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
V CONVOLUTED URETER						
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

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

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 NECROPSY OBSERVATIONS

		CONTROL PLACEBO	30 MG/KG	60 MG/KG	120 MG/KG	300 MG/KG
Litters Evaluated	N	15	16	15	18	16
Pups Evaluated	N	136	79	90	107	85
HEART						
Litter Incidence	N	1	6	4	5	12
Pup Incidence	N	1	7	8	6	15
V ARTERIA INNOMINATA NOT PRESENT/ABSENT						
Pup Incidence	N	1 f	5*	7**	2	1
	%	0.7	6.3	7.8	1.9	1.2
Litter Incidence	N	1 f	5	4	2	1
	%	6.7	31.3	26.7	11.1	6.3
V ARTERY ABNORMAL ORIGIN/ROUTE						
Pup Incidence	N	0 f	3*	0	2	8#
	%	0.0	3.8	0.0	1.9	9.4
Litter Incidence	N	0 f	3	0	1	7**
	%	0.0	18.8	0.0	5.6	43.8
V ARTERIA INNOMINATA SHORTENED						
Pup Incidence	N	0 f	0	1	2	8#
	%	0.0	0.0	1.1	1.9	9.4
Litter Incidence	N	0 f	0	1	2	7**
	%	0.0	0.0	6.7	11.1	43.8

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

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 SKELETAL OBSERVATIONS (HEAD ONLY)

		CONTROL PLACEBO	30 MG/KG	60 MG/KG	120 MG/KG	300 MG/KG
Litters Evaluated	N	15	16	15	18	16
Pups Evaluated	N	136	79	90	107	86
V INTERNAL PTERYGOID PROCESS SLIGHTLY BENT						
unilateral + bilateral	N	0	3	12	48	67
Pup Incidence	N	0	3	12	48	67
	%	0.0	3.8	13.3	44.9	77.9
Litter Incidence	N	0	2	7	18	16
	%	0.0	12.5	46.7	100.0	100.0
V TYMPANIC ANNULUS ABNORMAL SHAPE						
unilateral + bilateral	N	0	0	0	0	12
Pup Incidence	N	0	0	0	0	12
	%	0.0	0.0	0.0	0.0	14.0
Litter Incidence	N	0	0	0	0	7
	%	0.0	0.0	0.0	0.0	43.8
V HYOID BONE ABNORMAL SHAPE						
Pup Incidence	N	0	0	4	14	42
	%	0.0	0.0	4.4	13.1	48.8
Litter Incidence	N	0	0	3	10	14
	%	0.0	0.0	20.0	55.6	87.5
V ADDITIONAL BONE ELEMENT						
Pup Incidence	N	0	0	0	4	7
	%	0.0	0.0	0.0	3.7	8.1
Litter Incidence	N	0	0	0	2	4
	%	0.0	0.0	0.0	11.1	25.0
V ZYGOMATIC ARCH ABNORMAL SHAPE						
unilateral + bilateral	N	0	0	0	0	5
Pup Incidence	N	0	0	0	0	5
	%	0.0	0.0	0.0	0.0	5.8
Litter Incidence	N	0	0	0	0	5
	%	0.0	0.0	0.0	0.0	31.3

OBSERVATION CODE: V VARIATION

Embryotoxicity and Teratogenicity Study in the Rabbit with Oral (Gavage) Administration of Ro 47-0203

Location of Study Report: Vol 39, pg 109

Study Facility:

Study No.: 009R94
Report No.: 153699
Study Dates: 01/10/1994 – 02/12/1994

GLP Compliance: Yes

Animals: Russian Himalayan rabbits , weighing from 2290-2361 g on gestation day 0 (20 female rabbits per treatment group) 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 rabbits on gestation days 7 through 18.

Dose Levels: 0, 150, 450, 1500 mg/kg/day given in two daily doses 5 hours apart

Pilot study for dose selection: Eight pregnant rabbits were given bosentan at 150, 450 or 1500 mg/kg/day in two divided daily doses. The mean fetus body weight and crown-rumb length were lower in rabbits given 1500 mg/kg/day than in concurrent controls.

Mating: Each female was placed with an untreated male. If copulation was not observed after 4-6 hours, the process was repeated with another untreated male. The day of copulation was designated gestation day 0.

Observations/Measurements

All dams were observed daily for mortality and clinical signs of toxicity. Body weight measurements were performed on days 0, 7-19, 29 of gestation. Dam weights were not corrected for gravid uterine weights. Dams were sacrificed on gestation day 29. The uteri were examined for numbers of live and dead fetuses, corpora lutea and implantations.

All live fetuses were sexed, weighed, measured for length, and examined for external abnormalities.
Dead and aborted fetuses on gestation day 29 were either discarded after macroscopic inspection or examined for terata, per the study director's discretion.

Live fetuses were sacrificed, x-rayed in the dorso-ventral and lateral positions for evaluation of the skeletons, and then decapitated. All fetal heads were fixed in formalin/acetic acid and serially sectioned for examination. Fetal trunks (unstained) were examined for soft tissue anomalies. If the skeletons could not be judged on the basis of the x-rays, the trunks were then dissected and subjected to a skeletal examination.

Plasma Drug Levels: Not determined.

Drug Associated Findings

Maternal mortality at 450 and 1500 mg/kg/day was greater than concurrent control. The sponsor attributed excess deaths to dosing errors associated with the high viscosity of the dosing suspension. The sponsor did not provide evidence to support this conclusion.

Maternal body weight gains during drug treatment were significantly lower than concurrent control at 450 and 1500 mg/kg/day. Whereas body weights increased in concurrent control dams, body weights decreased in dams given 450 and 1500 mg/kg/day. Effects were dose-related. The NOAEL for maternal body weight was 150 mg/kg/day.

Fetal body weights were lower than concurrent control at 1500 mg/kg/day. The number of corpora lutea, implantations, and pre-implantation and post-implantation losses were unrelated to drug treatment. Fetal sex ratios were comparable across dose groups. The NOAEL for reduction of fetal body weight was 450 mg/kg/day.

~~Fetal skeletal variations were more common at 1500 mg/kg/day than in the concurrent control group.~~ Extra thoracic ribs were more common (litter incidences of 1, 0, 0 and 3 at 0, 150, 450 and 1500 mg/kg/day) as were supernumerary vertebra (observed in a single litter at 1500 mg/kg/day, but absent in concurrent control and lower dose groups).

Soft tissue and skeletal abnormalities were not observed in rabbits given bosentan at doses up to 1500 mg/kg/day.

There were no drug-related external anomalies in the trunk.

EMBRYOTOXICITY AND TERATOGENICITY STUDY IN RABBITS WITH ORAL ADMINISTRATION OF RO 47-0203/015. SEGMENT II-STUDY
Maternal survival and pregnancy status

		CONTROL PLACEBO		150 MG/KG	450 MG/KG	1500 MG/KG
No. of females at start	N	20		20	20	20
No. of females mated	N	20		20	20	20
Females with defined day 0 p.c.	N	20		20	20	20
Pregnant	N	19		19	19	20
- Died/sac'd during gestation	N	1		1	2	4
- Died delivering	N	0		0	0	0
- Died/sac.mor. post partum	N	0		0	0	0
- Aborted	N	0		1	0	1
- Delivered prematurely	N	0		0	0	0
Nonpregnant	N	1		1	1	0
- Died/sacrificed moribund	N	0		0	0	0
Total no. of females died/ sacrificed moribund	N	1	f	1	2	4
	%	5.0		5.0	10.0	20.0
Females pregnant and used for analysis at scheduled c-section	N	18		17	17	15
- with total fetal death	N	0	f	0	0	1
	%	0.0		0.0	0.0	6.7
- with viable fetuses	N	18	f	17	17	14
	%	100.0		100.0	100.0	93.3

Statistical key: f-Chi-square + Fishers exact test

EMBRYOTOXICITY AND TERATOGENICITY STUDY IN RABBITS WITH ORAL ADMINISTRATION OF RO 47-0203/015. SEGMENT II-STUDY
MEDIAN MATERNAL BODY WEIGHT CHANGE DURING GESTATION - grams

		CONTROL PLACEBO	150 MG/KG	450 MG/KG	1500 MG/KG
DAYS 0 TO 7	MEDIAN	81.0 d	74.5	91.0	77.0
	Q1	57.0	44.3	59.0	31.0
	Q3	138.0	99.0	127.0	133.0
	N	19	18	19	19
DAYS 7 TO 19	MEDIAN	50.0 d	29.0	-17.0**	-48.0#
	Q1	22.8	-31.0	-95.8	-151.0
	Q3	126.5	127.0	12.3	-32.0
	N	18	17	18	15
DAYS 19 TO 29	MEDIAN	197.5 d	211.0	215.0	158.0
	Q1	155.0	178.5	177.5	119.0
	Q3	244.0	245.0	246.0	259.0
	N	18	17	17	15

Statistical key: d=ANOVA + Dunnett-test ** = p<0.01 # = p<0.001

EMBRYOTOXICITY AND TERATOGENICITY STUDY IN RABBITS WITH ORAL ADMINISTRATION OF RO 47-0203/015. SEGMENT II-STUDY
SUMMARY OF REPRODUCTION DATA (C-SECTION)

		CONTROL PLACEBO		150 MG/KG	450 MG/KG	1500 MG/KG
Pregnant, used for calculation	N	18		17	17	15
Corpora Lutea	N	175		163	147	125
No. per animal	MEDIAN	10.5	d	10.0	9.0	8.0
	Q1	8.0		8.5	7.0	7.0
	Q3	11.3		11.0	10.5	11.0
Preimplantation Loss	N	35		30	24	19
% per group	%	20.0		18.4	16.1	15.2
% per animal	MEDIAN	25.0	u	18.2	18.2	12.5
	Q1	10.6		10.1	5.0	0.0
	Q3	27.6		27.9	29.3	25.0
Implantation Sites	N	140		113	121	106
No. per animal	MEDIAN	8.0	d	8.0	7.0	7.0
	Q1	6.0		7.0	6.0	5.0
	Q3	9.0		9.0	9.0	9.0
Fetuses	N	135		118	118	90
No. per animal	MEDIAN	8.0	d	7.0	7.0	6.0
	Q1	5.8		6.0	5.5	5.0
	Q3	9.0		8.0	8.5	8.0
Alive	%	100.0		100.0	100.0	100.0
Dead	%	0.0		0.0	0.0	0.0
Live Fetuses	N	135		118	118	90
No. per animal	MEDIAN	8.0	d	7.0	7.0	6.0
	Q1	5.8		6.0	5.5	5.0
	Q3	9.0		8.0	8.5	8.0
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

EMBRYOTOXICITY AND TERATOGENICITY STUDY IN RABBITS WITH ORAL ADMINISTRATION OF BO 47-0203/015. SEGMENT II-STUDY
SUMMARY OF REPRODUCTION DATA (C-SECTION)

		CONTROL PLACEBO		150 MG/KG		450 MG/KG		1500 MG/KG	
Pregnant, used for calculation	N	18		17		17		15	
Resorptions: Total	N	5		15		5		16	
No. per animal	MEDIAN	0.0	d	1.0		0.0		1.0	*
	Q1	0.0		0.0		0.0		0.0	
	Q3	1.0		1.5		0.5		1.0	
% of impl. per group	%	3.6		11.3		4.1		15.1	
% of impl. per animal	MEDIAN	0.0	u	10.0		0.0		14.3	
	Q1	0.0		0.0		0.0		0.0	
	Q3	8.0		20.8		5.6		18.2	
Resorptions: Early	N	5		13		3		11	
% of reserp. per group	%	100.0		86.7		60.0		68.8	
Resorptions: Late	N	0		2		2		5	
% of reserp. per group	%	0.0		13.3		40.0		31.3	
Postimplantation Loss	N	5		15		5		16	
No. per animal	MEDIAN	0.0	d	1.0		0.0		1.0	*
	Q1	0.0		0.0		0.0		0.0	
	Q3	1.0		1.5		0.5		1.0	
% of impl. per group	%	3.6		11.3		4.1		15.1	
% impl. per animal	MEDIAN	0.0	u	10.0		0.0		14.3	
	Q1	0.0		0.0		0.0		0.0	
	Q3	8.0		20.8		5.6		18.2	
Viable Male Fetuses	N	53	f	56		44		40	
	%	39.3		47.5		37.3		44.4	
Female Fetuses	N	82	f	62		74		50	
	%	60.7		52.5		62.7		55.6	
Fetal Body Weight (g)	MEDIAN	38.4	d	37.9		34.6		34.8	*
	Q1	34.8		35.2		30.8		30.2	
	Q3	39.9		40.7		38.4		37.2	
	N LITTERS	18		17		17		14	
Crown-rump length (cm)	MEDIAN	9.1	d	9.1		9.0		8.8	
	Q1	8.9		9.0		8.6		8.5	
	Q3	9.3		9.4		9.1		9.1	
Litters with dead fetuses	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 u=Kruskal-Wallis + Mann-Whitney U * = p<0.05

Supplementary Oral (Gavage) Study for Effects of Ro 47-0203 on Embryo-Fetal Development in the Rabbit

Location of Study Report: Vol 39, pg 244

Study Facility:

Study No.: 175R94
Report No.: 163251
Study Dates: 10/10/1994 – 11/09/1994

GLP Compliance: Yes

Animals: Mated female Himalayan rabbits (group median weight on gestation day 0: control, 2731g; 1500 mg/kg/day, 2827g) were housed individually and allowed feed and water *ad libitum.*

Drug Administration: Ro 47-0203 (Lot No. GMP 0024) was suspended in aqueous carboxymethyl cellulose and given orally by gavage to female rabbits on gestation days 7 through 18.

Dose Levels: 0, 1500 mg/kg/day given in two daily doses in the morning and the afternoon. The time between doses was not provided.

Mating: Each female was placed with one untreated male for 4-6 hours. If copulation was not observed after 4-6 hours, this process was repeated with another male. The day of copulation was designated gestation day 0.

Observations/Measurements: All dams were observed daily for mortality and clinical signs of toxicity. Body weight and food intake measurements were performed on days 0, 7-19, 29 of gestation. Dam weights were not corrected for gravid uterine weights. Dams were sacrificed on gestation day 29. The uteri were examined for numbers of live and dead fetuses, corpora lutea and implantations.

All live fetuses were weighed and examined for external abnormalities. Dead and aborted fetuses on gestation day 29 were discarded after macroscopic inspection. Following sacrifice of live fetuses, fetal heads were removed, fixed in formalin/acetic acid and serially sectioned for examination. Fetal trunks (unstained) were not examined. The sponsor performed this study to confirm the absence of craniofacial anomalies in rabbits.

Plasma Drug Levels: Not determined.

Drug Associated Findings

Mortality of dams was not drug related. Maternal body weight gain and food intake were lower in dams given 1500 mg/kg/day than in concurrent controls, consistent with the previous Segment II study in rabbits.

There were no drug-related effects on corpora lutea, implantation sites, pre- and post-implantation losses. Fetal weights and lengths were lower at 1500 mg/kg/day than in concurrent control, consistent with the previous Segment II study in rabbits.

Variations in the skull were significantly more common in litters at 1500 mg/kg/day than in concurrent control. (nasal and frontal bones were more often split into two bones or exhibited an additional small bone element with the central suture of the left and right frontal and nasal bone). These findings were considered to be variations rather than abnormalities since they did not seem to impair the normal function or shape of the skull.

There were no drug-related external anomalies in the trunk.

RO 47-0203/015 SEGMENT II: SUPPLEMENTARY ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL DEVELOPMENT IN THE RABBIT (STUDY 175R94)
MEDIAN MATERNAL BODY WEIGHT CHANGE DURING GESTATION - grams

		CONTROL (PLACEBO)	1500 MG/KG/D (2x750 MG/KG/D)
DAYS 0 TO 7	MEDIAN	61.0 d	43.0
	Q1	20.0	-13.5
	Q3	83.5	102.0
	N	17	17
DAYS 7 TO 19	MEDIAN	31.0 d	-85.5**
	Q1	-54.0	-164.0
	Q3	51.3	-33.3
	N	16	16
DAYS 19 TO 29	MEDIAN	221.5 d	155.0
	Q1	127.0	95.0
	Q3	250.8	255.0
	N	16	15

Statistical key: d-ANOVA + Dunnett-test ** = p<0.01

RO 47-0203/015 SEGMENT II: SUPPLEMENTARY ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL DEVELOPMENT IN THE RABBIT (STUDY 175R94)
MATERNAL FOOD CONSUMPTION DURING GESTATION -- SUMMARY

		CONTROL (PLACEBO)	1500 MG/KG/D (2x750 MG/KG/D)
MATERNAL FOOD CONSUMPTION -- GRAMS/KG/DAY			
DAYS 0 TO 7	MEDIAN	43 d	41
	Q1	35	36
	Q3	51	56
	N	17	17
DAYS 7 TO 14	MEDIAN	34 d	9#
	Q1	25	7
	Q3	36	13
	N	17	16
DAYS 14 TO 21	MEDIAN	28 d	8**
	Q1	18	5
	Q3	32	12
	N	16	15
DAYS 21 TO 29	MEDIAN	36 d	29*
	Q1	32	24
	Q3	43	40
	N	16	15

Statistical key: d=ANOVA + Dunnett-test * = p<0.05 ** = p<0.01 # = p<0.001

RO 47-0203/015 SEGMENT II: SUPPLEMENTARY ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL DEVELOPMENT IN THE RABBIT (STUDY 175R94)
SUMMARY OF REPRODUCTION DATA (C-SECTION)

		CONTROL (PLACEBO)	1500 MG/KG/D (2x750 MG/KG/D)
Pregnant, used for calculation	N	16	15
Corpora Lutea	N	141	146
No. per animal	MEDIAN	9.0 d	9.0
	Q1	7.3	8.0
	Q3	9.8	11.0
Preimplantation Loss	N	13	20
% per group	%	9.2	13.7
% per animal	MEDIAN	10.6 u	9.1
	Q1	0.0	0.0
	Q3	13.8	25.0
Implantation Sites	N	128	126
No. per animal	MEDIAN	8.0 d	9.0
	Q1	7.0	8.0
	Q3	9.0	9.0
Fetuses	N	111	109
No. per animal	MEDIAN	7.0 d	8.0
	Q1	6.0	7.0
	Q3	8.0	9.0
Alive	%	100.0	100.0
Dead Fetuses	N	0	0

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

RO 47-0203/015 SEGMENT II: SUPPLEMENTARY ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL DEVELOPMENT IN THE RABBIT (STUDY 175R94)
SUMMARY OF REPRODUCTION DATA (C-SECTION)

		CONTROL (PLACEBO)	1500 MG/KG/D (2x750 MG/KG/D)
Pregnant, used for calculation	N	16	15
Resorptions: Total	N	17	17
No. per animal	MEDIAN	0.5 d	0.0
	Q1	0.0	0.0
	Q3	1.8	2.0
% of impl. per group	%	13.3	13.5
% of impl. per animal	MEDIAN	5.6 u	0.0
	Q1	0.0	0.0
	Q3	16.1	22.2
Resorptions: Early	N	13	12
% of resorp. per group	%	76.5	70.6
Resorptions: Late	N	4	5
% of resorp. per group	%	23.5	29.4
Fetal Body Weight (g)	MEDIAN	39.6 d	30.6#
	Q1	37.7	28.9
	Q3	41.9	33.3
	N LITTERS	15	15
Crown-rump length (cm)	MEDIAN	9.0 d	8.6#
	Q1	8.7	8.0
	Q3	9.4	8.6

Statistical key: d=ANOVA + Dunnett-test f=Chi-square + Fishers exact test u=Kruskal-Wallis + Mann-Whitney U # = p<0.001

RO 47-0203/015 SEGMENT II: SUPPLEMENTARY ORAL STUDY FOR EFFECTS ON EMBRYO-FETAL DEVELOPMENT IN THE RABBIT (STUDY 175R94)
SUMMARY OF FE AL SKELETAL OBSERVATIONS

		CONTROL (PLACEBO)	1500 MG/KG/D (2x750 MG/KG/D)
Litters Evaluated	N	15	15
Fetuses Evaluated	N	59	58
SKULL			
Litter Incidence	N	13	15
Fetal Incidence	N	37	46
V HYOID BONE, VARIATION			
Fetal Incidence	N	21 f	18
	%	35.6	31.0
Litter Incidence	N	10 f	10
	%	66.7	66.7
R HYOID BONE INCOMPL.OSSIF.			
Fetal Incidence	N	6 f	11
	%	10.2	19.0
Litter Incidence	N	5 f	8
	%	33.3	53.3
V FRONTAL/PARIETAL BONE VARIATION			
Fetal Incidence	N	4 f	6
	%	6.8	10.3
Litter Incidence	N	3 f	5
	%	20.0	33.3
R PRESPHENOID INCOMPL.OSSIF.			
Fetal Incidence	N	5 f	11
	%	8.5	19.0
Litter Incidence	N	4 f	9
	%	26.7	60.0
R MAXILLARY BONE INCOMPL.OSSIF.			
Fetal Incidence	N	3 f	2
	%	5.1	3.4
Litter Incidence	N	2 f	2
	%	13.3	13.3
V NASAL/FRONTAL BONES VARIATION			
Fetal Incidence	N	2 f	18 f
	%	3.4	31.0
Litter Incidence	N	2 f	9 *
	%	13.3	60.0

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

Ro 47-0203 Oral Toxicokinetic Study in Pregnant Himalayan Rabbits

Location of Study Report: Vol 39, pg 313

Study Facility:

Study No.: 088R94
Report No.: 163313
Study Dates: 05/09/1994 – 05/31/1994

GLP Compliance: Yes

Animals: Pregnant Himalayan rabbits , weighing 2454-3369g on gestation day 0 (3 rabbits per treatment group) were housed individually and allowed feed and water *ad libitum*.

Drug Administration: Ro 47-0203 (Lot No. GMP 0038) was suspended in aqueous carboxymethyl cellulose and given orally by gavage to female rabbits on gestation days 7-18, 21 and 22. The sponsor did not indicate why rabbits were dosed during gestation days 21 and 22, and not on gestation days 19 and 20.

Dose Levels: 150, 450, 1500 mg/kg/day given in two daily doses 5-6 hours apart

Observations/Measurements: Pregnancy was confirmed by evaluating the uteri. Blood samples were taken from the marginal ear vein on gestation days 7 and 18, predose (only day 7) and at 1, 2, 3 and 5 hours after the first dosing, and at 1, 2, 3 and 18 hours after the second daily dose, and plasma bosentan levels determined.

Drug-Related Findings: Plasma AUCs were dose-related in pregnant Himalayan rabbits, and lower than AUCs observed in pregnant rats given 200, 600 or 2000 mg/kg/day. AUCs in pregnant rabbits given bosentan at 1500 mg/kg/day are lower than AUCs observed in pregnant rats given doses similar to those which are teratogenic in rats.

Note that the table below incorrectly lists the doses administered to rats as b.i.d. doses. In the original report of this pilot rat study in the appendix to Report No. 153693, the rat doses are listed as 200, 600 and 2000 mg/kg/day; see page 32 of this review). Note also that AUCs for rabbits are reported as ng.h/ml whereas those for rats are reported as µg.h/ml.

Rabbits (2 -3 pregnant/dose)

Doses (oral, mg/kg, b.i.d.)	150	450	1500
C_{max} (ng/ml)			
Day 1 (DG 7)	227	1380	1450
Day 12 (DG 18)	532	2071	1435
AUC (ngh/ml)			
Day 1 (DG 7)	2722	12070	17740
Day 12 (DG 18)	7402	27160	27700

Rats (3 pregnant/dose)

Doses (oral, mg/kg, b.i.d.)	200	600	2000
C_{max} (µg/ml)			
Day 1 (DG 6)	21	46	64
Day 10 (DG 12)	15	20	38
AUC (µgh/ml)			
Day 1 (DG 6)	87	209	230
Day 10 (DG 12)	44	82	132

C_{max} Values after the second administration on Day 1 or 10/12

In Vitro Study on the Effects of Ro 47-0203 on the Development of Cultivated Mouse Palatal Explants

Location of Study Report: Vol 39, pg 461

Study Facility:

Study No.: 908R95
Report No.: 163254
Study Dates: Not provided

GLP Compliance: No

Test System: Explanted palates from mouse embryos (mice, gestation day 13) with brain, tongue and lower jaw dissected. Palates were cultured for 76 hours in at 37°C and observed for closure. Sample sizes are shown in the data table on the following page. Each cultured palate was exposed to a single bosentan concentration.

Drug Concentrations: 0, 0.01, 0.1, 1, 3, 10, 30 and 100 μg/ml of Ro 47-0203
Lot and batch numbers were not provided.

Observations/Measurements: The following morphological features were assessed. [3]

Morphological Findings of Palate	Fusion of the Palate	Final Classification of Palatial Closure
Elevated, but not fused	0-25% of length	cleft
Elevated and partially fused	25-75% of length	cleft
Elevated and fused	75-100% of length	fused

[3] The sponsor did not provide an explanation of the system other than to indicate that this assay provided for an in vitro assessment of palatial closure.

Drug-Related Findings: Bosentan (Ro 47-0203; Ro-1) concentration-dependently prevented closure of explanted mouse palatal cultures. At the highest bosentan concentration of 100 µg/ml, the laboratory observed the jaw to be abnormally shaped.

Effect of Code Ro-1 on mouse palates in vitro (76 h)

test group	n	Clefts				total (%)
		elevated and fused	elevated and partially fused		elevated, but not fused		
control	24	22	1	(4.2%)	1	(4.2%)	8.3
control + 0.1% DMSO	48	44	3	(6.3%)	1	(2.1%)	8.3
Code Ro-1:							
100 µg/ml*	12**	9	1	(8.3%)	2	(16.7%)	25
30 µg/ml	36	11	11	(30.6%)	14	(38.9%)	69.4
10 µg/ml	48	30	13	(27.1%)	5	(10.4%)	37.5
3 µg/ml	24	18	5	(20.8%)	1	(4.2%)	25
1 µg/ml	24	20	3	(12.5%)	1	(4.2%)	16.7
0.1 µg/ml	12	11	1	(8.3%)	0	(0%)	8.3
0.01 µg/ml	12	11	1	(8.3%)	0	(0%)	8.3

* Ro-1 fell out immediately by adding the stock solution into the culture medium.

** After culturing all the organs showed an usual shape of the upper jaw.

Ro-1 = Ro 47-0203 = bosentan
Fell out = precipitated from solution

GENOTOXICITY STUDIES

Ames Bacterial Mutagen Assay

Test Agent: Ro 47-0203

Lot Number: Batch C (WS 10906/105/2)

Study Facility:

Study Number: 75M92

Report Number: B-159615

Study Dates: June 19, 1992- June 29, 1992

GLP Compliance: Yes

Test System: *Salmonella typhimurium* strains TA 97, TA 98, TA 100, TA102, TA 1535, TA 1537, TA 1538

Metabolizing system: Phenobarbital and β-naphthoflavone induced rat liver (S-9 fraction)

Procedure: Ro 47-0203 was dissolved in DMSO and added to culture plates containing the bacterial tester strains using the standard plate incorporation method. Ro 47-0203 was evaluated at doses of 100, 333, 1000, 3333 and 5000 μg/plate (four replicate plates/concentration) in the absence and presence of rat liver S-9. The test system was also exposed to Ro 47-0203 at doses of 33, 100, 333, 1000 and 3333 μg/plate using the liquid preincubation method (30 minute preincubation at 37° C). Doses of Ro 47-0203 were chosen on the basis of a dose range-finding study.

A positive result is defined by the sponsor as a doubling in the mean number of revertants per plate for strains TA 1535, TA 1537, TA 1538 and TA 98, and a 1.5 fold increase for strains TA97, TA100 and TA 102. Toxicity is evaluated by a decrease in background lawn growth or a decrease in the number of spontaneous revertants relative to concurrent control.

The following positive controls were utilized (two replicate plates per positive control): sodium azide with strains TA1535 and TA100, ICR 191 with strains TA1537 and TA97, 2-nitrofluorene with strains TA1538 and TA98 and mitomycin C with strain TAI02. 2-Aminoanthracene was used with all strains with and without metabolic activation to examine the activity of the S9 mix.

Results: No positive findings were noted with any Ro 47-0203 dose in any tester strain. Positive controls increased revertant frequencies as expected. Data is shown for both bosentan and positive control assays.

TABLE 1 Salmonella mutagenicity test (Ames standard assay), Mean values and standard deviations

Experiment No	01	01	01	01	01	01
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
Concentration in µg /plate	Test substance: Ro 47-0203/001					
0.00	45 ± 4	52 ± 6	187 ± 10	184 ± 11	217 ± 12	204 ± 20
100.00	48 ± 9	56 ± 4	183 ± 27	217 ± 14	193 ± 20	209 ± 21
333.00	45 ± 9	58 ± 5	185 ± 31	204 ± 27	216 ± 21	224 ± 39
1000.00	42 ± 7	47 ± 12	188 ± 21	185 ± 18	192 ± 28	199 ± 21
3333.00	42 ± 4	46 ± 4	144 ± 28	159 ± 14	220 ± 9	208 ± 23
5000.00	40 ± 7	41 ± 3	152 ± 18	159 ± 27	227 t ± 20	180 ± 5

Experiment No	02	02	02	02	02	02	02	02
Activation	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538	TA97	TA97
Concentration in µg /plate	Test substance: Ro 47-0203/001							
0.00	26 ± 2.6	12 ± 3	14 ± 4	16 ± 2	35 ± 6	36 ± 2	260 ± 20	298 ± 4
100.00	24 ± 4	11 ± 3	13 ± 6	15 ± 3	39 ± 5	44 ± 9	230 ± 30	262 ± 8
333.00	21 ± 3	10 ± 3	16 ± 4	13 ± 4	35 ± 8	42 ± 5	245 ± 13	270 ± 14
1000.00	25 ± 2	9 ± 3	15 ± 4	14 ± 2	33 ± 7	35 ± 9	236 ± 8	279 ± 25
3333.00	20 ± 5	9 ± 4	7 ± 1	13 ± 2	28 ± 8	29 ± 3	255 ± 27	246 ± 36
5000.00	18 ± 6	12 ± 3	8 t ± 2	8 t ± 3	26 ± 3	23 t ± 3	235 ± 40	263 ± 9

TABLE 2 Salmonella mutagenicity test (Liquid preincubation assay).
Mean values and standard deviations.

Experiment No	03	03	03	03	03	03
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
Concentration in µg /plate	Test substance: Ro 47-0203/001					
0.00	43 ± 2	36 ± 3	178 ± 12	171 ± 9	95 ± 2	107 ± 10
33.00	31 ± 5	36 ± 5	176 ± 13	178 ± 12	100 ± 9	103 ± 5
100.00	44 ± 8	44 ± 6	184 ± 19	173 ± 15	98 ± 7	110 ± 5
333.00	36 ± 5	40 ± 5	172 ± 16	172 ± 15	97 ± 7	107 ± 2
1000.00	40 ± 6	42 ± 5	158 ± 15	167 ± 9	51 ± 13	123 ± 8
3333.00	19 t ± 9	19 ± 5	43 t ± 8	147 ± 15	9 t ± 2	100 ± 20

Experiment No	04	04	04	04	04	04	04	04
Activation	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538	TA97	TA97
Concentration in µg /plate	Test substance: Ro 47-0203/001							
0.00	18 ± 5	8 ± 4	12 ± 2	15 ± 4	29 ± 3	33 ± 10	206 ± 20	253 ± 20
33.00	22 ± 2	13 ± 3	9 ± 4	12 ± 3	35 ± 8	30 ± 6	207 ± 8	259 ± 11
100.00	23 ± 8	5 ± 3	14 ± 2	16 ± 8	32 ± 5	16 ± 3	180 ± 11	235 ± 34
333.00	20 ± 2	8 ± 3	8 ± 4	11 ± 4	39 ± 7	39 ± 1	193 ± 24	247 ± 20
1000.00	15 ± 5	9 ± 1	9 ± 7	13 ± 8	28 ± 6	34 ± 7	189 ± 21	283 ± 20
3333.00	15 ± 4	10 ± 3	6 t ± 5	10 ± 5	T	18 t ± 4	151 ± 6	248 ± 7

TABLE A1 Salmonella mutagenicity test (Ames standard plate incorporation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphthoflavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	01	01	01	01	01	01
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
	Reference substance(s)					
Sodium azide 1.00 µg			511 468			
Mean			490			
SD			± 30.4			
MMC 0.40 µg					403 404	
Mean					404	
SD					± 0.7	
2-Aminoanthr. 4.00 µg	46 53	1877 1971	240 242	1869 1949	124 150	801 896
Mean	50	1924	241	1909	137	849
SD	± 4.9	± 66.5	± 1.4	± 56.6	± 18.4	± 67.2
2-NF 0.50 µg	189 186					
Mean	188					
SD	± 2.1					

TABLE A2 Salmonella mutagenicity test (Ames standard plate incorporation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphtho-flavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	02	02	02	02	02	02
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538
Reference substance(s)						
Sodium azide 1.00 µg	845					
	860					
Mean	853					
SD	± 10.6					
ICR 191 1.00 µg			144			
			185			
Mean			165			
SD			± 29.0			
2-Aminoanthr. 4.00 µg	11	419	19	446	71	2546
	22	391	20	496	54	2596
Mean	17	405	20	471	63	2571
SD	± 7.8	± 19.8	± 0.7	± 35.4	± 12.0	± 35.4
2-NF 0.50 µg					302	
					252	
Mean					277	
SD					± 35.4	

TABLE A2 Salmonella mutagenicity test (Ames standard plate incorporation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphtho-flavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	02	02
Activation	-S9	+S9
Strain	TA97	TA97
Reference substance(s)		
ICR 191 1.00 µg	774	
	733	
Mean	754	
SD	± 29.0	
2-Aminoanthr. 4.00 µg	270	1892
	238	1891
Mean	254	1892
SD	± 22.6	± 0.7

Ames Bacterial Mutagen Assay

Test Agent: Ro 47-0203

Lot Number: Batch A (GPul 920092)

Study Facility:

Study Number: 20M93

Report Number: B-159635

Study Dates: January 22, 1993 to February 19, 1993

GLP Compliance: Yes

Test System: *Salmonella typhimurium* strains TA 97, TA 98, TA 100, TA102, TA 1535, TA 1537, TA 1538

Metabolizing system: Phenobarbital and β-naphthoflavone induced rat liver (S-9 fraction)

Procedure: Ro 47-0203 was dissolved in DMSO and added to culture plates containing the bacterial tester strains using the standard plate incorporation method. Ro 47-0203 was evaluated at doses of 100, 333, 1000, 3333 and 5000 μg/plate (four replicate plates/concentration) in the absence and presence of rat liver S-9. The test system was also exposed to Ro 47-0203 at doses of 33, 100, 333, 1000 and 3333 μg/plate using the liquid preincubation method (30 minute preincubation at 37°C). Doses of Ro 47-0203 were chosen on the basis of a dose range-finding study.

A positive result is defined by the sponsor as a doubling in the mean number of revertants per plate for strains TA 1535, TA 1537, TA 1538 and TA 98, and a 1.5 fold increase for strains TA97, TA100 and TA 102. Toxicity is evaluated by a decrease in background lawn growth or a decrease in the number of spontaneous revertants relative to concurrent control.

The following positive controls were utilized (two replicate plates per positive control): sodium azide with strains TA1535 and TA100, ICR 191 with strains TA1537 and TA97, 2-nitrofluorene with strains TA1538 and TA98 and mitomycin C with strain TAI02. 2-Aminoanthracene was used with all strains with and without metabolic activation to examine the activity of the S9 mix.

Results: No positive findings were noted with any Ro 47-0203 dose in any tester strain. Positive controls increased revertant frequencies as expected.

TABLE 1 Salmonella mutagenicity test (Ames standard assay).
Mean values and standard deviations

Experiment No	01	01	01	01	01	01	01	01
Activation	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538	TA97	TA97
Concentration in µg /plate	Test substance: Ro 47-0203/010							
0.00	19 ± 2	8 ± 1	13 ± 4	12 ± 2	20 ± 5	23 ± 6	212 ± 4	249 ± 21
100.00	23 ± 11	7 ± 1	10 ± 3	11 ± 4	23 ± 4	31 ± 2	190 ± 14	237 ± 12
333.00	23 ± 4	8 ± 3	12 ± 2	8 ± 4	24 ± 6	25 ± 2	200 ± 15	226 ± 17
1000.00	31 ± 2	6 ± 2	15 ± 8	12 ± 4	29 ± 4	27 ± 4	214 ± 9	232 ± 16
2500.00	31 ± 5	8 ± 2	10 ± 3	10 ± 2	26 ± 6	26 ± 3	196 ± 7	230 ± 18
5000.00	21 ± 3	8 ± 2	8 t ± 3	9 ± 2	14 t ± 5	14 t ± 3	207 ± 14	231 ± 18

Experiment No	02	02	02	02	02	02
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
Concentration in µg /plate	Test substance: Ro 47-0203/010					
0.00	34 ± 16	33 ± 4	176 ± 14	172 ± 17	200 ± 20	141 ± 37
100.00	28 ± 14	26 ± 3	175 ± 15	185 ± 10	176 ± 25	128 ± 54
333.00	23 ± 3	29 ± 5	175 ± 10	184 ± 14	189 ± 8	108 ± 4
1000.00	27 ± 11	19 ± 3	185 ± 16	173 ± 13	181 ± 12	189 ± 15
2500.00	21 ± 4	28 ± 4	158 ± 8	187 ± 11	208 ± 13	207 ± 12
5000.00	24 ± 4	15 ± 3	155 ± 8	153 ± 10	246 ± 8	216 ± 43

TABLE 2 Salmonella mutagenicity test (Liquid preincubation assay).
Mean values and standard deviations.

Experiment No	03	03	03	03	03	03	03	03
Activation	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538	TA97	TA97
Concentration in µg /plate	Test substance: Ro 47-0203/010							
0.00	26 ± 8	11 ± 2	11 ± 3	10 ± 3	25 ± 4	29 ± 6	225 ± 9	272 ± 16
33.00	26 ± 2	9 ± 3	9 ± 2	10 ± 3	18 ± 3	27 ± 6	236 ± 24	250 ± 19
100.00	26 ± 5	11 ± 2	9 ± 3	11 ± 2	26 ± 5	27 ± 3	233 ± 10	261 ± 10
333.00	23 ± 4	9 ± 3	10 ± 4	12 ± 2	34 ± 3	32 ± 3	244 ± 14	262 ± 17
1000.00	22 ± 4	6 ± 2	9 ± 6	10 ± 2	28 ± 4	29 ± 9	239 ± 6	259 ± 26
3333.00	13 t ± 3	10 ± 5	5 t ± 4	9 ± 3	13 t ± 3	16 t ± 6	193 ± 14	282 ± 8

Experiment No	04	04	04	04	04	04
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
Concentration in µg /plate	Test substance: Ro 47-0203/010					
0.00	30 ± 8	32 ± 4	181 ± 10	139 ± 14	232 ± 43	306 ± 14
33.00	34 ± 4	39 ± 11	165 ± 10	145 ± 14	277 ± 30	308 ± 26
100.00	35 ± 3	38 ± 5	161 ± 8	149 ± 16	262 ± 11	328 ± 44
333.00	34 ± 5	42 ± 9	172 ± 19	156 ± 9	218 ± 10	300 ± 33
1000.00	30 ± 6	36 ± 3	159 ± 8	151 ± 8	191 ± 23	352 ± 29
3333.00	16 t ± 5	23 ± 1	59 t ± 27	126 ± 11	83 t ± 22	354 ± 22

TABLE A1 Salmonella mutagenicity test (Ames standard plate incorporation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphthoflavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	01	01	01	01	01	01
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538
	Reference substance(s)					
Sodium azide 1.00 µg	955 924					
Mean	940					
SD	± 21.9					
ICR 191 1.00 µg			46 45			
Mean			46			
SD			± 0.7			
2-Aminoanthr. 4.00 µg	24 24	308 314	12 23	377 356	NG NG	2331 2253
Mean	24	311	18	367		2292
SD	± 0.0	± 4.2	± 7.8	± 14.8		± 55.2
2-NF 0.50 µg					236 210	
Mean					223	
SD					± 18.4	

NG No growth (no bacteria plated)

TABLE A2 Salmonella mutagenicity test (Ames standard plate incorporation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphtho-flavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	02	02	02	02	02	02
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
Reference substance(s)						
Sodium azide 1.00 µg			650			
			609			
Mean			630			
SD			± 29.0			
MMC 0.40 µg					670	
					691	
Mean					681	
SD					± 14.8	
2-Aminoanthr. 4.00 µg	29	2375	279	2449	139	909
	30	2252	198	2393	182	937
Mean	30	2314	239	2421	161	923
SD	± 0.7	± 87.0	± 57.3	± 39.6	± 30.4	± 19.8
2-NF 0.50 µg	194					
	176					
Mean	185					
SD	± 12.7					

TABLE A3 Salmonella mutagenicity test (Liquid preincubation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphthoflavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	03	03	03	03	03	03
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA1535	TA1535	TA1537	TA1537	TA1538	TA1538
Reference substance(s)						
Sodium azide 1.00 µg	728 759					
Mean	744					
SD	± 21.9					
ICR 191 1.00 µg			497 235			
Mean			366			
SD			±185.3			
2-Aminoanthr. 4.00 µg	13 27	293 256	16 14	435 412	35 26	2078 2052
Mean	20	275	15	424	31	2065
SD	± 9.9	± 26.2	± 1.4	± 16.3	± 6.4	± 18.4
2-NF 0.50 µg					277 298	
Mean					288	
SD					± 14.8	

TABLE A3 Salmonella mutagenicity test (Liquid preincubation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphthoflavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	03	03
Activation	-S9	+S9
Strain	TA97	TA97
Reference substance(s)		
ICR 191 1.00 µg	1691 1375	
Mean	1533	
SD	±223.4	
2-Aminoanthr. 4.00 µg	264 223	1697 1373
Mean	244	1535
SD	± 29.0	±229.1

TABLE A4 Salmonella mutagenicity test (Liquid preincubation assay) in absence (-S9) and in presence (+S9) of a phenobarbital-beta-naphthoflavone-induced rat S9 liver homogenate fraction. Number of revertant colonies per plate.

Experiment No	04	04	04	04	04	04
Activation	-S9	+S9	-S9	+S9	-S9	+S9
Strain	TA98	TA98	TA100	TA100	TA102	TA102
	Reference substance(s)					
Sodium azide 1.00 µg			603 602			
Mean SD			603 ± 0.7			
MMC 0.40 µg					1267 1225	
Mean SD					1246 ± 29.7	
2-Aminoanthr. 4.00 µg	29 33	2138 2038	206 188	2167 2194	232 222	605 523
Mean SD	31 ± 2.8	2088 ± 70.7	197 ± 12.7	2181 ± 19.1	227 ± 7.1	564 ± 58.0
2-NF 0.50 µg	247 241					
Mean SD	244 ± 4.2					

Ames Bacterial Mutagen Assay

Rationale for Study: This study was performed to assess the mutagenic effects of impurities observed in clinical batches of bosentan.

Test Agent: Ro 47-0203

Batch Number: 41003A40
Contains three major impurities: Ro 47-4056 (0.6%), Ro 47-005 (0.1%) and Ro 47-9931 (0.3%)

Study Facility:

Study Number: 212M94

Report Number: B-163247

Study Dates: December 12, 1994 – January 9, 1995

GLP Compliance: Yes

Test System: *Salmonella typhimurium* strains TA 97, TA 98, TA 100, TA102, TA 1535, TA 1537, *E. coli* WP2 uvrA

Metabolizing system: Phenobarbital and β-naphthoflavone induced rat liver (S-9 fraction)

Procedure: Ro 47-0203 was dissolved in DMSO and added to culture plates containing the bacterial tester strains using the standard plate incorporation method. Ro 47-0203 was evaluated at doses of 50, 166, 500, 1666 and 5000 μg/plate (three replicate plates/concentration) in the absence and presence of rat liver S-9. The test system was also exposed to Ro 47-0203 at doses of of 50, 166, 500, 1666 and 5000 μg/plate (three replicate plates/concentration) in the absence and presence of rat liver S-9 using the liquid preincubation method (30 minute preincubation at 37° C).

A positive result is defined by the sponsor as a doubling in the mean number of revertants per plate for strains TA 1535, TA 1537, TA 98, and *E. coli* WP2 uvrA and a 1.5 fold increase for strains TA97, TA100 and TA 102. Toxicity is evaluated by a decrease in background lawn growth or a decrease in the number of spontaneous revertants relative to concurrent control.

The following positive controls were utilized (two replicate plates per positive control): sodium azide with strains TA1535 and TA100, ICR 191 with strains TA1537 and TA97, 2-nitrofluorene with strain TA98, mitomycin C with strain TAI02 and 4NQ with *E. coli* WP2 uvrA. 2-Aminoanthracene was used with all strains with and without metabolic activation to examine the activity of the S9 mix.

Results: No positive findings were noted with any Ro 47-0203 dose in the presence of S-9 in any tester strain. While TA98 in the absence of S-9 showed 1.5-2 fold increases over concurrent control, this was likely due to a low concurrent control measurements since the number of revertants with drug fell well within the historical control values. Additionally, the two-fold increase observed in one study was neither statistically significant nor reproducible. Positive controls increased revertant frequencies as expected.

TABLE 1A Summary of the results of the reverse mutation assay using bacteria of the indicated strains. Mean values and standard deviations.

Test Compound: Ro 47-0203/029 Method: AME

Strain Activation	TA97 -S9	TA97 +S9	TA98 -S9	TA98 +S9	TA100 -S9	TA100 +S9
Concentration µg/plate						
0.	193 ± 21	237 ± 9	11 ± 4	34 ± 4	51 ± 3	67 ± 5
50.	212 ± 6	243 ± 5	20 ± 3	27 ± 6	50 ± 5	74 ± 0
166.	189 ± 12	250 ± 27	22 ± 4	28 ± 4	50 ± 5	49 ± 0
500.	210 ± 7	236 ± 15	18 ± 5	22 ± 2	48 ± 4	50 ± 8
1666.	200 ± 10	250 ± 11	22 ± 4	26 ± 5	53 ± 1	50 ± 0
5000.	203 ± 2	259 ± 6	18 ± 3	27 ± 4	50 ± 5	29 ± 2

Strain Activation	TA102 -S9	TA102 +S9	TA1535 -S9	TA1535 +S9	TA1537 -S9	TA1537 +S9
Concentration µg/plate						
0.	308 ± 29	343 ± 12	9 ± 5	9 ± 4	7 ± 2	7 ± 2
50.	317 ± 7	317 ± 9	9 ± 1	7 ± 2	7 ± 3	6 ± 4
166.	308 ± 27	331 ± 24	15 ± 4	8 ± 4	6 ± 3	12 ± 14
500.	314 ± 16	335 ± 2	11 ± 2	8 ± 1	6 ± 3	5 ± 0
1666.	328 ± 20	391 ± 9	12 ± 1	10 ± 8	9 ± 2	7 ± 4
5000.	298 ± 36	365 ± 22	7 ± 2	7 ± 1	6 ± 4	6 ± 4

TABLE 18 Summary of the results of the reverse mutation assay using bacteria of the indicated strains. Mean values and standard deviations.

Method: AMES

Test Compound: Ro 47-0203/029

Strain Activation	WP2uvrA -S9	WP2uvrA +S9
Concentration µg/plate		
0.	13 ± 3	17 ± 7
50.	15 ± 3	14 ± 8
166.	14 ± 4	15 ± 6
500.	11 ± 2	15 ± 2
1666.	12 ± 4	14 ± 1
5000.	14 ± 1	15 ± 5

Study No.: 212894 Experiment No.: 4
Experiment Start: 06.01.95 Method: AMES

Test Compound: Ro 47-0203/029

Strain Activation	TA100 -S9	TA100 +S9
Concentration µg/plate		
0.	69 ± 4	71 ± 6
50.	70 ± 2	65 ± 9
166.	72 ± 15	68 ± 8
500.	67 ± 12	66 ± 3
1666.	86 ± 12	72 ± 6
5000.	68 ± 5	59 ± 5

TABLE 2A Summary of the results of the reverse mutation assay using bacteria of the indicated strains. Mean values and standard deviations.

Test Compound: Ro 47-0203/029 Method: PRE

Strain Activation	TA97 -S9	TA97 +S9	TA98 -S9	TA98 +S9	TA100 -S9	TA100 +S9
Concentration µg/plate						
0.	197 ± 16	222 ± 33	12 ± 4	20 ± 8	70 ± 4	69 ± 6
50.	221 ± 11	217 ± 21	11 ± 3	25 ± 4	63 ± 10	66 ± 13
166.	216 ± 6	228 ± 17	13 ± 3	23 ± 3	66 ± 3	60 ± 12
500.	208 ± 4	242 ± 18	13 ± 5	25 ± 4	68 ± 1	66 ± 6
1666.	174 ± 17	267 ± 5	18 ± 1 *	33 ± 10	28 ± 6	47 ± 5
5000.	125 ± 9 t	208 ± 14	17 ± 3 t	22 ± 12	25 ± 16 t	38 ± 13

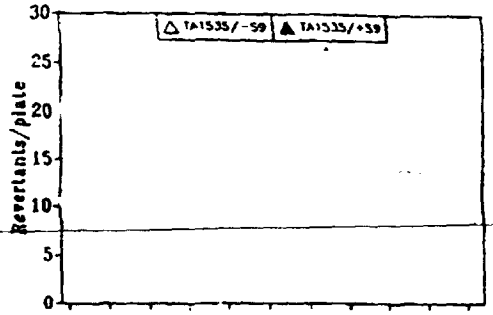
Strain Activation	TA102 -S9	TA102 +S9
Concentration µg/plate		
0.	296 ± 32	389 ± 22
50.	339 ± 22	366 ± 35
166.	324 ± 10	393 ± 16
500.	304 ± 21	415 ± 30
1666.	233 ± 4 t	434 ± 13
5000.	162 ± 9 t	356 ± 7 t

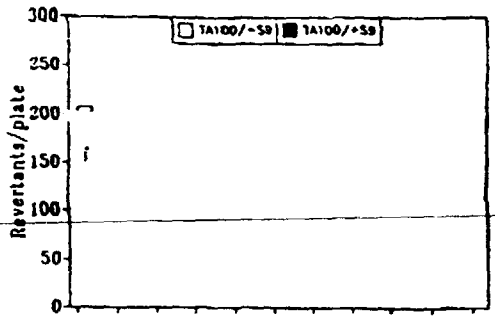
TABLE 28 Summary of the results of the reverse mutation assay using bacteria of the indicated strains. Mean values and standard deviations.

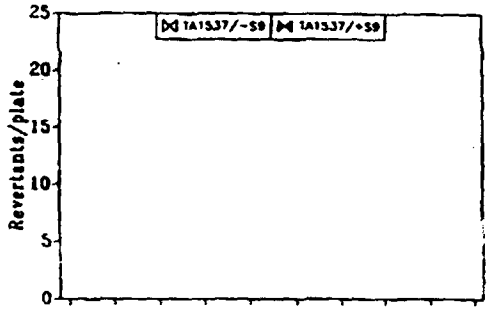
Test Compound: Bo 47-0203/029 Method: PRE

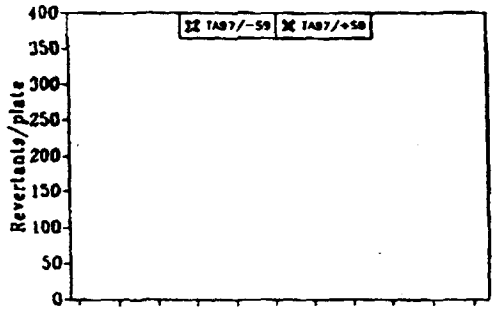
Strain Activation	TA1535 -S9	TA1535 +S9	TA1537 -S9	TA1537 +S9	WP2uvrA -S9	WP2uvrA +S9
Concentration µg/plate						
0.	20 ± 1	5 ± 2	11 ± 3	12 ± 3	36 ± 9	37 ± 7
50.	17 ± 4	7 ± 4	11 ± 3	11 ± 2	31 ± 5	32 ± 2
166.	18 ± 2	10 ± 5	10 ± 1	12 ± 4	32 ± 4	34 ± 4
500.	21 ± 6	9 ± 4	6 ± 3	15 ± 2	36 ± 1	40 ± 2
1666.	15 ± 2	9 ± 3	7 ± 2	11 ± 4	38 ± 8	34 ± 8
5000.	12 ± 3 t	9 ± 3	5 ± 4 t	10 ± 5	19 ± 6 t	30 ± 10

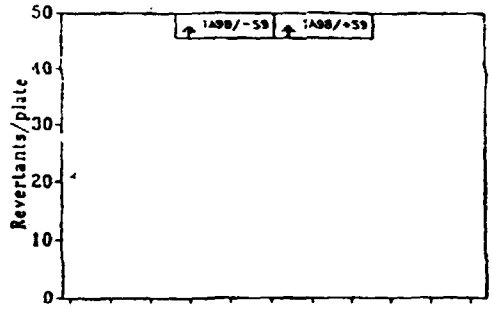
Historical control data
Negative control values of tests recently performed in our laboratory. Spontaneous rates (revertants per plate) for seven routinely used tester strains in absence (-S9) as well as in presence (+S9) of an exogenous metabolic activation system are plotted.

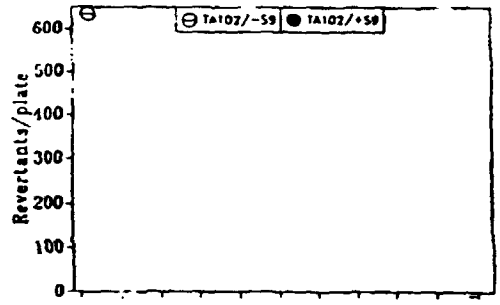


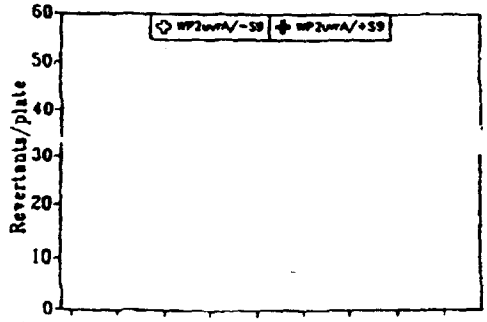












Unscheduled DNA Synthesis (UDS) Assay with Ro 47-0203 Using Primary Rat Hepatocytes

Test Agent: Ro 47-0203

Lot Number: G PM 0017

Study Facility:

Study Number: 020M94

Report Number: B-161147

Study Dates: 01/27/94 – 04/13/94

GLP Compliance: Yes

Test System: Hepatocytes from male albino rats weighing 184-200 g (age not provided) were exposed to test agent and ^{3}H-methyl-thymidine for 18 hours in vitro. DNA repair synthesis is assessed by counting the number of silver grains in nuclei of non-replicating cells (100 cells/dose) in a blinded manner. The number of cells containing more than 5 nuclear grain counts are tabulated as are mean nuclear grain count (NG), cytoplasmic grain counts (CG) and mean net nuclear grain count (NNG). Cytoplasmic grain counts (CG) are determined to assess indirect cytotoxicity and NNG is determined by subtracting CG from NG.

A test article is considered positive if there is a statistically significant and dose-related increase in mean net grain count and the values for at least two consecutive doses are above the threshold level of NNG.

Based on a preliminary toxicity study in rat hepatocytes, Ro 47-0203 was evaluated for UDS at doses of 1.1, 3.2, 10.6, 31.9 and 106.4 μg/ml.

Results: Ro 47-0203 was negative for UDS in rat hepatocytes at doses up to 106.4 μg/ml. At higher doses of 266 and 532 μg/ml, no viable cells were available due to cytotoxicity. The positive control 2-acetylaminofluorene (2AAF) was positive in two of three assays.

Table 1a : Unscheduled DNA Synthesis (UDS) assay with freshly isolated rat hepatocytes after 18-hours exposure to Ro 47-0203/010 (Experiment 020M94/0)

Test chemical	Dose µg/ml	No.Cell Analysed	Grain Counts / Cell Nuclear > 5 (%)	Nuclear Mean ± SD		Cytoplasmic Mean ± SD		Net Nuclear Mean ± SD	
Negative control: DMSO	0.00	100	6	2.0	± 2.1	1.8	± 1.2	0.2	± 1.6
Ro 47-0203/010	1.10	100	2	2.1	± 1.6	3.5**(+)	± 1.5	-1.4**(-)	± 2.0
Ro 47-0203/010	3.20	100	6	2.3	± 1.8	2.8 *(+)	± 1.5	-0.6 *(-)	± 1.8
Ro 47-0203/010	10.60	100	5	1.8	± 1.7	1.8	± 1.1	0.02	± 1.7
Ro 47-0203/010	31.90	100	0	1.5	± 1.1	1.6	± 1.1	-0.1	± 1.6
Ro 47-0203/010	106.40	75	3	1.8	± 1.3	1.4	± 0.8	0.4	± 1.5
Reference substance : 2AAF	0.40	103	13	3.2	± 2.3	1.0	± 0.7	2.2	± 2.3
Reference substance : 2AAF	1.00	101	15	3.3	± 2.3	1.5	± 1.2	1.8	± 2.5

Statistical significance: * for $p < 0.05$, ** for $p < 0.01$

Table 1b : Unscheduled DNA Synthesis (UDS) assay with freshly isolated rat hepatocytes after 18-hours exposure to Ro 47-0203/010 (Experiment 020M94/1)

Test chemical	Dose µg/ml	No.Cell Analysed	Grain Counts / Cell						
			Nuclear			Cytoplasmic		Net Nuclear	
			> 5 (%)	Mean ± SD		Mean ± SD		Mean ± SD	
Negative control: DMSO	0.00	100	0	0.8	± 1.0	0.7	± 0.7	0.02	± 1.1
Ro 47-0203/010	6.25	75	1	1.4	± 1.5	1.5	± 1.1	-0.1	± 1.5
Ro 47-0203/010	12.50	100	0	1.0	± 1.2	0.9	± 0.7	0.1	± 1.1
Ro 47-0203/010	25.00	100	0	0.8	± 1.0	0.7	± 0.6	0.1	± 1.1
Ro 47-0203/010	50.00	87	0	0.7	± 1.1	0.9	± 0.8	-0.2	± 1.2
Ro 47-0203/010	100.00	100	0	0.6	± 0.7	0.7	± 0.6	-0.1	± 0.8
Reference substance : 2AAF	0.40	100	62	7.8	± 5.5	1.8	± 1.1	6.0	± 4.9
Reference substance : 2AAF	1.00	100	79	9.7	± 4.7	2.4	± 1.5	7.3	± 3.8

Statistical significance: * for $p < 0.05$, ** for $p < 0.01$

Table 1c : Unscheduled DNA Synthesis (UDS) assay with freshly isolated rat hepatocytes after 18-hours exposure to Ro 47-0203/010 (Experiment 020M94/3)

Test chemical	Dose µg/ml	No.Cell Analysed	Grain Counts / Cell					
			Nuclear			Cytoplasmic	Net Nuclear	
			> 5 (%)	Mean ± SD		Mean ± SD	Mean ± SD	
Negative control: DMSO	0.00	101	1	1.9	± 1.4	2.6	± 1.2	-0.7 ± 1.6
Ro 47-0203/010	1.00	100	5	2.2	± 1.9	2.6	± 1.4	-0.4 ± 2.0
Ro 47-0203/010	3.00	100	8	2.2	± 1.9	2.6	± 1.8	-0.4 ± 2.0
Ro 47-0203/010	10.00	101	5	2.2	± 1.7	2.6	± 1.4	-0.4 ± 1.8
Ro 47-0203/010	30.00	40	0	1.5	± 1.2	0.9	± 0.5	0.6 ± 1.3
Ro 47-0203/010	100.00	100	1	1.3	± 1.4	1.4	± 0.9	-0.03 ± 1.6
Reference substance : 2AAF	0.40	25	92	8.7	± 3.4	1.0	± 0.5	7.8 ± 3.4
Reference substance : 2AAF	1.00	76	88	11.8	± 5.0	1.5	± 1.1	10.3 ± 4.7

Statistical significance: * for $p < 0.05$, ** for $p < 0.01$

Gene Mutation Test with Ro 47-0203 in Cultured Mammalian Cells (V79/HPRT Test)

Test Agent: Ro 47-0203

Lot Number: G PM 0017

Vehicle: DMSO

Study Facility:

Study Number: 019M94

~~Report Number: RRB 161146~~

Study Dates: January 24, 1994 – February 23, 1994

GLP Compliance: Yes

Test System: Chinese Hamster Lung Cells (V79/HPRT assay)

Metabolizing System: Phenobarbital and β-naphthoflavone induced rat liver (S-9 fraction)

Procedure: Ro 47-0203 was dissolved in DMSO and added to culture plates containing Chinese hamster lung cells in the presence and absence of rat liver S-9. Exposure times were 5 hours in the presence and 16 hours in the absence of rat liver S-9. Cytotoxicity was evaluated by measuring relative cell viability. This assay is based on mutations at the hypoxanthine phosphoribosyl transferase locus (HPRT-mutants). Mutations at the HPRT locus confers resistance to 6-thioguanine, which is metabolized by HPRT to a toxic metabolite. Mutation at this site is evaluated by counting the number of 6-thioguanine resistant clones.

Ro 47-0203 was tested at concentrations of 0, 1.1, 3.3, 10.8, 32.5 and 108 μg/ml (expt 1) and 0, 26.6, 53.3, 106.5 and 213 μg/ml (expt 2) in the absence of rat liver S-9. Ro 47-0203 was tested at concentrations of 0, 1.1, 3.2, 10.6, 31.8, 106 and 108 μg/ml (expt 1), and 0, 81, 162, and 324 μg/ml (expt 2) in the presence of rat liver S-9. EMS at 80 μg/ml served as a positive control in the absense of metabolic activation. DMBA (0.5 μg/ml) served as a positive control in the presence of metabolic activation.

The sponsor considers a test as positive if the number of HPRT-mutant clones was dose-related, the number of clones in the drug-treated groups (for more than one dose) was significantly greater than the negative control, and the effects were reproducible.

Results: Ro 47-0203 did not increase the number of HPRT–mutant clones at any dose level in the absence or in the presence of rat liver S-9. Concentration-dependent cytotoxicity was observed, with relative cell viability of 13-21% in cells exposed to 213 μg/ml in the absence of rat liver S-9, and 49-57% in cells exposed to 324 μg/ml in the presence of rat liver S-9. The positive controls increased mutant frequency as expected.

Micronucleus Test in Mouse Bone Marrow after Oral Administration of Ro 47-0203

Test Agent: Ro 47-0203

Batch Number: 213120

Study Facility:

Study Number: 004M94

Report Number: B-161116

Study Dates: 03/21/94 – 06/20/94

GLP Compliance: Yes

Test System: Male and female mice weighing 37 and 33 g, respectively.

Procedure: Mice were given a single oral dose of 500, 1000 or 2000 mg/kg orally by gavage (5 mice/sex/dose). The doses evaluated were based on the OECD guideline (OECD Guideline for testing of chemicals, # 474, 1987) and recommendations of MacGregor etal. (Mutation Research 189; 103-112, 1987). The mode of dosing was not provided. Positive control animals (n=5) received a single dose of procarbazine (50 mg/kg). Animals given bosentan at 500, 1000 and 2000 mg/kg/day were sacrificed at 24 hours after dosing, as were concurrent negative and positive controls. Additional animals receiving bosentan at 2000 mg/kg and negative controls were sacrificed 48 hours after dosing. Bone marrow cells collected from both femora were analyzed for micronuclei (MN). 2000 polychromatic nuclei (PCE) per animal were evaluated for MN.

The test article is considered to induce a positive response when the number of polychromatic erythrocytes with micronuclei (MN-PCE) is statistically significantly increased at any dose or sampling time and it exceeds the normal range of historical controls.

Plasma Drug Levels: Plasma bosentan levels were determined in male and female mice (2 mice/sex/time point) given bosentan at 2000 mg/kg. Concurrent control animals were also evaluated for bosentan levels. Blood samples were taken at 1, 3 and 6 hours after dosing. AUCs were not determined.

Results: Ro 47-0203 did not increase the the number of PCEs with micronuclei at doses evaluated. In comparison, the positive control procarbazine increased the number of PCEs with micronuclei as expected. Plasma bosentan levels were 11.7±9.7, 8.5 ±6.4 and 9.6±7.6 μg/ml at 1, 3 and 6 hours, respectively in mice given bosentan at 2000 mg/kg. Plasma bosentan levels were undectable in concurrent control mice.

Adequacy of Doses Tested: Plasma bosentan levels observed in mice given 2000 mg/kg in the micronucleus assay (the highest dose tested) were similar to or greater than those observed in mice given 2000 or 4500 mg/kg/day (mid and high dose levels) in the two-year carcinogenicity study.

It seems unlikely that oral doses higher than 2000 mg/kg would yield markedly increased plasma AUCs because, in the mouse carcinogenicity study, plasma bosentan AUCs at 2000 mg/kg/day were approximately 75% of those observed at the maximum dose of 4500 mg/kg/day. Consequently, the dose evaluated in the mouse micronucleus assay appears adequate.

Table 1a : Micronucleus Test with . Mice

Treated with RO 47-0203/010. Mode of application: ORAL

Sampling time: 24 h

Single Dose mg/kg	Animal No.	Sex	NCE with MN No.	%	Ratio PCE/NCE	Median	PCE with MN No.	%	Median + Significance Levels
0	111	m			1.24				
	112	m			1.09				
	113	m			0.88				
	114	m			0.81				
	115	m			1.16				
						1.20			0.12
	116	f			1.07				
	117	f			2.12				
	118	f			1.70				
	119	f			2.32				
	1110	f			1.34				
500	211	m			1.10				
	212	m			1.05				
	213	m			1.15				
	214	m			1.87				
	215	m			1.42				
						1.48			0.05 n.s.
	216	f			1.86				
	217	f			1.87				
	218	f			1.31				
	219	f			2.27				
	2110	f			1.54				
1000	311	m			0.99				
	312	m			1.60				
	313	m			1.82				
	314	m			1.96				
	315	m			1.42				
						1.55			0.05 n.s.
	316	f			1.49				
	317	f			2.29				
	318	f			1.11				
	319	f			1.75				
	3110	f			1.29				
2000	411	m			1.80				
	412	m			1.95				
	413	m			1.29				
	414	m			1.64				
	415	m			1.74				
						1.71			0.05 n.s.
	416	f			2.28				
	417	f			2.04				
	418	f			1.44				
	419	f			1.68				
	4110	f			1.42				

Experiment Number: 004M94/

No.of PCE scored per animal: 2000

n.s. = no significance

* for P =< 0.05 ** for P =< 0.01

Trend : (+) increasing / (-) decreasing

Table 1b : Micronucleus Test with Mice

Treated with RO 47-0203/010. Mode of application: ORAL

Sampling time: 48 h

Single Dose mg/kg	Animal No.	Sex	NCE with MN No.	x	Ratio PCE/NCE	Median	PCE with MN No.	x	Median + Significance Levels
0	121	m			1.12				
	122	m			1.27				
	123	m			1.92				
	124	m			1.40				
	125	m			1.31	1.29			0.12
	126	f			1.98				
	127	f			1.22				
	128	f			1.97				
	129	f			0.63				
	1210	f			1.11				
2000	421	m			0.80				
	422	m			1.26				
	423	m			1.65				
	424	m			1.28				
	425	m			1.62	1.27			0.07 n.s.
	426	f			1.14				
	427	f			1.07				
	428	f			0.81				
	429	f			1.84				
	4210	f			1.72				

Experiment Number: 004M94/2

No.of PCE scored per animal: 2000

n.s. = no significance

* for P =< 0.05 ** for P =< 0.01

Trend : (+) increasing / (-) decreasing

APPEARS THIS WAY ON ORIGINAL

Table 2 : Micronucleus Test with Mice

Treated with RO 04-6467/001. Mode of application: ORAL

Sampling time: 24 h

Single Dose mg/kg	Animal No.	Sex	NCE with MN No.	%	Ratio PCE/NCE	Median	PCE with MN No.	%	Median + Significance Levels
0	111	m			1.24				
	112	m			1.09				
	113	m			0.88				
	114	m			0.81				
	115	m			1.16				
						1.20			0.12
	116	f			1.07				
	117	f			2.12				
	118	f			1.70				
	119	f			2.32				
	1110	f			1.34				
50	811	m			1.48				
	812	m			0.92				
	813	m			1.01	1.02			1.65 **(+)
	814	m			1.02				
	815	m			1.38				

Experiment Number: 004M94/2

No.of PCE scored per animal: 2000

n.s. = no significance

* for P =< 0.05 ** for P =< 0.01

Trend : (+) increasing / (-) decreasing

Ro 04-6467/001 = procarbazine

APPEARS THIS WAY ON ORIGINAL

Chromosome Analysis in Human Peripheral Blood Lymphocytes Treated in vitro with Ro 47-0203

Test Agent: Ro 47-0203

Lot Number: 14467-007B-09F

Study Facility:

Study Number: 002 M 94

Report Number: B 161884

Study Dates: January 17, 1994 – May 30, 1994

GLP Compliance: Yes

Test System: Human peripheral blood lymphocytes

Metabolizing system: Phenobarbital and β-naphthoflavone induced rat liver (S-9 fraction)

Procedure: CHO cells were exposed to Ro 47-0203 dissolved in DMSO in the presence and absence of rat liver S-9. In the absence of S-9, cells were exposed to Ro 47-0203 at doses of 0, 1.5, 5.0 and 15 μg/ml for 24 and 48 hours. In the presence of S-9, cells were exposed to Ro 47-0203 at doses of 50, 100 and 200 μg/ml for 3 hours. In another experiment cells were exposed to Ro 47-0203 at 20, 67.7 and 200 μg/ml in the absence and presence of S-9 for 3 hours. Doses were chosen on the basis of cytotoxicity as reflected by a decrease in mitotic index. (~50% at the highest dose). 100 cells from each dose were evaluated for chromosomal aberrations.

A test article is considered to induce a positive response when it produces a statistically significant increase (Fisher's Exact test) in structural or numerical chromosomal aberrations at one or more concentrations compared to concurrent control, and the incidence of aberrations exceeds the normal range for this assay.

Results: Ro 47-0203 in the presence and absence of rat liver S-9 did not increase structural or numerical aberrations in human lymphocytes at both noncytotoxic and cytotoxic (~50% reduction of mitotic index) concentrations. The positive controls increased structural chromosomal aberrations as expected.

Test substance	Dose µg/ml	AGT h	M-I %	S-cells %	U-cells %	P-cells %
Treatment 24 h without S9						
Concurrent negative controls	0	15.0	6.5	3.0	0.5	0.5
Ro 47-0203/010	1.5	16.8	3.3	2.0	0.0	0.5
"	5.0	15.9	4.3	1.5	0.0	0.5
"	15.0	18.6	1.7	2.0	1.0	1.0
Positive control Bleomycin	4.0	-	3.6	44.0**	6.0*	-
Treatment 48 h without S9						
Concurrent negative controls	0	-	4.2	1.5	1.5	0.5
Ro 47-0203/010	15.0	-	1.5	2.5	2.0	0.0
Positive control Colcemid	0.06	-	-	-	-	58.0**

- : Not tested
* : Significant at the 5% level
**: Significant at the 1% level

Average generation time (AGT), mitotic index (M-I), cells with structural aberrations excluding gaps (S-cells), cells with gaps only (U-cells), cells with numerical aberrations (P-cells).

Test substance	Dose µg/ml	AGT h	M-I %	S-cells %	U-cells %	P-cells %
Treatment 3 h with S9; Recovery 20 h						
Concurrent negative controls	0	16.5	3.4	3.0	1.0	1.0
Ro 47-0203/010	50	16.4	3.0	1.5	2.0	0.5
"	100	18.2	3.1	3.0	1.5	0.5
"	200	17.8	0.9	2.3	1.2	0.6
Positive control Cyclophosphamide	6	-	1.3	42.0**	8.0**	-

- : Not tested
**: Significant at the 1% level

Average generation time (AGT), mitotic index (M-I), cells with structural aberrations excluding gaps (S-cells), cells with gaps only (U-cells), cells with numerical aberrations (P-cells).

Test substance	Dose µg/ml	M-I %	S-cells %	U-cells %	P-cells %
		Treatment 3 h without S9; Recovery 21 h			
Concurrent negative controls	0	8.7	3.0	1.0	1.0
Ro 47-0203/010	20.0	7.3	2.0	1.0	0.5
"	67.7	6.1	3.0	0.5	0.5
"	200.0	3.6	2.0	1.0	0.0
Positive control Bleomycin	16	3.1	30.0**	2.0	-
		Treatment 3 h with S9; Recovery 21 h			
Concurrent negative controls	0	8.5	2.5	1.0	1.0
Ro 47-0203/010	20.0	7.0	2.0	0.0	0.0
"	67.7	5.6	2.0	1.0	0.5
"	200.0	4.0	2.5	1.0	0.5
Positive control Cyclophosphamide	12	7.0	28.0**	4.0	-

- : Not tested
**: Significant at the 1% level

Mitotic index (M-I), cells with structural aberrations excluding gaps (S-cells), cells with gaps only (U-cells), cells with numerical aberrations (P-cells).

Chromosome Analysis in Human Peripheral Blood Lymphocytes Treated in vitro with Ro 47-0203

Rationale for Study: This study was performed to assess the clastogenic effects of impurities observed in clinical batches of bosentan.

Test Agent: Ro 47-0203

Lot Numbers: G PM 0017 (Ro 47-0203/010),
410003A40 (Ro 47-0203/029), contains major impurities: Ro 47-4056 (0.6%), Ro 47-005 (0.1%) and Ro 47-9931 (0.3%).

Study Facility:

Study Number: 015 M 95

Report Number: B 165300

Study Dates: January 16, 1995 – May 19, 1995

GLP Compliance: Yes

Test System: Human peripheral blood lymphocytes

Metabolizing system: Phenobarbital and β-naphthoflavone induced rat liver (S-9 fraction)

Procedure: CHO cells were exposed to Ro 47-0203 dissolved in DMSO in the presence and absence of rat liver S-9. The effects of 3 hours of treatment were studied, both with and without metabolic activation, at concentrations of 200 and 300 μg/ml. The effects of 24 hours of treatment were studied, only in the absence of metabolic activation, at concentrations of 10 and 15 μg/ml. Doses were chosen on the basis of cytotoxicity as reflected by a decrease in mitotic index (~50% at the highest dose). 100 cells from each dose were evaluated for chromosomal aberrations.

A test article is considered to induce a positive response when it produces a statistically significant increase (Fisher's Exact test) in structural or numerical chromosomal aberrations at one or more concentrations compared to concurrent control, and the incidence of aberrations exceeds the normal range for this assay.

Results: The test articles (bosentan plus impurities) in the presence and absence of rat liver S-9 did not increase structural or numerical aberrations in human lymphocytes at concentrations that were cytotoxic (~50% reduction of mitotic index). The positive control increased structural chromosomal aberrations as expected.

Occurrence of cells in mitosis (MI), with structural chromosomal aberrations excluding gaps (S-cells) and significance levels (n.s.: no significance, **: $p < 0.01$), with gaps only (U-cells) and with numerical chromosome changes (P-cells) in cultured human peripheral blood lymphocytes.

Test articles	concentration µg/ml	MI %	S-cells %		U-cells %	P-cells %
Treatment 24 hrs in the absence of metabolic activation						
Concurrent negative controls	0	13.0	0.5		0.5	0.9
Ro 47-0203/010	15	4.6	1.0	n.s.	0.0	0.5
Ro 47-0203/029	10	6.4	1.0	n.s.	0.0	0.8
"	15	5.1	1.5	n.s.	0.5	0.0
Positive control Bleomycin	6		36.0	**	2.0	
Treatment 3 hrs in the absence of metabolic activation						
Concurrent negative controls	0	14.4	1.0		0.0	0.4
Ro 47-0203/010	300	8.0	0.5	n.s.	1.0	0.0
Ro 47-0203/029	200	7.3	2.0	n.s.	0.5	0.0
"	300	6.9	1.5	n.s.	1.5	0.5
Positive control Bleomycin	16		22.3	**	8.0	
Treatment 3 hrs in the presence of metabolic activation						
Concurrent negative controls	0	13.6	1.0		0.0	0.9
Ro 47-0203/010	300	7.2	1.5	n.s.	0.0	0.5
Ro 47-0203/029	200	5.1	1.5	n.s.	0.0	0.3
"	300	6.6	1.0	n s.	0.0	0.0
Positive control Cyclophosphamide	18		24.0	**	4.0	

104 Week Carcinogenicity Study in Rats

Location of Data: Volumes 1.30-1.35

Testing Facility:

Test site (Histopathology):

Study Number: Project 165915

Study Dates: May 1996 to June 1998

GLP Compliance: Studies were performed in accordance with GLP regulations.[4]

Protocol Concurrence: The sponsor evaluated bosentan in the two-year rat carcinogenicity study at the maximal dose recommended by the EC-CAC. The sponsor did not concur with the EC-CAC's recommendation for individual animal housing. See attached EC-CAC minutes.

Animals: rats were approximately 5 week of age at the start of the study; male rats weighed approximately 134 g and female rats weighed approximately 113 grams at onset of dosing. Rats received pelleted rat diet and tap water, *ad libitum*. Rats were housed 5 animals per cage in cages throughout the study period.

Mode of Administration of Test Agent: Oral by dietary administration in pelleted feed.

Dose Levels: 0, 0, 125, 500, 2000 and 3000 mg/kg/day. Doses were based on the most recent body weight.

Test Article: Ro 47-0203/029; batches 55206/F, 55206/G, 55206/H, 55206/J

Basis for Doses Evaluated: The dose of 3000 mg/kg/day is the maximum feasable dose when given in diet.

Analysis of Diets for Test Agent: Samples of all diets prepared during weeks 1, 2, 3, 4, 5 and 13 and approximately every 13 weeks thereafter were analyzed by to check the accuracy and homogeneity of preparation.

Number of Animals: 50/sex/group (main study animals)
Additional 10 rats /sex/group for determination of systemic exposure at week 51.

Observations/Measurements: Clinical signs were recorded for main study animals once weekly. All rats were evaluated twice daily for morbidity and mortality. Individual body weights were recorded weekly for the first 18 weeks and every second week thereafter. Food consumption was determined weekly. Rats were palpitated weekly for tissue masses, and the size of masses recorded. Rats were evaluated for respiratory noises in weeks 19, 51, 70, 86 and 100. The incidence of sneezing per cage over a period of 10 minutes was scored in weeks 70, 73 and 100. The

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[4] The following were not performed per GLP.
- Pathology Peer Review of the nasal cavity of satellite animals: performed by
- Endoscopy of the nasal cavity

nasal cavity was evaluated endoscopically in week 94 in two female rats (one given 2000 mg/kg/day and one given 3000 mg/kg/day).

Blood was taken from all animals killed in extremis and from all surviving animals at terminal necropsy for hematology and clinical chemistry measurements.

The following hematology parameters were evaluated (week 52, satellite animals; week 104, main study animals): erythrocyte count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, platelet count, red cell distribution width and total leukocyte count. Prothrombin time and partial thromboplastin times were determined. Additionally, differential leukocyte counts were to be performed when requested by the study pathologist (not clear if this was done)

The following clinical chemistry parameters were determined (week 52, satellite animals only): alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, sorbitol dehydrogenase, total bilirubin, bile, cholesterol, triglycerides, creatinine, glucose, urea, total protein, albumin , sodium, potassium, chloride, calcium and phosphorus.

Interim Sacrifices: None

Plasma Drug Concentrations: Plasma bosentan concentrations were determined in all satellite animals during weeks 3, 9, 27 and 51. Plasma metabolites were determined during weeks 3, 9 and 27 in rats given bosentan at 3000 mg/kg/day. Blood was taken at 8.00 a.m., 16.00 p.m., and 24.00 p.m. according to the following schedule:

Concurrent control group 1: 1 animal/sex, at each of the sampling times,
125, 500, 2000 and 3000 mg/kg/day dose groups: 3 animals per sex per group, at each of the sampling times.
The animals used at each time point were the same for all four sampling sessions when possible.

Plasma bosentan concentrations were determined in a sampling of main study animals during week 103 at the same time points as indicated for the satellite animals:

Control group 1: 1 rat/sex at each of the sampling times.
Drug-treated: 5 rats/sex/dose group, at each of the sampling times.

Necropsy: All satellite and main study animals surviving to the end of the observation period and all animals killed *in extremis* were subjected to a full post mortem examination. Rats found dead were subjected to a full post mortem examination as soon as possible after death. All tissues from all main study animals were evaluated macroscopically and histopathologically. Additionally, the nasal cavities (levels 1-3) of all main study animals were evaluated.

Adrenal glands	Liver	Spleen
Aorta	Lungs, infused with formalin	Sternum with bone marrow
Brain	Lymph nodes - mandibular, mesenteric	Stomach
Cecum	Ovaries	Testes
Colon	Esophagus	Thymus
Duodenum	Pancreas	Thyroid including parathyroid
Epididymides	Pituitary gland	Tongue
Eyes with optic nerve and Harderian gland	Prostate gland	Trachea
	Rectum	Urinary bladder
Male and Female mammary glands	Salivary glands - mandibular, sublingual, parotid	Uterus
Femur including knee joint		Vagina
Heart	Sciatic nerve	Zymbal gland
Ileum	Seminal vesicles including coagulation gland	(Tattoo and ears: for identification only)
Jejunum		
Kidneys	Skeletal muscle	All gross lesions, tissue masses and tumors
Larynx	Skin	
	Spinal cord -cervical, midthoracic, lumbar	

The head was fixed from all animals of the main and satellite groups

Liver sampling for electron microscopy: The livers of concurrent control groups and rats given 2000 and 3000 mg/kg/day (5 animals/sex/dose group) were sampled for electron microscopy at 104 weeks.

Organ weights: The following organ weights were recorded for all surviving main study animals surviving until 104 weeks.

Adrenal glands	Pituitary (after fixation for at least 24 hours)
Brain	Spleen
Heart	Testes
Kidneys	Thyroid (after fixation for at least 24 hours)
Liver	Ovaries
Lungs	

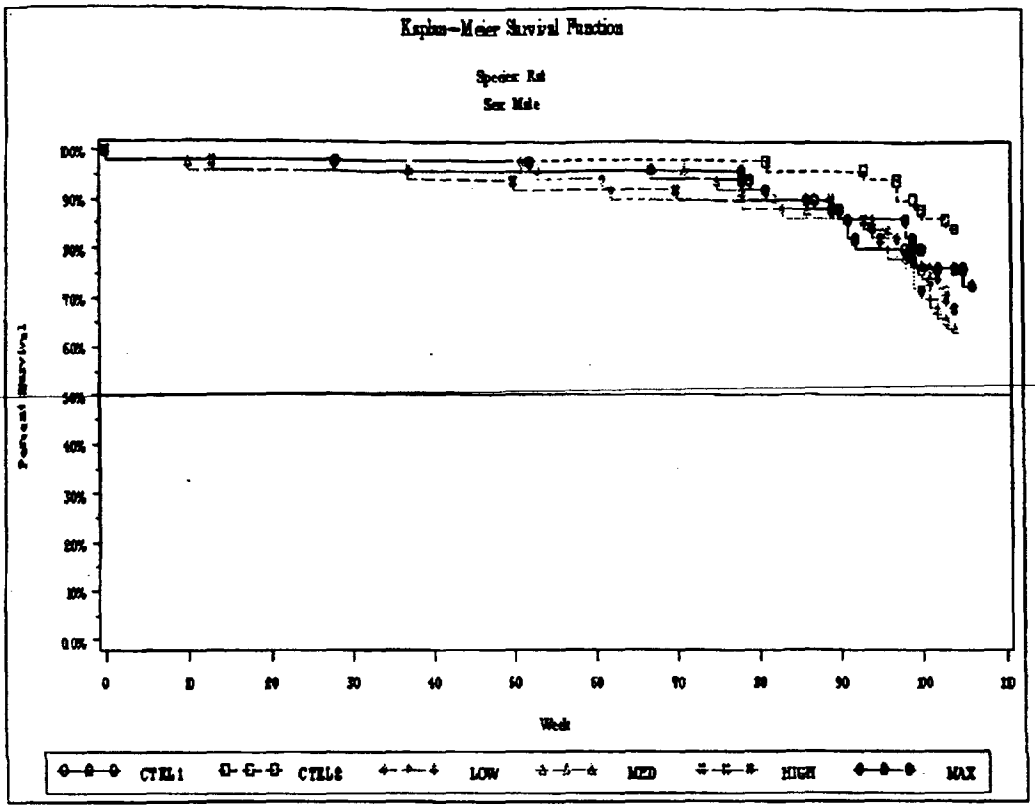
Results

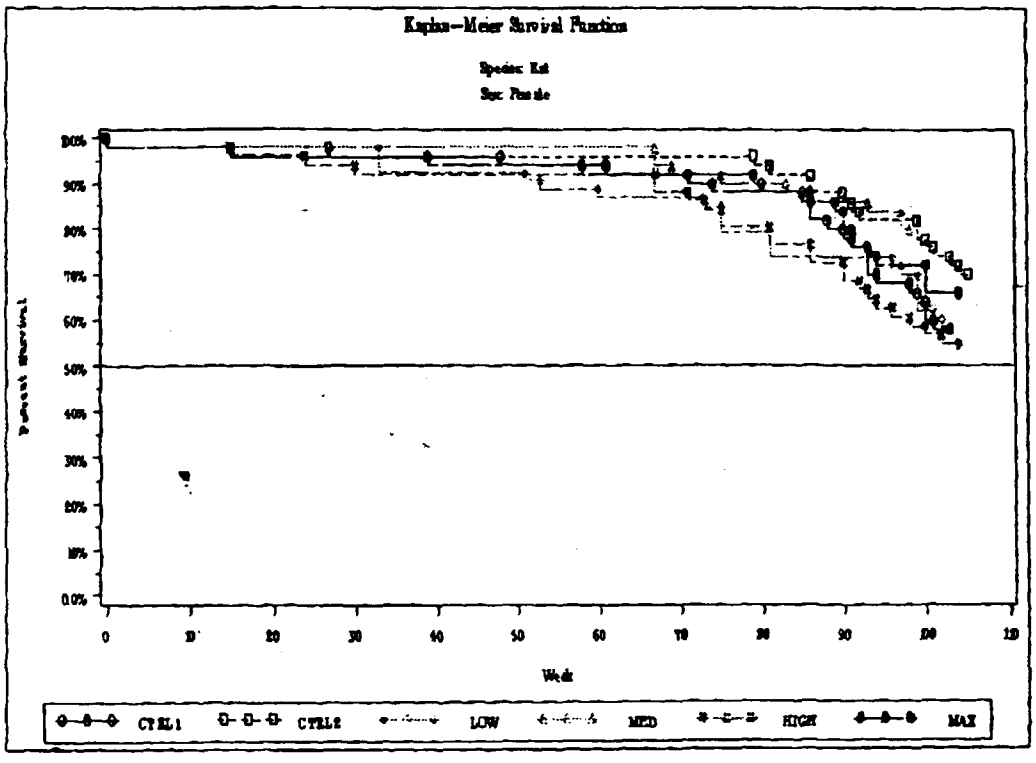
Achieved doses: Based on food intake and measured dietary concentration of test agent, the intake of test agent by the respective dose groups appeared reasonably close to the target doses.

Target Dose (mg/kg/day)	Achieved Dose (mg/kg/day)	
	Male	Female
125 mg/kg/day	125	124
500 mg/kg/day	501	499
2000 mg/kg/day	1985	2015
3000 mg/kg/day	2723^	2995

^For 3000 mg/kg/day males, the dietary concentration was maximized at 5.6% (56000 ppm) from week 37 onwards. Although higher concentrations were required to reach the 3000 mg/kg intake, the sponsor did not increase the dietary test agent concentration, in order to avoid nutritional imbalance of the diet.

Mortality: Mortality was not drug-related for either male or female rats. At 104 weeks, survival (at least 32 males and 28 females per group) was sufficient for evaluation of tumorigenic effects.


Kaplan-Meier Survival Function
Species Rat
Sex Male
Percent Survival
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0.0%
0
10
20
30
40
50
60
70
80
90
100
110
Week
CTRL1
CTRL2
LOW
MED
HIGH
MAX


Kaplan-Meier Survival Function
Species Rat
Sex Female
Percent Survival
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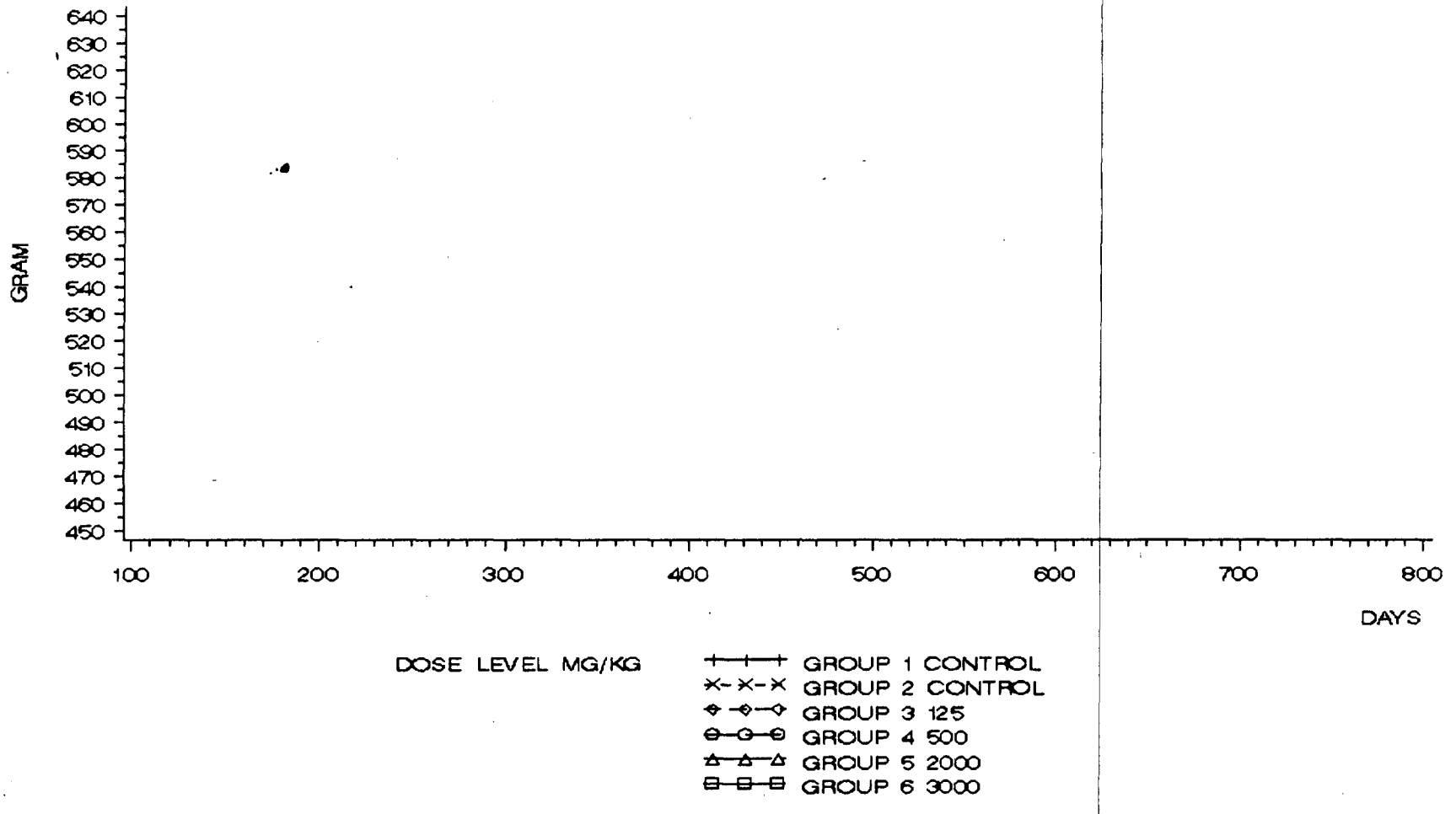
20%
10%
0.0%
0
10
20
30
40
50
60
70
80
90
100
110
Week
CTRL1
CTRL2
LOW
MED
HIGH
MAX

Body Weights

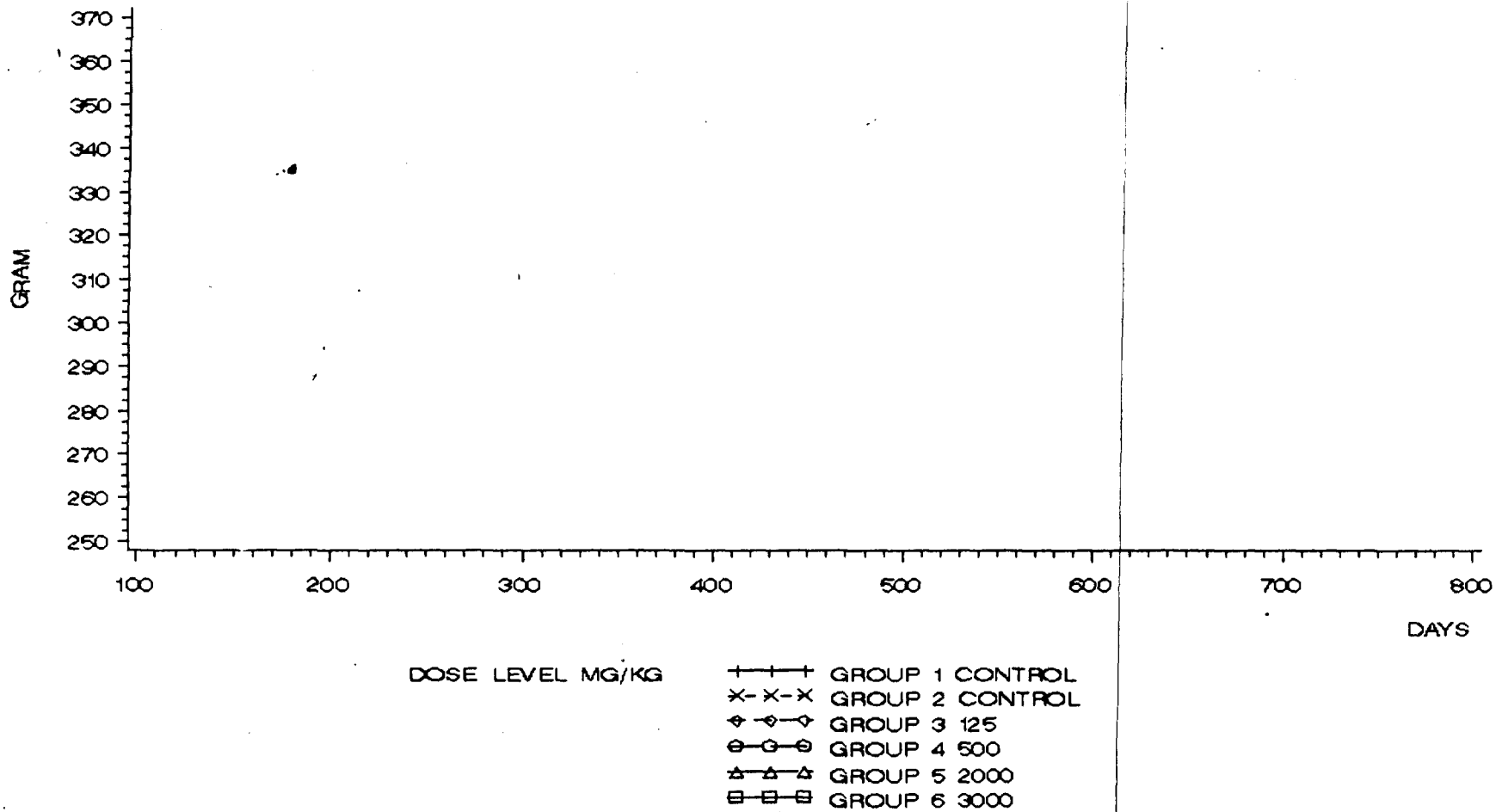
Body weights were significantly lower than concurrent control for male mice given 2000 and 3000 mg/kg/day, but only during the last two weeks of treatment.

Body weights were significantly lower than concurrent control for female mice given doses ≥500 mg/kg/day. Differences were significant from week 58 until the end of study for female rats given 500 mg/kg/day, and from 44 weeks until the end of study for female rats given 2000 or 3000 mg/kg/day.

Body Weights of Male Rats Given Bosentan for 104 Weeks



Body Weights of Female Rats Given Bosentan for 104 Weeks



Food Consumption: Food intake was significantly greater than concurrent control for male rats given doses ≥125 mg/kg/day. These differences were evident throughout the entire study duration. In contrast, food intake was significantly less than concurrent control for female rats given doses ≥500 mg/kg/day. In males, average food intake was 104%, 104%, 108% and 113% of concurrent control for rats given 125, 500, 2000 and 3000 mg/kg/day, respectively. In females, average food intake was 95% of concurrent control for all dose groups.

Clinical Findings: Dose-related increases in the incidence of abnormal respiratory noises were observed in male and female rats. Respiratory noises appeared earlier in animals given higher doses.

Cage Incidences of Rats with Abnormal Respiratory Noises
(Number of Abnormal Cages /Number of Cages Evaluated)

Gender	Week Evaluated	Dose (mg/kg/day)					
		0	0	125	500	2000	3000
Male	19	1/12	0/10	0/12	6/12	12/12	11/12
	51	0/12	0/10	0/12	10/12	11/12	11/12
	70	0/10	0/10	0/12	10/10	9/10	9/10
	86	0/10	0/10	0/12	7/10	10/10	10/10
	100	0/10	0/10	0/12	8/10	9/10	10/10
Female	19	0/12	0/10	1/12	11/12	12/12	12/12
	51	0/12	0/10	2/12	11/12	12/12	12/12
	70	0/10	0/10	3/10	10/10	10/10	10/10
	86	0/10	1/10	2/10	10/10	10/10	10/10
	100	0/10	0/10	2/10	10/10	10/10	9/10

Dose-related increases in sneezing incidences were observed in male and female rats. Two cages per dose group were evaluated for sneezing incidence in weeks 73 and 100.

Sneezing Incidence (two individual cages/dose group)

Gender	Week Evaluated	Dose (mg/kg/day)					
		0	0	125	500	2000	3000
Male	73	0, 0	0, 0	1, 0	0, 0	9, 10	8, 11
	100	0, 0	2, 0	0, 0	12, 3	1, 7	8, 7
Female	73	2, 0	1, 0	0, 0	15, 7	17, 14	19, 13
	100	0, 0	0, 1	0, 6	5, 4	8, 10	13, 8

Dose-related increases in incidence of respiratory rales were observed in female rats given ≥2000 mg/kg/day.

Incidence of Respiratory Rales

Gender	Dose (mg/kg/day)					
	0	0	125	500	2000	3000
Male	0	0	0	5-15% from week 77	<5%	0
Female	<5%	<5%	<5%	<5%	Up to 30% of animals from week 73 and onward	Up to 30% of animals from week 61 and onward

A dose-related increase in hunched posture was observed in male rats given ≥2000 mg/kg/day and in female rats given ≥500 mg/kg/day. Up to 40% of male rats and 60% of the female rats were affected.

Drug-related, but not dose-related decreases in erythrocyte counts, hemoglobin and hematocrit were observed in male rats at weeks 52 and 104 and in female rats at week 104. In males, decreases were similar at both time points; only 104 week data is shown. Other hematological parameters, including white cell counts, eosinophils, basophils and platelets, were similar in drug-treated and concurrent control animals.

Gender	Parameter	Dose (mg/kg/day)				
		0	125	500	2000	3000
Males	RBC (T/l)	7.53 ±0.44 (38)	7.25 ±0.46 (36)	6.95* ±0.72 (33)	7.04* ±0.45 (33)	7.10* ±0.53 (38)
	HG (mmol/l)	9.6 ±0.4 38	9.3 ±0.4 36	8.9* ±1.2 33	9.0* ±0.5 36	9.1* ±0.7 38
	HCT (l/l)	0.434 ±0.020 38	0.425 ±0.018 36	0.415* ±0.046 33	0.415* ±0.021 36	0.419 ±0.029 38
Females	RBC (T/l)	6.71 ±0.40 37	6.28* ±0.47 30	6.44 ±0.56 40	6.64 ±0.53 28	6.74 ±0.69 36
	HG (mmol/l)	9.2 ±0.5 37	8.5* ±0.6 30	8.7* ±0.6 40	8.7* ±0.9 29	8.9 ±0.8 36
	HCT (l/l)	0.421 ±0.021 37	0.367* ±0.025 30	0.392* ±0.032 40	0.406 ±0.043 29	0.391* ±0.050 36

* Indicates significant difference from control at P<0.05.

No consistent drug-related changes in clinical chemistry parameters were observed.

Liver weights and liver weight/body weight ratios were higher in drug treated than in concurrent control rats. These liver findings were not dose-related.

Organ weights and organ weight/body weight ratios of other organs, including thyroid gland (male and female) and testes, were not drug-related.

Liver weights at 104 Weeks of Drug Treatment

Gender	Organ	Dose (mg/kg/day)				
		0	125	500	2000	3000
Male	Liver wt (g)	15.38 ±2.40	17.33* ±2.47	16.54 ±2.35	17.11* ±3.37	16.10 ±3.66
	Liver wt/body wt	2.66 ±0.33	2.87* ±0.35	2.91* ±0.31	3.07* ±0.41	2.97* 0.44
Female	Liver wt (g)	11.72 ±2.41	11.43 ±1.91	10.68 ±1.85	11.24 ±2.56	10.45 ±1.68
	Liver wt/body wt	3.41 ±0.59	3.44 ±0.47	3.47 ±0.43	3.92* ±0.84	3.66 ±0.33

Drug-related non-neoplastic histopathology

The incidence of tubular atrophy of the testes was higher in bosentan-treated rats at 104 weeks of treatment than in concurrent controls. This finding was not dose-related (see table on following page for overall incidence).

Bosentan increased the incidence of both bilateral and unilateral atrophy of the seminiferous tubules.

Tubular Atrophy Incidences	Dose (mg/kg/day)					
	0	0	125	500	2000	3000
Total	4	2	12	16	15	14
Unilateral	3	0	5	11	9	7
Bilateral	1	2	7	5	6	7

Although bosentan did not increase the average severity of testicular tubular atrophy, bosentan increased the incidence of testicular atrophy at all severity levels.

Average severity of testicular atrophy in rats with lesions

Dose mg/kg/day					
0	0	125	500	2000	3000
2.5	2	1.9	1.9	2.0	1.8

Number of Animals with Severity Grade

Severity Grade	Dose (mg/kg/day)					
	0	0	125	500	2000	3000
Grade 1 Mild	1	1	5	8	5	9
Grade 2 Mild	1	0	5	4	6	2
Grade 3 Moderate	1	1	0	2	2	1
Grade 4 Marked	1	0	2	2	2	1
Grade 5 Severe	0	0	0	0	0	1

The incidence of mineralization of the testes was greater in drug treated rats at 104 weeks of treatment than in concurrent controls. This finding was not dose-related. However, average severity of testicular mineralization was dose-related (1, 1, 1, 1.3, 1.5, and 2.0 for 0, 0, 125, 500, 2000 and 3000 mg/kg/day).

The incidence of chronic inflammation of the prostate was higher in rats given 3000 mg/kg/day than in concurrent controls; severity of this finding was not drug-related.

Drug-related pathology was not observed in the epididymides and seminal vesicles of these treated rats. Testes from satellite animals (sacrificed in week 52) were not analyzed histopathologically.

NUMBER OF ANIMALS WITH NON-NEOPLASTIC LESIONS BY ORGAN/GROUP/SEX
STATUS AT NECROPSY: KO, INCL. DEATHS
TERMINAL SACRIFICE

SEX :						MALE
DOSE GROUP:	01	02	03	04	05	06
NO.ANIMALS:	50	50	50	50	50	50
TESTES :	50	50	50	50	50	50
- TUBULAR ATROPHY :	4	2	12	26	15	14
- MINERALISATION :	4	2	7	14	8	7
- LEYDIG HYPERPL (FOC):	3	4	3	5	4	1
- DILATED TUBULES :	1	-	-	1	-	-
- ARTERITIS :	1	-	-	-	-	-
- MESOTHEL HYPERPLASIA:	-	-	-	1	-	-
- EDEMA :	-	1	-	-	-	-
- HEMORRHAGE :	-	-	-	-	1	-
- ADHESIONS :	-	-	-	-	1	-
EPIDIDYMIDES :	50	50	50	50	50	50
- REDUCED SPERMATOZOA :	3	1	3	2	4	5
- ATROPHY :	1	-	-	-	1	-
- DILATED TUBULES :	-	-	1	-	-	1
- SUPPURATIVE INFLAMM :	-	-	-	-	1	-
- SPERMATOCELE :	-	-	1	1	1	-
- ARTERITIS :	-	-	-	-	1	-
- EPITHEL INCLUSIONS :	-	-	-	1	-	-
SEMINAL VESICLES :	50	50	50	50	50	50
- RETAINED SECRETION :	2	-	2	-	4	4
- ATROPHY :	4	5	9	3	8	4
- HYPERPLASIA (FOCAL) :	1	2	-	1	2	-
- SUPPURATIVE INFLAMM :	-	2	-	-	3	-
- CHRONIC INFLAMMATION:	-	-	1	1	-	-
PROSTATE :	50	50	50	50	50	50
- HYPERPLASIA (FOCAL) :	6	10	3	7	3	8
- SUPPURATIVE INFLAMM :	7	9	7	7	5	2
- CHRONIC INFLAMMATION:	3	3	2	2	1	7
- ATROPHY :	3	3	6	2	5	2

Groups 1, 2: concurrent control; Groups 3, 4, 5 and 6 refer to 125, 500, 2000 and 3000 mg/kg/day dose groups, respectively.