	N	0	0	0	0	1
Pup Incidence	N	0	0	0	0	1

Statistical key: f=Chi-square + Fishers exact test * = p<0.05 ** = p<0.01
OBSERVATION CODE: A-ABNORMALITY V-VARIATION R-RETARDATION