EPA Reviewer: Timothy F. McMahon, Ph.D. _____, Date 3/18/98
Senior Scientist, RASSB/AD (7510W)
EPA Secondary Reviewer: Winston Dang, Ph.D. _____, Date 4/6/98
Team Leader, Team One, RASSB/AD

DATA EVALUATION RECORD

STUDY TYPE: Liver Biochemical Induction -hamster- non-guideline

DP BARCODE:D240164 SUBMISSION CODE:S532102
P.C. CODE:054901

TEST MATERIAL (PURITY): Irgasan DP300, 99.5% a.i.

SYNONYMS: Triclosan

CITATION: Thomas, Rer. Nat. H. (1994): The Effect of FAT 80'023/Q and the model inducers Phenobarbitone, 3-Methylcholanthrene, Pregnenolone 16∝-carbonitrile and Nafenopin on Selected Biochemical and Morphological Liver Parameters in the Syrian Hamster. Study conducted by Ciba-Geigy Limited. Study number CB 93/40. Submitted under MRID # 44389706. Unpublished.

SPONSOR: Ciba Geigy Corporation

EXECUTIVE SUMMARY:

In this special study, Irgasan DP300 technical (purity 99.5%) was administered to 4 groups of young adult male and female Syrian Hamsters (five/sex/group) in a pelleted standard hamster diet (Nafag 924) at concentrations of 0, 700, 5000, and 15000ppm [approximately 0, 49.9, 309.8, 799.0 mg/kg/day (males) and 46, 314.3, 958.8 mg/kg/day (females)] for 14 days. Separate recovery groups of five males and five females received either 0 or 15000 ppm Irgasan DP300 in the diet for 14 days followed by a 28 day recovery period.

Significant treatment-related effects were observed in male and female hamsters at the 5000 (309.8 mg/kg/day in males, 314.3 mg/kg/day in females) and 15000 ppm (799 mg/kg/day in males, 958.8 mg/kg/day in females) treatment levels. At 5000 ppm Irgasan, significant induction of total hepatic microsomal cytochrome P-450 and activities of ethoxyresorufin-o-deethylase (EROD) and pentoxyresorufin-o-depentylase (PROD) was observed, as was an increase in Mab clo4 immunoreactive protein in male hamster liver. At 15000 ppm, the above effects were also observed, and in addition, abnormal histopathology of the kidneys in females (randomly distributed spots or white patches of white pigmentation on the surface of the kidney) was observed after 14 days of treatment. Total activity towards testosterone was not affected by Irgasan feeding in the diet, but specific hydroxylation reactions were affected. Of note in males, formation of androstenedione was increased in a dose-related manner, as was the formation of the 16-β metabolite. A noticeable dose-response was observed only for androstenedione formation, however.

In female hamsters, a dose-related increase in formation of both the 7-∝ and 15-∝ hydroxy metabolites was noted as a result of Irgasan treatment (formation of the 7-∝ metabolite: activities of 22.16, 35.74, 39.33, and 46.66 nmol/min/g at the 0,700, 5000, and 15000 ppm dose levels, respectively; formation of the 15-∝ metabolite: 8.61, 12.78, 18.29, and 29.87 nmol/min/g at 0, 700, 5000, and 15000 ppm Irgasan, respectively). Androstenedione formation was also slightly increased with dose of Irgasan, with a doubling of activity at the high dose (42.72 nmol/min/g) in relation to control activity (21.14 nmol/min/g).
Significant treatment-related increases in lauric acid hydroxylation were observed in male and female hamsters at 15000 ppm Irgasan, as were significant decreases in activity of cytosolic glutathione-S- transferase and increases in bilirubin and morphine glucuronyltransferase activity. Total immunoreactive protein towards the Mab clo4 antibody (indicative of induction of CYP4A P-450, a peroxisome proliferator inducible form) was observed in male and female hamsters at 15000 ppm. Together with the data presented on the effects of the model inducers phenobarbital, 3-methylcholanthrene, nafenopin, and pregnenolone 16 ∝-carbonitrile, the data suggest that Irgasan acts as a peroxisome proliferator, as observed in other work with rats and mice. Hamsters, however, appear less sensitive to Irgasan treatment relative to rats and mice.

Based on the results of this study, a Systemic NOEL of 700 ppm can be established, with a Systemic LOEL of 5000 ppm, based on induction of total cytochrome P-450, EROD, and PROD in male and female hamsters, and induction of Mab clo4 immunoreactive protein (CYP4A peroxisome proliferator inducible P-450) in male hamsters).

This study is classified as **acceptable (non-guideline)**. The study provides important information on the mechanistic basis of Irgasan induced liver toxicity, and also provides information on the relative sensitivity of the hamster to the hepatic effects of Irgasan.

MATERIALS AND METHODS

Test Materials:

1) Irgasan DP300 (2,4,4' -trichloro-2'-hydroxy-diphenyl ether. Lot No. EN 275927.26. Purity: 99.5%. Expiration date: December 1999.

2) Phenobarbital Sodium. Lot No. 275971.1190. Purity: 98%. Expiration date: July 1996.

3) 3-Methylcholanthrene. Lot No. 102H2605. Purity 96.3%. Expiration date: May, 1998

4) Pregnenolone 16∝-carbonitrile. Lot No. 50H0576. Purity: >99%. Expiration date: not stated

5) Nafenopin. Batch No.CDF:2147. Purity: 99.8%. Expiration date: not stated

Irgasan and Nafenopin were supplied by the chemicals division of Ciba-Geigy. Phenobarbital was obtained from Fluka Chemie AG. 3-Methylcholanthrene and Pregnenolone 16∝-carbonitrile were obtained from Sigma Chemicals, St.Louis, MO.

Test Animals:

Species and Strain: Syrian Hamster, A.U.R.A. strain
Source: Zentralinstitut fur Versuchstierforschung, Hannover, Germany
Age and weight: 4-6 weeks, weight range stated as 79-125.1g
Environmental Conditions: Hamsters were housed individually in standard macrolon cages on softwood bedding with a temperature range of 22±3 Celsius an a 12 hour light/dark cycle. Relative humidity was stated as 55±15%.
Acclimation: approximately 2 weeks.

Study Design and Treatment:

The Syrian Hamster was, according to the report, selected for the purpose of providing "insight into the mechanistic properties and potential species-specific action of FAT 80'023 by characterizing the effects of the test article on biochemical and ultrastructural parameters in the liver after dietary administration...for 14 days."

Fifty male and 30 female hamsters were randomly assigned to 16 treatment groups as outlined in the following table. Four groups of 5 male and 5 female animals each received Irgasan DP300 ad libitum in pelleted standard hamster chow for 14 days. Targeted dose levels were 0, 50, 350, and 1000 mg/kg/day.

	Treatment Groups				Recovery Groups	
Concentration (ppm)	0	700	5000	15000	0/0	15000/0
MALES						
Group No.	1	2	3	4	5	6
Animal No.	1-5	6-10	11-15	16-20	21-25	26-30
FEMALES						
Group No.	7	8	9	10	11	12
Animal No.	31-35	36-40	41-45	46-50	51-55	56-60

data taken from page 48 of the report.

16
Animals given the reference compounds were treated in the following manner:

	Phenobarbitone 80 mg/kg/day	Nafenopin 250 mg/kg/day	3-methyl-cholanthrene 25 mg/kg/day	pregnenolone 16∝ carbonitrile 100 mg/kg/day
MALES				
Group No.	13	14	15	16
Animal No.	61-65	66-70	71-75	76-80

data taken from page 48 of the report

These satellite animals were given intraperitoneal injections of the above treatments at the

indicated dose levels for four consecutive days, except for the nafenopin group, which received this material by oral gavage once daily for 14 days.
Taking two samples

Dose Selection Rationale

Doses for this study were selected on the basis of results obtained from a 13-week oral toxicity study in hamsters, where 700ppm and 15000ppm were also used as the low and high dose levels.
Dietary Preparation and Analysis

The exact preparation procedure for mixing of the test article into the pelleted diet was not specified. It was stated that two samples of 500g from the control diet and 200g from all other

dietary mixtures were taken at the time of diet preparation and deep frozen for later analysis of concentration and homogeneity by Ciba-Geigy. Dietary mixtures used in this study were stored at room temperature. Analysis of concentration of Irgasan in the diet was also performed at the end of the study. All analyses were carried out using HPLC.
Results of testing of dietary mixtures was presented on page 50 of the report. Homogeneity analysis showed relative standard deviations of 1.98%, 1.29%, and 3.30% for the mean of three independent samples analyzed in duplicate at the 700, 5000, and 15000ppm dose levels, respectively. Actual test article concentration were observed to be 657, 4490, and 13317 ppm, corresponding to 94%, 90%, and 89% of nominal concentration. No significant decline in test article concentration in the diet was observed at the end of the study at any dose level.

Experimental Observations

Body Weight: Individual body weights were recorded daily during the 14 day dosing period, and twice per week during the recovery period.

Food Consumption: Mean daily food consumption was determined for each animal on a daily basis, and twice per week during the recovery phase. Mean daily target dose was also calculated using the following formula:

$$\text{Target dose} = \frac{\text{Test article concentration} \times \text{Food consumption}}{\text{Body weight}}$$

Sacrifice and Pathology

Animals were fasted overnight prior to sacrifice. Sacrifice was accomplished by carbon dioxide anesthesia. Immediately after sacrifice, livers from all animals were removed and weighed.

Electron microscopy was performed on small liver tissue blocks from three randomly selected animals of all treatment groups. Following the sampling for electron microscopy, livers were dissected, divided into two weighed aliquots and immediately frozen in liquid nitrogen until needed for biochemical measurements

Biochemical Measurements

Liver samples from all animals of all experimental groups were characterized biochemically for the effects of the various treatments. Differential centrifugation was performed on thawed liver samples homogenized in Tris/HCl buffer containing 250mM sucrose. The following measurements were made:

A) protein content of 100g supernatants and microsomal and cytosolic fractions

B) cyanide-insensitive peroxisomal fatty acid beta-oxidation in the 100g supernatant

C) microsomal hydroxylation of lauric acid

D) microsomal cytochrome P-450 content

E) microsomal 7-ethoxyresorufin O-de-ethylase and 7-pentoxyresorufin O-dependentylase

F) regio- and stereoselective microsomal testosterone hydroxylation

G) microsomal UDP-glucuronyltransferase activities

H) cytosolic glutathione S-transferase

Immunoblot Analysis

In order to evaluate the induction of specific cytochrome P-450 isozymes at the protein level, Western Blots were performed with microsomal fractions from treated and control hamster livers using monoclonal antibodies against and specific for rat liver isozymes of the gene subfamilies CYP1A, CYP2B, CYP3A, and CYP4A.

RESULTS

Clinical Observations: The report stated that there were no observed signs of toxicity in either male or female hamsters at any dose level of Irgasan tested, or in any of the animals receiving the model inducer compounds.

Body Weight: Group mean body weight and weight gain in male and female hamsters given Irgasan in the diet are summarized below:

MALES	Treatment Groups (ppm)			
	0ppm	657ppm	4490ppm	13317ppm
b.w. day 0	103.4±5.4	95.0±10.5	97.4±6.8	94.9±4.7
b.w. day 14	106.5±3.8	102.2±6.2	98.5±6.2	85.7±5.1
b.w. gain	3.1	7.2	1.1	-9.2
FEMALES				
b.w. day 0	100.4±4.9	97.0±4.1	88.9±3.4	88.6±5.6

b.w. day 14	109.0±2.3	100.4±4.6	92.9±5.4	80.4±8.2
b.w. gain	8.6	3.4	4.0	-8.2

Data taken from pages A41-A52 of the report.

As noted, effects of Irgasan treatment on body weight were observed at the high dose of test chemical, where losses in body weight were observed over the 14 day treatment period for both sexes. In those male animals receiving the model inducer compounds, treatment was only for four days except for nafenopin. Body weight loss was not observed in any of these satellite groups.

Food Consumption and Target Dose

Mean daily food consumption and test article intake in male hamsters given Irgasan in the diet are summarized in the following table:

Treatment Groups MALES	Mean Food Consumption (kg food/kg b.w.)	Mean Daily Dose (mg/kg/day)
0 ppm	0.069±0.004	0
657 ppm	0.076±0.011	49.9±6.9
4490 ppm	0.069±0.004	309.8±16.8
13317 ppm	0.060±0.008	799.0±100.9
Recovery Groups, Days 1-14		
0 ppm	0.071±0.004	0
13317 ppm	0.049±0.009**	652.5±123.3
Recovery Groups, Days 15-42		
0 ppm	0.065±0.007	0
13317 ppm	0.075±0.004	0

data from page 51 of the report.

As noted above, effects on food consumption were observed at the high dose of 15000 ppm (13317 ppm actual dose). At the end of the 14 day treatment period with Irgasan at this dose,

food consumption was significantly reduced (0.049 vs 0.071 in control as noted above). However, by the end of the recovery period, food consumption in the high dose group had returned to control levels. Actual daily target doses of Irgasan in the diet are also noted above.

For female hamsters, data were presented on page 52 of the report, and show similar food intake effects as in males, i.e. a significant decrease in intake at the high dose at the end of the 14 day treatment period, followed by a rebound to control levels by the end of the recovery period. Mean daily target dose in females was 0, 46.0, 314.3, and 958.8 mg/kg/day for the 0, 657, 4490, and 13317 ppm dose groups, respectively.

When taken together, the observed decreases in body weight and food consumption were approximately equivalent (~10% decrease), suggesting palatibility as the cause of the decreased body weight at the high dose.

Necropsy and Organ Weights

The report presented results of gross necropsy examinations and organ weights at sacrifice for treated animals (pages 53-55 of the report). The following observations were noted with respect to organ pathology:

1) At 15000 ppm, 5 of 5 male hamsters displayed randomly distributed spots or white patches of white pigmentation on the surface of the kidney. These discolorations of the kidney were considered treatment-related.

2) The urinary bladder in one male at the high dose was observed with a mucous white precipitate.

2) At 15000 ppm, all female hamsters were observed with similar kidney discolorations as male hamsters. However, in contrast to males, one female hamster at 5000 ppm was observed with this lesion, while one female hamster in the recovery group was observed with "light aereal connective tissue-like capsule thickenings,' attributed by the report as an "incomplete recovery from the treatment-related white pigmentation of the kidney.'

Absolute liver weight in male hamsters was decreased in a dose-related fashion (final weights of 3.15, 3.11, 3.06, and 2.85 grams for the 0, 700, 5000, and 15000 ppm dose groups respectively), but the high dose liver weight was decreased only by 10% from control. Relative liver weight at the high dose was increased by %, but was based on a decreased final body weight.

In female hamsters, decreased absolute weight of the liver was also observed at the 15000 ppm dose, but the magnitude of the decrease was larger than in males (2.41g vs 3.05g in control, decrease of %). Relative liver weight was again increased and again was based upon a decrease in final body weight.

Treatment with the model inducers phenobarbitone, 3-methylcholanthrene, and nafenopin resulted

in absolute increases in liver weight of 28%, 42%, and 72%, respectively. Treatment with pregnenolone 16∝ carbonitrile did not result in a liver weight increase.

Biochemical Measurements

a. Cytochrome P-450

The effects of treatment with Irgasan and model inducer compounds on microsomal cytochrome P-450 content is summarized as follows:

1) In male hamsters treated with Irgasan, microsomal cytochrome P-450 showed significant increases at the 5000 and 15000 ppm dose levels (48.5 and 59.6 nmol/g liver vs 36.6 nmol/g in controls).

2) In female hamsters, a similar result was obtained (P-450 content of 36.6 and 46.2 nmol/g at 5000 and 15000 ppm vs 26.9 nmol/g in control). Statistically significant increases in EROD and PROD activities were observed in both sexes at these dose levels. Summary of these effects is made in the following table:

Dose (ppm)	P-450 (nmol/g)	EROD (nmol/min/g)	PROD (nmol/min/g)
Main Treatment Groups (MALES)			
0	36.6±5.9	8.78±1.93	0.42±0.12
700	36.0±1.7	9.12±1.46	0.59±0.14
5000	48.5±2.5***	13.83±2.00	1.03±0.20***
15000	59.6±4.7***	22.63±4.66	1.80±0.34***
Treatment / Recovery Groups			
0/0 ppm	34.0±2.9	8.48±1.16	0.54±0.06
15000/0 ppm	33.4±3.6	7.84±1.33	0.48±0.05
Reference Compound Treatment Groups			
phenobarbitone	62.5±6.2***	17.91±5.77	1.70±0.47
Nafenopin	38.0±6.6	12.99±1.70	0.41±0.06
3-methylcholanthrene	71.8±4.4	85.36±11.32	1.25±0.26
Pregnenolone	28.9±1.1	8.71±1.65	0.51±0.09

data taken from page 58 of the report. *** $p < 0.001$ by Dunnett's test.

Dose (ppm)	P-450 (nmol/g)	EROD (nmol/min/g)	PROD (nmol/min/g)
Main Treatment Groups (FEMALES)			
0	26.9±4.6	6.86±1.21	0.82±0.20
700	27.4±1.9	8.09±0.66	0.93±0.08
5000	36.6±4.4*	11.35±2.10***	1.60±0.35***
15000	46.2±4.2***	20.51±2.24***	2.60±0.51***
Recovery Groups			
0/0	22.3±3.1	6.11±0.68	0.67±0.09
15000/0	24.6±1.2	5.20±1.18	0.69±0.17

As noted, cytochrome P-450 content was elevated in both sexes at the 5000 and 15000 ppm dose levels for Irgasan treated hamsters after 14 days of treatment. The recovery groups showed a return to control levels of cytochrome P-450 after 4 weeks of untreated diet.

No increase in microsomal cytochrome P-450 content was observed after nafenopin treatment of male hamsters. Treatment with pregnenolone 16∝-carbonitrile reduced cytochrome P-450 content to 79% of control in males, whereas phenobarbital and 3-methylcholanthrene caused the expected increase in P-450 content (171% and 196% of control, respectively).

The above data show the content of cytochrome P-450 on a per gram of liver basis. It is useful to evaluate the effects of Irgasan treatment on the specific activity of cytochrome P-450 (that is, *nmol/mg protein).*

Microsomal Testosterone Hydroxylation

According to the report, determination of regio- and stereoselective microsomal testosterone hydroxylation has been established as a diagnostic method to assess simultaneously treatment related alterations in the activity of several constitutively and/or inducibly expressed isoenzymes of the cytochrome P-450 system in rodents. The effects of Irgasan treatment as well as the model inducers on these activities are summarized in the following tables:

Metabolite identified	Effect of Irgasan on P-450 Dependent Testosterone Hydroxylation (nmol/g/min)

	0 ppm	700 ppm	5000 ppm	15000 ppm	0/0 ppm	15000/0
2-β-OH	106.62±38.7	109.87±20.5	123.53±34.5	76.71±17.52	108.65±11.4	110.09±22.6
6-β-OH	191.99±70.6	200.19±42.3	221.41±58.2	138.50±32.4	198.75±23.2	202.64±39.0
15-β-OH	57.37±19.5	58.91±13.5	69.99±18.3	48.25±12.1	62.15±8.15	62.54±12.43
7-α-OH	66.53±18.81	71.82±16.1	69.61±15.2	52.33±12.55	79.85±18.17	72.56±10.4
15α-OH	15.71±3.36	15.6±4.67	25.79±6.02	36.26±11.64	20.47±4.41	17.29±4.5
Androstene-dione	21.51±5.87	23.08±6.09	32.74±8.02	41.27±11.36	24.68±3.98	24.56±5.37
16-β-OH	15.65±4.38	16.55±4.46	19.86±3.78	21.52±5.56	17.20±1.80	15.97±3.75
Total Activity	475±159	496±104	563±134	415±99	512±57	506±94

data taken from Tables 13-15, pages 60-62 of the report.

As shown, the total testosterone hydroxylation activity was not affected by treatment with Irgasan, but specific hydroxylation reactions appeared increased in a dose-related fashion. For instance, formation of androstenedione was increased in a dose-related manner, as was the formation of the 16-β metabolite. A noticeable dose-response was observed only for androstenedione formation, however.

In female hamsters, formation of testosterone hydroxylated metabolites was also largely unaffected by treatment with Irgasan. It is to be noted that in contrast to male hamsters, female hamsters formed considerably less of the 7-∝ hydroxy metabolite (22.16 nmol/min/g in controls) than male hamsters. A dose-related increase in formation of both the 7-∝ and 15-∝ hydroxy metabolites in female hamsters was noted as a result of Irgasan treatment (formation of the 7-∝ metabolite: activities of 22.16, 35.74, 39.33, and 46.66 nmol/min/g at the 0,700, 5000, and 15000 ppm dose levels, respectively; formation of the 15-∝ metabolite: 8.61, 12.78, 18.29, and 29.87 nmol/min/g at 0, 700, 5000, and 15000 ppm Irgasan, respectively). Androstenedione formation was also slightly increased with dose of Irgasan, with a doubling of activity at the high dose (42.72 nmol/min/g) in relation to control activity (21.14 nmol/min/g).

Of the model inducer compounds, phenobarbital was observed to cause an increase in formation of androstenedione and 15-∝ hydroxytestosterone, similar to the response caused by Irgasan.

Microsomal Lauric Acid Hydroxylase

The report noted, as background, that lauric acid hydroxylation at position 12 in rat liver is catalyzed almost exclusively by isoenzyme members of the peroxisome proliferator inducible cytochrome P-450 CYP4A gene family, while hydroxylation at position 11 is mediated by a number of constitutive and/or 3-methylcholanthrene or phenobarbital inducible isoenzymes of the CYP1A, CYP2B, and CYP2C gene families.

With this in mind, results of Irgasan treatment show the following:

Dose (ppm)	LA-11-OH	LA-12-OH
Main Treatment Groups		
0 ppm	45.1±8.0	52.7±16.3
700 ppm	45.8±5.7	58.0±7.9
5000 ppm	51.8±2.0	74.7±8.7
15000 ppm	71.4±12.8***	126.7±27.4***
Treatment / Recovery Groups		
0/0	39.3±5.2	51.8±9.1
15000/0	41.2±3.4	50.8±6.8
Reference Compound Treatments		
PB, 80 mg/kg/day	69.2±12.5***	50.9±15.2
Nafenopin, 250 mg/kg/day	92.1±18.2***	359.0±53.2***
3-MC, 25 mg/kg/day	31.6±2.5	40.1±6.4
Pregnenolone 16-∝ carbonitrile, 100 mg/kg/day	42.9±4.8	71.5±18.2

Data from page 66 of the report.

As shown, 11-hydroxylation of lauric acid in males was increased to 158% of control at the high dose of Irgasan, while 12-hydroxylation was increased to 142% and 240% of control at the 5000 and 15000 ppm dose levels, respectively. Female hamsters also showed an increase in both hydroxylation activities over control, but the response was at the high dose and was not as strong as that observed in males, despite the existence of similar control enzyme activities for both forms of hydroxylation in male and female hamsters.

The reference compound treatments resulted in the strongest response observed from nafenopin treatment, which resulted in a 2-fold and 6.8-fold increase in 11- and 12-hydroxylation of lauric acid. Nafenopin, it is noted, is a prototype peroxisome proliferator type compound.

Cyanide-insensitive peroxisomal fatty-acid beta-Oxidation

As noted in the report, measurement of cyanide-insensitive peroxisomal fatty acid beta-oxidation is a parameter for assessing peroxisome proliferation in rodents.

Irgasan treatment resulted in no significant changes in this enzyme activity in either male or female hamsters, whereas nafenopin treatment in males caused an increase in this enzyme activity of approximately 2.5-fold.

Cytosolic Glutathione-S-Transferase

Measurement of cytosolic glutathione-S-transferase (GST) was made based on the known depression of this enzyme activity by peroxisome proliferating agents.

Results of measurement of this enzyme activity (Tables 21 and 22, pages 68 and 69 of the report) showed depression of activity at the 15000 ppm Irgasan dose. In males, activity was decreased from a control value of 640±67 μmol/min/g to 522±30 μmol/min/g at 15000 ppm, while in females, activity was decreased from a control value of 639±49 μmol/min/g to 537±29 μmol/min/g. Both decreases were identified as statistically significant ($p < 0.05$ vs control).

Recovery groups of males did not show a decrease in glutathione-S-transferase after recovery, thus indicating the reversible nature of the effect of Irgasan treatment.

Of the model inducer test chemicals used, phenobarbital caused reduction in GST activity to 83% of control, while nafenopin and pregnenolone 16-∝ carbonitrile reduced enzyme activity to 64% and 77% of control, respectively.

Microsomal UDP-glucuronosyltrasnferases (UDPGT)

Previous investigations of UDPGT activities in rats, rabbits, and humans have established that there appear to be at least 2 gene families, one which is associated with induction by 3-MC (UDPGT1), and one associated with induction by phenobarbital (UDPGT2). Substrate specificity of UDPGT1 towards 1-naphthol and morphine has been previously demonstrated. In addition, a peroxisome-proliferator inducible UDPGT isozyme of the UDPGT1 gene family with substrate specificity towards bilirubin has been identified in the rat. Based on this information, the effects of Irgasan treatment on UDPGT activities towards morphine, 1-naphthol, and bilirubin were investigated.

Results of these experiments were presented in Tables 23 and 24 of the report, pages 70 and 71. Treatment with Irgasan produced effects on UDPGT at the 15000 ppm dose, specifically:

MALES

1) increased UDPGT bilirubin activity, from 43±29 nmol/min/g in control to 89±30 nmol/min/g at 15000 ppm Irgasan

2) increased UDPGT morphine activity, from 1564±386 nmol/min/g in control to 2602±562 nmol/min/g at 15000 ppm Irgasan

3) In the 15000 / 0 ppm treatment / recovery group, UDPGT morphine was significantly decreased vs control after the recovery period (1135±75 nmol/min/g, $p < 0.05$)

FEMALES

1) increased UDPGT morphine activity, from 1355±228 nmol/min/g to 2014±466 nmol/min/g at 15000 ppm Irgasan.

2) increased UDPGT 1-naphthol activity, from 947±150 nmol/min/g to 1478±232 nmol/min/g at 15000 ppm Irgasan.

All changes noted above were significant at the 0.05 level of probability.

Immunoblot Analysis

In order to evaluate the induction of individual cytochrome P-450 isozymes, Western blot analyses were performed with microsomal fractions from treated and control hamsters using monoclonal antibodies generated against specific rat liver isozymes of the gene subfamilies CYP1A, CYP2B, CYP3A, and CYP4A. For this analysis, mixtures of equal volumes from microsomal suspensions from all animals per treatment group were subjected to SDS-PAGE, and monoclonal antibodies (purified IgG fractions) were used for immunoblot analysis. Mab d15 was used for CYP1A1 and CYP1A2, Mab be4 used for CYP2B1 and CYP2B2, Mab p6 used for CYP3A, and Mab clo4 used for CYP4A.

The results of immunoblot analysis are presented below:

Immunochemical quantitation of P-450 subfamilies in Irgasan Treated Male Hamsters (% of control)

Dose (ppm)	CYP1A Related Protein	CYP3A Related Protein	CYP4A Related Proteins (relative absorption units)		
			HMW	LMW	Total
Main Treatment Groups					
0	100	100	100	100	100

700	133	99	65	64	65
5000	126	106	143	200	160
15000	88	88	229	278	244
Treatment / Recovery Groups					
0 /0	100	100	100	100	100
15000 / 0	117	101	58	52	57
Reference Compound Treatment					
PB, 80 mg/kg/ day	55	60	n.d.	N.d.	85
nafenopin, 250 mg/kg/day	24	44	n.d.	N.d.	267
3-MC, 25 mg/kg/day	1550	34	n.d.	N.d.	49
pregnenolone 16∝-carbonitrile, 100 mg/kg/day	93	124	n.d.	N.d.	148

data taken from Table 25, page 72 of the report. n.d., HMW and LMW forms not clearly distinguishable. LMW, low molecular weight protein; HMW, high molecular weight protein. Two signals were detected with the antibody against CYP4A. For the main dose groups, results are based on a single blot, while for reference compounds, results are means from two independent blotting experiments.

Immunochemical quantitation of P-450 subfamilies in Irgasan Treated Female Hamsters (% of control)

Dose (ppm)	CYP1A Related Proteins (relative absorption units)			CYP3A related protein	CYP4A Related Proteins (relative absorption units)		
	HMW	LMW	Total		HMW	LMW	Total
Main Treatment Groups							
0	100	100	100	100	100	100	100

700	123	175	130	62	87	89	87
5000	130	186	137	40	72	93	77
15000	118	174	126	15	109	160	121
Treatment / Recovery Groups							
0 /0	100	100	100	100	100	100	100
15000 / 0	106	119	108	151	94	73	91

data taken from Table 26, page 73 of the report. n.d., HMW and LMW forms not clearly distinguishable. LMW, low molecular weight protein; HMW, high molecular weight protein. (Two signals were detected with the antibodies against CYP1A and CYP4A). For the main dose groups, results are based on a single blot, while for reference compounds, results are means from two independent blotting experiments.

For clarification, **Mab be4** is specific for the major phenobarbital inducible rat liver cytochromes CYP2B1 and CYP2B2. **Mab p6** is diagnostic for steroid-inducible rat liver cytochromes CYP3A1 and CYP3A2. **Mab clo4** is diagnostic for the peroxisome proliferator-inducible rat liver isoenzymes of the CYP4A gene family.

Use of the Mab be4 antibody showed no detectable signal in any of the treated groups of hamsters (data not shown in the report). In male hamsters treated with Irgasan, there were no detectable changes in microsomal content of the CYP3A gene family. In female Irgasan treated hamsters, a dose-related decrease in the content of the CYP3A cytochrome was observed, with only 15% of control value observed at the 15000 ppm dose.

In male Irgasan treated hamsters, total Mab clo4 immunoreactive protein was increased 1.6- and 2.4 fold at the 5000 and 15000 ppm dose levels, respectively. Induction of the low molecular weight fraction appeared dominant over the high molecular weight fraction. In female Irgasan treated hamsters, total Mab clo4 immunoreactive protein was reduced to 87% and 77% of control at the 700 and 5000 ppm dose levels, while an increase to 121% of control was observed at 15000 ppm Irgasan. Again, as in males, induction of the low molecular weight component appeared to predominate over the high molecular weight component.

Treatment with model inducers in males showed that PB and 3-MC reduced expression of total Mab clo4 immunoreactive protein to 85% and 49% of control, respectively. An increase of 1.5- and 2.7-fold was observed after treatment with pregnenolone 16-∝ carbonitrile and nafenopin, respectively.

In the recovery groups, females showed return to normal of cytochrome P-450 isozymes. Males appeared to show a "rebound" of CYP1A and CYP3A isozymes after recovery to percentages

above control, while percentages of CYP4A were decreased to approximately 50% of control after recovery.

Electron Microscopy Examinations

According to data provided in the report, treatment of male and female hamsters with Irgasan did not elucidate any changes in the distribution, frequency, and morphology of hepatocyte organelles, except for a marginal reduction in the size of peroxisomes. By contrast, phenobarbital administration caused a marginal proliferation of smooth and rough endoplasmic reticulum membranes, with some mitochondria containing dilated inner mitochondrial cristae. Nafenopin treatment resulted in a striking increase in peroxisome number, with a marginal proliferation of rough endoplasmic reticulum membranes also observed. 3-methylcholanthene resulted in a marked proliferation of smooth endoplasmic reticulum with small myelin figures. Some mitochondria were observed to contain dilated inner mitochondrial cristae. Treatment with pregnenolone 16∝-carbonitrile resulted in a moderate increase in rough endoplasmic reticulum membranes and a moderate reduction in size of peroxisomes.

Discussion

In this study, Irgasan DP300 technical (purity 99.5%) was administered to 4 groups of young adult male and female Syrian Hamsters (five/sex/group) in a pelleted standard hamster diet (Nafag 924) at concentrations of 0, 700, 5000, and 15000ppm [approximately 0, 49.9, 309.8, 799.0 mg/kg/day (males) and 46, 314.3, 958.8 mg/kg/day (females)] for 14 days. Separate recovery groups of five males and five females received either 0 or 15000 ppm Irgasan DP300 in the diet for 14 days followed by a 28 day recovery period. Additional groups of male hamsters (5/group) received standard reference inducing compounds (phenobarbital, 80 mg/kg/day i.p. for 4 days; 3-methylcholanthrene, 25 mg/kg/day i.p. for 4 days, pregnenolone 16∝-carbonitrile, 100 mg/kg/day i.p. for 4 days, and nafenopin, 250 mg/kg/day in the diet for 14 days) for comparison of the endpoints measured from Irgasan administration to these reference compounds. Endpoints measured included cytochrome P-450 content, and activities of several hepatic enzymes related to peroxisome proliferation. Immunoblotting was also performed to characterize possible induction of specific isozymes of P-450 related to peroxisome proliferation.

The Syrian Hamster was, according to the report, selected for the purpose of providing "insight into the mechanistic properties and potential species-specific action of FAT 80'023 by characterizing the effects of the test article on biochemical and ultrastructural parameters in the liver after dietary administration...for 14 days." It is noted that similar studies have been conducted in both CD-1 mice (MRID #) and Sprague-Dawley rats (MRID #). Effects on body weight were observed in the present study at the high dose of 15000 ppm nominal in both sexes, where weight loss was observed over the 14 day treatment period. This weight loss appears to be the result of decreased food consumption at the high dose, as the percentage decreases in both body weight and food consumption are approximately equal (~10% from control).

Absolute and relative liver weight were decreased in both male and female hamsters at the 15000 ppm targeted dose level. Examination of organs for abnormal histopathology showed randomly distributed spots or white patches of white pigmentation on the surface of the kidney in 5 of 5 high dose males. These discolorations of the kidney were considered treatment-related. In females at the high dose, all were observed with similar kidney discolorations as male hamsters. However, in contrast to males, one female hamster at 5000 ppm was observed with this lesion, while one female hamster in the recovery group was observed with "light aereal connective tissue-like capsule thickenings,' attributed by the report as an "incomplete recovery from the treatment-related white pigmentation of the kidney.' Treatment with the model inducers phenobarbitone, 3-methylcholanthrene, and nafenopin resulted in absolute increases in liver weight of 28%, 42%, and 72%, respectively. Treatment with pregnenolone 16∝ carbonitrile did not result in a liver weight increase.

Measurement of total hepatic cytochrome P-450 content and activities of EROD and PROD showed inductive effects of Irgasan at the 5000 and 15000 ppm nominal dose levels. Both sexes demonstrated significant increases in these parameters. The recovery groups showed a return to control levels of cytochrome P-450 levels and EROD and PROD activity after 4 weeks of untreated diet.

No increase in microsomal cytochrome P-450 content was observed after nafenopin treatment of male hamsters. Treatment with pregnenolone 16∝-carbonitrile reduced cytochrome P-450 content to 79% of control in males, whereas phenobarbital and 3-methylcholanthrene caused the expected increase in P-450 content (171% and 196% of control, respectively).

With respect to microsomal testosterone hydroxylation, total activity towards testosterone was not affected by Irgasan feeding in the diet, but specific hydroxylation reactions were affected. Of note in males, formation of androstenedione was increased in a dose-related manner, as was the formation of the 16-β metabolite. A noticeable dose-response was observed only for androstenedione formation, however.

In female hamsters, a dose-related increase in formation of both the 7-∝ and 15-∝ hydroxy metabolites was noted as a result of Irgasan treatment (formation of the 7-∝ metabolite: activities of 22.16, 35.74, 39.33, and 46.66 nmol/min/g at the 0,700, 5000, and 15000 ppm dose levels, respectively; formation of the 15-∝ metabolite: 8.61, 12.78, 18.29, and 29.87 nmol/min/g at 0, 700, 5000, and 15000 ppm Irgasan, respectively). Androstenedione formation was also slightly increased with dose of Irgasan, with a doubling of activity at the high dose (42.72 nmol/min/g) in relation to control activity (21.14 nmol/min/g).

Hydroxylation of lauric acid was investigated in this study. Lauric acid hydroxylation at position 12 in rat liver is catalyzed almost exclusively by isoenzyme members of the peroxisome proliferator inducible cytochrome P-450 CYP4A gene family, while hydroxylation at position 11 is mediated by a number of constitutive and/or 3-methylcholanthrene or phenobarbital inducible isoenzymes of the CYP1A, CYP2B, and CYP2C gene families. Irgasan treatment resulted in significant increases in both 11- and 12- position hydroxylation in both male and female hamsters at the 15000 ppm dose level. The increase in hydroxylation at the 12 position by Irgasan is

supportive of its role as a peroxisome proliferating chemical.

Similar effects have been observed in rats and mice, but a lower dietary concentrations. Mice appear to be most sensitive to the biochemical effects of Irgasan, as significant changes in enzyme activities have been observed at doses as low as 18.4 mg/kg/day

Cyanide insensitive fatty acid hydroxylation was not significantly affected by Irgasan treatment in this study. Mice have been observed to have significant increases in this enzyme activity at doses as low as 53 mg/kg/day, whereas rats have been observed to be relatively insensitive to this induction at doses up to 6000 ppm.

Phase II enzymes measured in this study included cytosolic glutathione-S-transferase and microsomal UDPGT. Treatment with Irgasan produced significant reductions in GST activity and significant increases in UDPGT activity at the 15000 ppm dose level. Of the model inducer test chemicals used, phenobarbital caused reduction in GST activity to 83% of control, while nafenopin and pregnenolone 16-∝ carbonitrile reduced enzyme activity to 64% and 77% of control, respectively.

Immunoblot analyses of liver samples from treated hamsters showed that in male hamsters, treatment with Irgasan resulted in no detectable changes in microsomal content of the CYP3A gene family. In female Irgasan treated hamsters, a dose-related decrease in the content of the CYP3A cytochrome was observed, with only 15% of control value observed at the 15000 ppm dose.

In male Irgasan treated hamsters, total Mab clo4 immunoreactive protein was increased 1.6- and 2.4 fold at the 5000 and 15000 ppm dose levels, respectively. Induction of the low molecular weight fraction appeared dominant over the high molecular weight fraction. In female Irgasan treated hamsters, total Mab clo4 immunoreactive protein was reduced to 87% and 77% of control at the 700 and 5000 ppm dose levels, while an increase to 121% of control was observed at 15000 ppm Irgasan. Again, as in males, induction of the low molecular weight component appeared to predominate over the high molecular weight component.

Treatment with model inducers in males showed that PB and 3-MC reduced expression of total Mab clo4 immunoreactive protein to 85% and 49% of control, respectively. An increase of 1.5- and 2.7-fold was observed after treatment with pregnenolone 16-∝ carbonitrile and nafenopin, respectively.

The results of these experiments show Irgasan to be a phenobarbital-type inducing agent in the hamster liver, with effects also consistent with peroxisome proliferating agents (comparison of effects from nafenopin and phenobarbital treatment support this conclusion). In comparison to other species, the hamster appears relatively insensitive to the inducing effects of Irgasan, with the mouse being the most sensitive. It should also be mentioned that recent research into the relevance of peroxisome proliferating agents for human carcinogenesis has proposed that agents which produce such effects in animal species (i.e. rats in particular, which are widely used as

research animals) may not be relevant for human carcinogenesis. This is because the cause of peroxisome proliferation may be receptor-based, and the human has a much lower affinity for peroxisome proliferating agents than the rat. Further research into the species sensitivity differences observed with the rat, mouse, and hamster in submitted studies on Irgasan would add significant information to this hypothesis.
Based on the results of this study, a Systemic NOEL of 700 ppm can be established, with a Systemic LOEL of 5000 ppm, based on induction of total cytochrome P-450, EROD, and PROD in male and female hamsters, and induction of Mab clo4 immunoreactive protein (CYP4A peroxisome proliferator inducible P-450) in male hamsters).

This study is classified as **acceptable (non-guideline)**. The study provides important information on the mechanistic basis of Irgasan induced liver toxicity, and also provides information on the relative sensitivity of the hamster to the hepatic effects of Irgasan.

In the recovery groups, females showed return to normal of cytochrome P-450 isozymes. Males appeared to show a "rebound" of CYP1A and CYP3A isozymes after recovery to percentages above control, while percentages of CYP4A were decreased to approximately 50% of control after recovery.