RPA 201772 11/12/1996 Mechanistic Study

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DATA EVALUATION RECORD

STUDY TYPE: Mechanistic Study for Thyroid Effects-Rats Nonguideline

DP BARCODE: D224202
P.C. CODE: 123000
MRID NO.: 43904818

SUBMISSION CODE: S501233
TOX. CHEM. NO.: [New Chemical]

TEST MATERIAL (PURITY): RPA 201772 (99.7%)

CHEMICAL NAME: 5-Cyclopropyl-4-(2-methylsulfonyl-4-trifluoro-methylbenzoyl) isoxazole

SYNONYM: Isoxaflutole

CITATION: Chambers, P. R. (1995). RPA 201772. Effects on the Thyroid in Male Rats After Dietary Administration for 2 Weeks. Huntingdon Life Sciences Ltd., Huntingdon, Cambridgeshire, England; Report No. RNP 478/952145; December 11, 1995. MRID NUMBER: 43904818. (Unpublished)

SPONSOR: Rhöne-Poulenc Agriculture, Essex, England

EXECUTIVE SUMMARY: The mechanism of action of RPA 201772 on thyroid was investigated in male Sprague-Dawley rats (MRID# 43904818). In this study RPA 201772 (99.7% a.i.) was administered in the diet to male Crl:CD (SD) rats (14/dose) at dosage levels of 0 or 500 mg/kg/day for 14 days. A third group (positive control) of rats received 80 mg/kg/day sodium phenobarbital by gavage and an untreated diet. Following treatment period, the liver enzyme activities including cytochrome P-450 and p-nitrophenol uridine 5'-diphosphatase-glucuronyltransferase (UDPGT) as well as thyroxine levels were monitored and thyroid weights were determined. The rate of T4 disappearance from blood was measured in rats after intravenous administration of sodium ^{125}I-thyroxine. The effect on blood concentration half-life, thyroid gland iodine uptake and thyroid weights were measured.

RPA 201772 administration caused more than two-fold increase in cytochrome P-450 dependent mixed-function oxidase system and UDPGT activity which resulted in increased clearance of ^{125}I-thyroxine from the blood as indicated by shorter half-life and decreases in plasma T_4 level. In addition, there were increases in liver and

thyroid weights. The plasma T_3 level was unaffected. The significant reduction in the level of circulating T_4 was possibly the result of enhanced glucuronidation by hepatic UDPGT and a rapid systemic clearance of total radioactive ^{125}I-thyroxine in RPA 201772 treated group. Following intravenous administration of ^{125}I-thyroxine, the thyroid iodine uptake was slightly higher and thyroid weights were significantly higher than controls in RPA 201772 treated rats. The effects observed in this study are supportive of the hypothesis that RPA 201772 may have induced thyroid tumors in male rats (MRID# 43904806) through a disruption in the thyroid-pituitary hormonal feedback mechanisms.

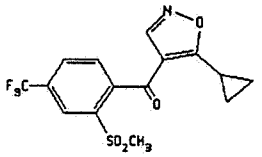
The study is classified as Acceptable (Nonguideline) as it is not a required guideline study. It is acceptable for the purposes for which it was intended as a special study.

COMPLIANCE: Signed and dated GLP, Quality Assurance, Data Confidentiality, and Flagging statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: RPA 201772
   Chemical Name: 5-Cyclopropyl-4-(2-methylsulfonyl-4-trifluoromethylbenzoyl) isoxazole
   Synonym: Isoxaflutole
   Description: Cream colored crystalline solid
   Batch #: 40 ADM 93
   Purity: 99.6%
   Storage: At room temperature in the dark
   Structure:



2. Vehicle: Basal diet

3. Reference Material: Phenobarbital (sodium salt)
   Purity: 100%
   Lot No.: 122 HO143
   Description: White powder
   Storage: At room temperature in the dark

4. Radioactive Compound: L-(3'5 ^{125}I) thyroxine
   Batch No.: B9534
   Radiochemical purity: >90.0% with,5% free ^{125}I-iodide
   Specific activity: 50 μCi/μg

5. Test Animals: Rat
   Strain: Crl: CD (SD) BR
   Age and weight at arrival: Approx. 28 days;
   Males - 170 to 198 g (at dosing)
   Source: Charles River U.K. Ltd, Kent, England
   Housing: Two per cage, selected at random
   Diet: SDS Rat and Mouse No.1 modified maintenance diet from Special Diet Services Ltd, Essex, England ad libitum
   Water: Tap water ad libitum

Environmental conditions: Temperature: 21 ± 2°C;
Relative Humidity: 55 ± 10%;
Air changes: Not reported;
Photoperiod: 12 hours light/dark
Acclimation period: Approx. 13 days

B. METHODS/STUDY DESIGN:

1. In Life Dates - start: August 29, 1995
end: September 12, 1995

2. Animal Assignment

Animals were assigned randomly to three groups on a weight basis (see Table 1).

Table 1: Study Design[a]

Dose Groups (mg/kg/day)	# of Male Rats
Control: 0	14
RPA 201772: 500	14
Phenobarbital: 80[b]	14

a 2 spare rats/group were included in each group to ensure that 6 rats/group would be available for liver enzyme assessment and an additional 6 rats/group for thyroxine kinetic studies.

b Positive control group received 80 mg phenobarbital/kg/day (5 ml/kg) by gavage in addition to basal diet; dosing solution was prepared fresh daily.

3. Diet Preparation and Analysis

The premix was prepared by grinding the test substance and mixing it with the basal diet using a Turbula mixer for at least 5 minutes. The test diet of desired concentration was prepared by incorporating the premix into the basal diet and a homogenous mixer was prepared by further mixing it for at least 5 minutes. The concentration was adjusted weekly based on body weight. Prior to initiation of the study, the concentration

of test substance in the diet was verified; homogeneity and stability data were analyzed. Test diets were prepared weekly and samples of control and test diets prepared for the first week were analyzed for accuracy of preparation. An additional samples prepared during second week were stored for future analysis.

Results - The analytical data indicated that the mixing procedure was adequate and that the variance between nominal and actual dosage to the animals was acceptable.

Analyses for homogeneity of the top, middle and bottom portions of the sample diet formulations was confirmed at a concentration of 4000 ppm; the test concentrations in the samples were within ± 10% of the intended concentration (C.V.: 2.38%; concentration range: 100.25%-110.0% of nominal; page 107 of the report).

The stability analyses of the 4,000 ppm diet samples revealed that the test compound was stable in the diet for up to 8 days when stored at room temperature (range: 107.25%, 105.5%–105.75% and 106.0%–106.25% at 0, 4 and 8 days of storage, respectively; page 108 of the report).

The concentration analyses of the sample test diets showed that the percents of the intended RPA 201772 concentration in each of the three test diet samples were within ±3% of the targeted concentration (97.7%; page 106 of the report).

4. Dose Selection

The dose level of 500 mg/kg/day was selected to replicate the high dose used in the carcinogenicity study. The study was conducted in male rats because the histopathological changes in the thyroid observed in the carcinogenicity study were limited to male rats only.

5. Statistics

The following procedures were utilized in analyzing the numerical data:

Body weight gain, food consumption, and organ weight data - Levene's test was conducted for heterogeneity of variance between groups followed by ANOVA for significant differences. If Levene's test revealed heterogeneous variances, then a logarithmic transformation was applied; if not an analysis of ranks followed by Student's t test and Wilcoxon rank sums test were used.

Relative liver weight, microsomal protein and cytochrome P-450 concentrations, 7-Pentoxyresorufin O-depentylase (PROD) as well as UDPGT activities - Levene's test was conducted for homogeneity of variances. Depending on the pattern of the variance heterogeneity, for significant differences, the original analysis was retained or non-parametric tests were applied. One-way analysis of variance was then applied followed by two-sided t tests for comparison between the treated and positive control groups with the negative control. Significant differences were considered at 5% and 1% levels only.

Thyroxine kinetics data were analyzed by One-way analysis of variance followed by Student's t-test.

C. METHODS:

1. Observations:

Animals were observed at least once daily after dosing, twice during weekdays and once during weekends and holidays for clinical signs of toxicity and mortality.

2. Body Weight

Animals were weighed prior to the beginning of treatment, on the first day of dosing, then once weekly thereafter and before necropsy.

3. Food Consumption and Efficiency/Water Consumption

Food consumption per cage was determined weekly and is presented as g food/animal/week. Food efficiency was calculated based on amount of food consumed and body weight gain. The report stated that water consumption was monitored daily by visual inspection of the water bottles throughout the study.

4. Clinical Chemistry:

On study day 14, blood was collected under anesthesia from the orbital sinus of six rats from each group for analysis of tri-iodothyronine (T3) and thyroxine (T4) by radioimmunoassay. Blood sampling was taken between 1300 and 1400 hours on the day of collection. Samples were collected into tubes containing heparin anticoagulant.

a. Tissue Processing for Liver Enzyme Assays:

At post mortem, liver samples from 6 rats/group were homogenized in 50mM Tris Hcl buffer (pH 7.4) containing 0.25 M sucrose. Each individual liver homogenate were centrifuged at 13000 rpm for 20 min at 4°C. The microsomal fractions of the supernatant were prepared by further centrifugation at 105,000 rpm for 1 hour. The supernatant was discarded and the microsomal pellet was suspended in Tris buffer (pH 7.4) at 25°C and stored on ice for measurement of cytochrome P-450 (1 ml suspension = approx. 300 mg original wet weight of liver). Duplicate aliquots of 0.1 ml microsomal suspensions were used for determination of protein and the remainder was stored (ca -75°C) for measurement of liver enzymes.

Protein concentrations (mg of protein per g of liver and total mg of protein per whole liver) were determined by the method of Lowry et al. (1951) while cytochrome P-450 concentration (nanomoles per mg microsomal protein and nanomoles per g of liver) and pentoxyresorufin-O-depentylase activity (rate of resorufin production per mg protein and per g of liver) were determined using method of Rutten et al (1987) and Lubet et al (1985), respectively.

UDPGT with p-nitrophenol (PNP) as a substrate, was assayed using modified method of Winsnes (1969). Assays were performed in duplicate and glucuronyltransferase activity was determined by the difference in PNP concentration between the blank and test incubations. UDPGT activity was expressed as the rate of PNP utilization per milligram of protein and per gram of liver.

b. Thyroxine Kinetics Study:

Rats (6/group) that were not chosen for blood sampling or liver enzyme investigations were assigned to the Kinetics study group. The dosing solution was prepared by adding sterile water to a volumetric flask (10 ml) containing 200 μCi ^{125}I-thyroxine (T4) to provide a solution of nominal concentration 20 μCi/g solution. After 14-days treatment, 0.4 ml ^{125}I-T4 dose solution (10 μCi) was administered intravenously into a lateral tail vein of each rat. The amount of radioactivity administered to each animal (dpm, μCi) was calculated by multiplying the weight of dose administered (g) by the specific activity of the dose solution (dpm/g dose solution). The blood (20μl) was withdrawn for measurement of total radioactivity prior to dosing, and at 1, 2, 4, 6, 8, 12, 24, 36, and 48 hours post-dosing. Following

collection of blood samples at 48 hour, animals were sacrificed; thyroid glands were removed and weighed and radioactivity content were measured by scintillation counting. The measurement of specific radioactivity (dpm/g dose solution) present in replicate aliquots (0.2 ml) of the ^{125}I-T4 dose solution was performed by Cobra II automatic gamma scintillation counter (Model 5005, Packard Instrument Co.). Blood samples collected in capillary tubes at various time intervals were subjected to direct measurement of radioactivity. To aid measurement and comparison of radioactivity, non-radiolabeled and radiolabeled reference standards were used.

Pharmacokinetic analysis was performed using KIN 5.1 program. The study report stated that "the areas under the whole-blood radioactivity concentration-time curves to infinite time (AUC) were calculated as $AUC = AUC_1 + C_{last/Kel}$, where AUC_1 is the area under the whole-blood concentration-time curves and K_{el} is the rate constant of the terminal phase of the whole-blood concentration-time curve determined by log-linear regression analysis of those sample points which constitute the terminal, linear phase of the concentration-time curve. Terminal half-life was calculated as $ln2/K_{el}$". Systemic clearance (CL) was calculated as Dose/AUC and expressed as ml/unit time.

5. Sacrifice and Pathology

After 14 days of treatment, liver, thyroid and pituitary were removed from 6 rats/group for T3 and T4 blood sampling. These organs were weighed and preserved in buffered 10% formalin. Samples of abnormal tissues were preserved for future examination. In addition, at necropsy, terminal body weights were recorded. The livers from each animal were removed and weighed and the weights were expressed as absolute and relative to body weights.

II. RESULTS

A. Observations :

1. Mortality - No mortalities were noted.

2. Toxicity - No clinical signs of toxicity were observed in group treated with RPA 201772. The only clinical sign of toxicity observed among all animals receiving phenobarbital consisted of unsteady gait starting 30 minutes after dosing and occasionally lasting up to 6 hours after dosing.

B. Body weight and weight gain:

There were no treatment-related effects on body weight or body weight gain observed in rats from all dose groups (Table 2). A slight increase in body weight gain in rats treated with RPA 201772 was not considered to be toxicologically significant. The body weight gain of animals receiving phenobarbital was comparable to that of control.

C. Food consumption and Food efficiency:

There were no significant differences between the RPA 201772 treated and control groups in mean daily food consumption (Table 2). The reduced mean cumulative food intake of rats treated with phenobarbital during week 2 of treatment was considered to be an isolated incident. The food efficiency of animals in all dose groups was unaffected.

D. Clinical Chemistry: Treatment-related decrease in T_4 levels and increase in microsomal protein and cytochrome P-450 concentrations as well as increases in PROD and UDPGT activities were observed in RPA 201772 treated and phenobarbital treated rats. These data are summarized in Tables 2 and 3 and are discussed below.

1. Effect on Thyroxine Levels: Following 2-weeks treatment, the T_4 levels in the RPA 201772 and phenobarbital treated rats were significantly decreased compared to control (Table 2). Rats receiving RPA 201772 had a larger decrease (56% of control) than phenobarbital treated rats (86% of control). The mean T_3 values for both treated groups were comparable to that of control.

2. Effect on Hepatic Enzymes: Treatment with RPA 201772 caused statistically significant increase in Phase I and Phase II liver enzymes as follows (see Table 3):

   - Microsomal protein concentration: There was significant increase in both the concentration (mg/g liver) and the total weight of hepatic microsomal protein in both RPA 201772 and phenobarbital treated rats.

   - Cytochrome P-450 concentration: RPA 201772 treatment resulted in 2-fold (per mg of protein) to 3.6-fold (per g of liver) increases in the concentration of microsomal cytochrome P-450 over control values; the increases in phenobarbital treated group were slightly lower compared to that of RPA 201772 treated group (1.9-fold per

Table 2

Effect of Body Weight, Food Consumption and Thyroid Hormone Concentrations in Rats Following Treatment with RPA 201772 or Phenobarbital for Fourteen Days[a]

Parameters Measured	Dose Groups		
	Control	RPA 201772	Phenobarbital
Body Weights (g)			
Week 0:	182	182	181
Week 1:	232	233	229
Week 2:	281	288	278
Body Weight Gain- Week 0-2	99	106	97
(% of control)b	- -	(108%)	(99%)
Food Consumption (g/rat/week)			
Week 0:	235	228	209
Week 2:	213	218	201
Total Consumption- Week 1-2	448	446	410
(% of control)b	- -	(100%)	(92%)
Food Efficiency- Week 1-2	4.6	4.2	4.2
Thyroid Hormone Concentrations:			
T3 in µg/dl: Week 2	74	68	69
T4 in ng/dl: Week 2	5.7	3.2 **	4.9**
(% of control)b	(- -)	(56%)	(86%)

a Extracted from Tables 1, 2, 12 and 13 (pages 27, 28, 37 and 38) of the study no. RNP 478/952145; **p<0.01

b Calculated by the reviewer

- 7-Pentoxyresorufin O-depentylase activity (PROD): The induction of this activity significantly increased in both RPA 201772 and phenobarbital treated rats compared to control. There was 14-fold increase in (expressed as per mg protein) in both treated groups; the increases in activity expressed as per g of liver in the above groups were 27-and 20-fold, respectively.

- p-Nitrophenol UDP-glucuronyltransferase activity (UDPGT): This Phase II enzyme activity increased by 2.2-fold (expressed as per mg protein) to 3.8-fold (expressed as per g liver) in RPA 201772

treated rats and by 2.2-fold (expressed as per mg protein) to 3-fold (expressed as per g liver) in phenobarbital treated rats.

F. Thyroxine kinetics: The mean whole blood radioactive (^{125}I) thyroxine concentrations decreased in both the RPA 201772 and phenobarbital treated groups than in the control group (Table 4). The terminal rate constants (K_{el}) for the control, RPA 201772 and phenobarbital treatment groups were 0.0407, 0.0520 and 0.0428 per hours, respectively. The rate constant for RPA 201772 was significantly higher than that of control while that for the phenobarbital group was not. The mean terminal half-lives for the control, RPA 201772 and phenobarbital treated groups were 17.0, 13.3 and 16.2 hours, respectively. The systemic clearance of total radioactivity for both RPA 201772 and phenobarbital groups was higher than the control group (0.065, 0.049 and 0.038 ml/min, respectively).

Table 3
Effect on Hepatic Enzyme Activity in Rats
Treated with RPA 201772 for Fourteen Days[a]

Hepatic Enzymes	Dose Groups		
	Control	RPA 201772	Phenobarbital
Microsomal Protein Concentration:			
mg/g liver:	18.1	31.6** (175%)b	24.6**(136%)
mg/total liver	249	665 (267%)	488 (196%)
Cytochrome P-450:			
nmoles/mg protein:	0.9	1.8** (203%)	1.7** (187%)
nmoles/g liver:	16.1	57.9** (360%)	41.3** (257%)
7-Pentoxyresorufin O-depentylase:			
nmoles/min/mg protein:	0.3	4.2** (1413%)	4.2** (1400%)
nmoles/min/g liver:	5.1	136.9** (2684%)	104.3**(2045%)
p-Nitrophenol UDP-glucuronyltransferase:			
μmoles/hr/mg protein:	3.1	6.8**(223%)	6.7**(220%)
μmoles/hr/g liver	57.0	216.0 (379%)	169.0**(296%)

a Extracted from Tables 7, 8, 9 and 10 (pages 42, 43, 44 and 45) of the study no. RNP 478/952145; *p<0.05; **p<0.01

b Value in parenthesis represent percent of control.

Table 4
Effect on Whole-blood Radioactivity in Rats
Following single intravenous dose of ^{125}I-Thyroxine to Rats Pretreated with RPA 201772 or Phenobarbital for Fourteen Days[a]

Parameters Measured	Dose Groups		
	Control	RPA 201772	Phenobarbital
Dose (μg)	0.142	0.137	0.145
Kel/hours	0.0407	0.0520***	0.0428
t1½ (hours)[b]	17.0	13.3	16.2
AUC (ng equiv.h/ml)	62.5	36.3	49.8
CL (ml/min)	0.038	0.065***	0.049*

a Extracted from Table 11 (page 46) of the study no. RNP 478/952145; *p<0.05; **p<0.01
b Calculated as ln2/min K^{el}

Thyroid Weights and Radioactive Concentration: Following intravenous administration of ^{125}I-thyroxine, the thyroid weights of animals in RPA 201772 and phenobarbital treated groups were significantly higher (146 and 139% of control, respectively) than controls (Table 5). The thyroid iodine uptake (% dose/g) in RPA 201772 treated rats was slightly higher than control; the concentration of radioactivity (ng/g) in phenobarbital treated group, on the other hand, was lower than the control group (Table 5).

G. Sacrifice and Pathology:

There was significant increase in the relative liver weights in RPA 201772 (149% of control) and phenobarbital (143% of control) treated rats (Table 6). Although thyroid weights in these groups were also increased, the increase in RPA 201772 treated rats was not statistically significant when compared with that of control. Pituitary weights of rats in these groups were unaffected. Macroscopic examination conducted on 6 animals/group necropsied following 2 weeks treatment, revealed liver enlargement among all 6 animals each from the two treated groups when compared with control.

Table 5
The Amount of radioactivity in Thyroid and Thyroid Weight Changes in Rats Receiving Intravenous Administration of ^{125}I-Thyroxine After Pretreatment with RPA 201772 or phenobarbital for Fourteen Days[a]

Parameters Measured	Dose Groups		
	Control	RPA 201772	Phenobarbital
Radioactivity in Thyroid: % dose (% dose /g): ng/g[b] (Total ng)[b]:	4.1 (241.6) 348.7 (5.95)	4.6 (182.1) 256.6 (6.6)	5.8 (238.3) 344.6 (8.3)
Thyroid Weights (mg): (% of control)[c]	17.5 (–)	25.6** (146%)c	24.3** (139%)

a Extracted from Tables 12 and 13 (pages 47 and 48) of the study no. RNP 478/952145
b Expressed as ng thyroxine equivalents/g thyroid
c Calculated by the reviewer; *p<0.05; **p<0.01

Table 6
Body Weights and Organ Weight Changes in Rats Treated with RPA 201772 for Fourteen Days[a]

Dose Groups	Parameters Measured				
	Body Wt. (g)	Absolute Pituitary Wt. (mg)	Absolute Thyroid Wt. (mg)[b]	Absolute Liver Wt (g)	Relative Liver Wt.(g/100 g body wt.)
Control	274	10.4	18.7	13.9	5.07
RPA 201772 (% of control)	280 (102%)	10.1 (97%)c	20.1 (107%)c	21.1 (152%)c	7.55** (149%)
Phenobarbital (% of control)	273 (99%)	10.6 (102%)c	23.6* (126%)c	19.8 (143%)c	7.26** (143%)

a Extracted from Table 4 and 6 (pages 39 and 41) of the study no. RNP 478/952145
b Absolute thyroid weight; no relationship between organ weight and body weight was noted for thyroid.
c Calculated by the reviewer *p<0.05; **p<0.01

IV. DISCUSSION

A. Reviewer's interpretation of study results: This special study shows that dietary administration of RPA 201772 to male Sprague-Dawley rats for 2 weeks induced de novo synthesis of microsomal protein, increased Phase I (cytochrome P-450 dependent mixed-function oxidase system) and Phase II (uridine 5'-diphosphatase-glucuronyltransferase) drug-metabolizing enzyme activities, caused increased clearance of ^{125}I-Thyroxine as seen by decrease in T_4 level and shorter half-life, and increased liver and thyroid weights. The plasma T_3 level was not significantly affected (Refer to Table 7). These measured effects in RPA 201772 treated rats were greater than those of phenobarbital treated rats, a well-known inducer of hepatic drug-metabolizing enzymes in mammalian species. RPA 201772, therefore, acts as a phenobarbital-type inducer of rat hepatic drug metabolizing enzymes.

As the report stated, "the significant reduction of T_4 was possibly a result of the enhanced glucuronidation of this hormone by hepatic UDPGT. This mechanism is a major pathway for the elimination of T_4 from the body." The effects observed in this study suggest that RPA 201772 may induce thyroid tumors through a disruption in the thyroid-pituitary hormonal feedback mechanisms.

Table 7. Summary of Results

Parameters Measured	Dose Groups[a]		
	Control	RPA 201772	Phenobarbital
Absolute Liver Wt. in g (% of control) Relative Liver Wt. in g (% of control)	13.9 5.07	21.1 (152%) 7.5 (149%)	19.8 (143%) 7.3 (145%)
Microsomal Protein (mg/g liver) (% of control)	18.1	31.6**(175%)	24.6**(136%)
P-450 (nmol/g liver)	16.1	57.9**	41.3**
Absolute Thyroid Wt. in mg (% of control)	18.7	20.1 (107%)	23.6*(126%)
UDPGT (mmol/hr/g liver)	57	216*	169**
PROD (nmol/hr/g liver)	5.1	137**	104**
T4 (ng/dl)	5.7	3.2*	4.9*
T3 (μg/dl)	74	68	69
125I-Thyroxine Kel (per hr)	0.0407	0.0520***	0.0428
125I-Thyroxine t1/2 (hr)	17.0	13.3	16.2
125I-Thyroxine Clearance (ml/min)	0.038	0.065***	0.049*

a Dose: RPA 201772 = 500 mg/kg/day; Phenobarbital = 80 mg/kg/day;* $p<0.05$; ** $p<0.01$; *** $p<0.001$;

B. Study deficiencies: The following deficiencies were noted:

1. The analysis for homogeneity, stability and concentration of the test compound in the diet was conducted on sample diet rather than the test diet used in the study.

2. Histopathology of the liver and thyroid glands was not performed.

However, these deficiencies do affect the outcome of the study results.