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UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

WASHINGTON, D.C. 20460

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1997

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OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Chlorothalonil: Review of cell proliferation studies conducted by the registrant.

EPA Identification Numbers:

MRIDs: 44223001; 44223002; 44240901; 44247501

DP Barcode: D235134 P.C. Code: 081901
Submission: S521281

TO: Walter Waldrop / Andrew Ertman
Product Manager # 71
Special Review and Reregistration Division (7508W)

and

Mary Clock
Risk Characterization and Analysis Branch (7509C)

FROM: Timothy F. McMahon, Ph.D. 5/24/97
Pharmacologist, Review Section I
Toxicology Branch II, Health Effects Division (7509C)

THRU: Jess C. Rowland, M.S. J. C. Rowland for 6/2/97
Acting Head, Review Section I
Health Effects Division (7509C)

and

Yiannakis M. Ioannou, Ph.D. Y. M. Ioannou 6/2/97
Acting Chief, Toxicology Branch II
Health Effects Division (7509C)

Registrant: ISK Biosciences

Action Requested: Review of cell proliferation studies in support of the re-classification of the carcinogencity of chlorothalonil.

Background / Data Summary: In previous meetings with the registrant and as a result of the recent re-evaluation of the carcinogenicity of chlorothalonil (cancer peer review document dated February 28, 1996), it was determined that, based on the available evidence, the mechanism of carcingenicity of chlorothalonil was likely non-genotoxic. However, it was also recognized that several mechanisms might be possible in the production of renal and forestomach tumors observed in long-term studies with chlorothalonil. Thus, the registrant submitted studies on cell proliferation associated with repeated oral dosing of chlorothalonil in rats. Executive summaries are shown below.

1) Citation: Mizens, M. (1996): A 90-Day Pilot Study for the Evaluation of Cell Proliferation in the Kidneys of Male Rats Following the Oral Administration of Technical Chlorothalonil. Study performed by Ricerca, Inc. MRID # 44223002. Unpublished.

Executive Summary: In a cell proliferation study, twenty-eight male Fischer 344 rats received technical chlorothalonil (97.9% a.i.) in the diet at 175 mg/kg/day for up to 91 days. Scheduled sacrifices occurred on days 7 (14 rats), 28 (7 rats), and 91 (7 rats) for the purpose of assessing the effect of chlorothalonil administration on cell proliferation in the kidney. Rats were implanted with Alzet minipumps containing bromodeoxyuridine 3.5 and 6.5 days prior to sacrifice (day 7), or 3.5 days prior to sacrifice (days 28 and 91). Mean labeling index was statistically increased in the kidneys of male rats treated with 175 mg/kg/day chlorothalonil at all scheduled sacrifice times. From day 7 to day 28, the fold increase in labeling index was relatively stable (approximately 10-fold over control), with a decrease to approximately 3.5-fold over control on day 91. Increased cell proliferation correlated with histopathological lesions of degeneration of the proximal convoluted tubules and epithelial hyperplasia. The results of this study demonstrate a sustained cell proliferative response as a result of dietary administration of technical chlorothalonil at a dose of 175 mg/kg/day. The apparent lack of cytotoxicity compared to the hypertrophic response in this study is not readily explained by the available data

This study is classified as **acceptable** (non-guideline). The study does not satisfy a particular guideline requirement, but demonstrates a cell proliferative effect of chlorothalonil on the kidney at a dose which also produces kidney tumors.

2) Citation: Hironaka, M. (1996): Analysis of Hyperplastic Changes in the Stomach and Kidney of Male Rats After 28-Day Induction by Chlorothalonil Technical. Study performed by the Center for Safety Assessment of Food, Agricultural Chemicals and Medical Drugs, Sumitomo. MRID # 44240901. Unpublished.

Executive Summary: In this study, 96 male SPF rats were divided into test groups of 6 animals per group. Rats received technical chlorothalonil (98.98% a.i.) in the diet at dose levels of 0, 1.5, 15, or 175 mg/kg/day for either 7, 14, 21, or 28 days (total of 24 rats per time point). Histological examination of kidney and stomach tissue was performed for each group after the appropriate exposure. In addition, kidneys were subjected to PCNA staining and stomach to BrdU staining, and the labeling index and labeling count of cell nuclei performed. Duodenum was used as a negative control for PCNA and BrdU staining. Increased absolute and relative weight of the kidneys was observed at 175 mg/kg/day at all time points, and in one animal at 15 mg/kg/day on Day 28. Increased incidence of vacuolization of the epithelium of the proximal convoluted tubules was observed at all time points at 175 mg/kg/day and on Days 7, 14, and 21 at 15 mg/kg/day. PCNA immunostaining of the proximal convoluted tubule epithelial cells showed increased labeling of cells at the 175 mg/kg/day dose level at all time points, and increased labeling at 15 mg/kg/day on Days 7, 14, and 21. BrdU labeling of the rat forestomach showed marked labeling at 175 mg/kg/day at all time points, and increased labeling on Day 28 at 15 mg/kg/day. The results of this study demonstrate a toxic response of the kidney and forestomach to repeated dietary administration of chlorothalonil at doses of 15 and 175 mg/kg/day.

This study is classified as **acceptable** (non-guideline). This study does not satisfy a specific guideline requirement, but provides scientific data demonstrating a toxic response of the kidney and forestomach at repeated dietary administration of 15 and 175 mg/kg/day technical chlorothalonil.

The remaining two submissions, MRID#'s 44223001 and 44247501, represent summary data for the two cell proliferation studies reviewed above and a published article in which the relationship between biotransformation and toxicity of chlorothalonil was examined. These data do not require review.

Reviewed by: Timothy F. McMahon, Ph.D Date: 5/22/97
Section I, Toxicology Branch II (7509C)
Secondary Reviewer: Nancy McCarroll Date: 5/22/97
Section II, Toxicology Branch II (7509C)

Data Evaluation Record

Study type: Cell Proliferation - rats
Guideline: non-guideline

EPA ID Numbers: MRID number: 44240901 DP Barcode: D235134
P.C. Code: 081901 Submission: S521281

Test material: Chlorothalonil, 98.98% a.i.

Study number(s): not stated; Report No. 3561

Sponsor: ISK Biosciences Corporation

Citation: Hironaka, M. (1996): Analysis of Hyperplastic Changes in the Stomach and Kidney of Male Rats After 28-Day Induction by Chlorothalonil Technical. Study performed by the Center for Safety Assessment of Food, Agricultural Chemicals and Medical Drugs, Sumitomo. MRID # 44240901. Unpublished.

Executive Summary:

In this study, 96 male SPF rats were divided into test groups of 6 animals per group. Rats received technical chlorothalonil (98.98% a.i.) in the diet at dose levels of 0, 1.5, 15, or 175 mg/kg/day for either 7, 14, 21, or 28 days (total of 24 rats per time point). Histological examination of kidney and stomach tissue was performed for each group after the appropriate exposure. In addition, kidneys were subjected to PCNA staining and stomach to BrdU staining, and the labeling index and labeling count of cell nuclei performed. Duodenum was used as a negative control for PCNA and BrdU staining. Increased absolute and relative weight of the kidneys was observed at 175 mg/kg/day at all time points, and in one animal at 15 mg/kg/day on Day 28. Increased incidence of vacuolization of the epithelium of the proximal convoluted tubules was observed at all time points at 175 mg/kg/day and on Days 7, 14, and 21 at 15 mg/kg/day. PCNA immunostaining of the proximal convoluted tubule epithelial cells showed increased labeling of cells at the 175 mg/kg/day dose level at all time points, and increased labeling at 15 mg/kg/day on Days 7, 14, and 21. BrdU labeling of the rat forestomach showed marked labeling at 175 mg/kg/day at all time points, and increased labeling on Day 28 at 15 mg/kg/day. The results of this study

demonstrate a toxic response of the kidney and forestomach to repeated dietary administration of chlorothalonil at doses of 15 and 175 mg/kg/day.

This study is classified as **acceptable** (non-guideline). This study does not satisfy a specific guideline requirement, but provides scientific data demonstrating a toxic response in the kidney and forestomach after repeated dietary administration of 15 and 175 mg/kg/day technical chlorothalonil.

Compliance

Signed and dated statements of GLP, Quality Assurance, and Data Confidentiality were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Technical chlorothalonil
Purity: 98.98% batch no: GC30M2 (I-680)
Description: powder, gray-white
CAS # 1897-45-6
Storage: In the dark at room temperature.
Stability: Test material was stated to be stable in a cool, dark place.

2. Vehicle: dietary preparation.

3. Test Animals: Male Fischer 344 rats. Source: Nippon Charles River, K.K. Age: 4 weeks old at receipt and 5-6 weeks old at study initiation. Rats were housed in a barrier system rearing room in stainless steel mesh rearing cage with aluminum front, sides, and floor. Food (radiation sterilized modified NIH open formula rat and mouse food; Oriental Kobo Kogyo K.K.) and tap water were available *ad libitum*. Temperature and humidity were maintained at 21-25 °C, and 45-65%, respectively with at least 20 air changes per hour and a 12 hour light/dark cycle. The dose groups are summarized as follows :

B. STUDY DESIGN

1. Animal Assignment:

Test Group	1	2	3	4
Dose (mg/kg/day)	0	1.5	15	175
No. male rats	24	24	24	24
Duration of treatment and animal numbers				
7 Days Dosing	1001-1006	1101-1106	1201-1206	1301-1306
14 Days Dosing	1007-1012	1107-1112	1207-1212	1307-1312
21 Days Dosing	1013-1018	1113-1118	1213-1218	1313-1318
28 Days Dosing	1019-1024	1119-1124	1219-1224	1319-1324

2. Dietary Mixtures:

Test substance was mixed with feed for 20 minutes using a ribbon mixer. Dietary mixes were prepared once weekly and stored refirigerated. Food left more than one week after preparation was incinerated. Dietary mixtures were given to the animals and changed once daily. When stored refrigerated, the dietary mixtures were stable for 1 week, according to the report.
Homogeneity and stability data for dietary mixtures of chlorothalonil were presented on pages 108-109 of the report. Data indicated homogenous mixing of the test chemical with the diet, and Dietary mixtures in this study were not adjusted for purity. From page 19 of the report: "Appropriate amounts of test material were mixed fresh weekly with the feed. A premix was prepared using a mortar and pestle. The test material and rodent feed were ground together until the test material was uniformly dispersed in the mixture (10 minutes). The final mix was prepared by mixing the premix with the balance of the feed in a Patterson-Kelly 1 cubic foot blender for ten minutes. Seven-kilogram batches were prepared weeks 1 and 2. For all subsequent weeks, 4-kg batches were prepared. Concentrations of the test material were adjusted weekly to achieve desired dietary intake of test material. The adjustment was based on the most recent body weight and food consumption data.
Homogeneity and stability analyses were performed on batches of food used for dose groups 2 and 4. Homogeneity analyses were performed on 3 food samples collected from the ribbon mixer for test diets in dose groups 2 and 4. Results of homogeneity analyses are shown below:

Table 1
Homogeneity of Chlorothalonil in Rodent Feed[a]

Homogeneity Analysis at 13 ppm		Homogeneity Analysis at 1560 ppm	
Sample ID	ppm in feed	Sample ID	ppm in feed
top	14.0, 14.3	top	1400, 1560
middle	13.3, 13.6	middle	1480, 1360
bottom	13.0, 13.0	bottom	1480, 1440
Mean	13.5±0.5	**Mean**	1450±70.0

[a]Data from page 108 of the report.

A variety of stability scenarios were explored in this study: Storage at 4 days room temperature; storage for 4 days in a regfirgerator followed by 3 days at room temperature; storage at room temperature for 1,2,3, and 4 days at room temperature; and storage for 4 and 7 days in a refrigerator.

Stability data are summarized below:

Table 2a

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Stability of Chlorothalonil in Rodent Feed

	Mean observed conc. under the following conditions[a]:		
Nominal Conc. (ppm)	Day 0, rm. temp.	Storage at rm. temp. 4 days	Refrig. 4 days, rm. temp. 3 days
13.0	13.3 (100%)	9.75 (73.3%)[b]	10.6 (79.7%)[b]
1560	1460 (100%)	1230 (84.2%)	1150 (78.8%)

[a]Data from page 109 of the report.
[b]Percent Day 0 concentration

Table 2b

	Mean observed conc. under the following conditions[a]:				
Nominal Conc. (ppm)	Day 0, rm. temp.	Day 1, rm. temp.	Day 2, rm. temp.	Day 3, rm. temp.	Day 4, rm. temp.
13.0	13.3 (100%)	12.4 (95.4%)[b]	10.2 (78.5%)[b]	10.4 (80.0%)[b]	10.6 (81.5%)[b]
1560	1480 (100%)	1300 (87.8%)	1110 (75.0%)	1010 (68.2%)	1130 (76.4%)

[a]Data from page 110-111 of the report.
[b]Percent Day 0 concentration

Table 2c

	Mean observed conc. under the following conditions[a]:		
Nominal Conc. (ppm)	Day 0, refrigerated	Day 4, refrigerated	Day 7, refrigerated
13.0	13.0 (100%)	12.7 (97.7%)[b]	11.2 (86.2%)[b]
1560	1480 (100%)	1340 (90.5%)	1330 (89.9%)

[a]Data from page 112 of the report.
[b]Percent Day 0 concentration

The above data on homogeneity and stability show that dietary mixtures of

chlorothalonil were homogenously mixed, and were most stable when kept in a refrigerator, which was the procedure adopted by the study.

3. Statistical Analysis

Body weight, organ weight, and food consumption data were statistically analyzed to compare the treatment group means to the control group mean by use of Dunnett's multiple comparison test. Gross and microscopic histologic observations, PAS staining, and PCNA immunostaining data were analyzed by student's t-test.

C. METHODS

1. Observations:

Rats in this study were observed twice daily for the presence of toxicity, behavioral abnormalities, and viability.

2. Body Weight

Individual body weights were taken daily from the beginning of the study until Day 28, the last day of sacrifice.

3. Food consumption and compound intake

Food consumption was calculated by measuring the remaining quantity of food every day. Food efficiency and test substance intake were also calculated.

4. Pathological Examinations

On Days 7, 14, 21, and 28, food was witheld for 16 hours from animals designated to be sacrificed. Rats were then killed by exsanguination through the abdominal aorta under ether anesthesia. Examination involved gross observations, organ weights of selected organs, and histopathological examination.
For histopathological examination, kidneys, stomach, duodenum, and organs with gross changes were removed and fixed in 10% neutral buffered formalin for 24 hours. From fixed tissue, 2 paraffin sections were prepared. once section was stained using H&E, while the other was subjected to PCNA and BrdU staining.

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5. Immunohistology

Using PC10 as the PCNA antibody, labeled nuclei in approximately 2000 epithelial cells were counted and the percentage of labeled nuclei calculated. Stomach tissue was subjected to BrdU immunostaining by administration of 0.1 g/kg BrdU 1 hour prior to sacrifice. The labeling count in the anterior stomach (forestomach) was calculated.

II. OBSERVATIONS AND RESULTS

A. **Mortality and Clinical Observations**

There were no treatment-related deaths reported in this study, and no treatment related clinical abnormalities were reported in any treated group of rats.

B. **Body Weights**

Group mean body weights, as summarized on page 69 of the report, were not significantly changed in treated rats vs. control. Body weight gain was also unaffected in treated rats in this study.

C. **Food Consumption and Efficiency**

Food consumption and efficiency (as presented on pages 72-73 of the report), were not significantly affected in treated groups of rats in this study.

D. **Intake of Chlorothalonil**

Test substance intake summary is presented below:

Table 3
Compound Consumption in Male Rats Administered Chlorothalonil in the Diet for 13 Weeks[a]

Dose level (mg/kg/day)	Day 7	Day 14	Day 21	Day 28	Mean
1.5	1.51±0.07	1.39±0.08	1.56±0.13	1.52±0.12	1.48±0.11
15	15.05±0.93	14.36±0.78	15.14±1.06	15.16±0.81	14.87±0.95
175	169.94±8.21	168.36±7.43	191.06± 10.79	185.64±8.82	175.26± 12.58

[a]Data from page 25 of the report.

As shown, mean compound intake over the course of the study was within an acceptable range of nominal for each dose level.

E. Ophthalmoscopic Examination

Ophthalmoscopic examination was not performed in this study.

F. Clinical Pathology

This study did not specifically examine clinical pathology, as this was not the purpose of the study.

G. Organ Weights

Absolute and relative organ weight data were shown on pages 75-82 of the report. The findings are summarized below.

Table 4
Organ Weights (in GRAMS) in Chlorothalonil Treated Male Rats[a]

	Males (mg/kg/day)			
7 Days (N = 14)	**0**	**1.5**	**15**	**175**
kidney weight (abs.)	0.90±0.07	0.98±0.06	1.01±0.03*	1.09±0.09**
kidney weight (g/100g)	0.825±0.035	0.865±0.023	0.886±0.027**	0.932±0.035**
stomach weight	0.77±0.08	0.70±0.06	0.68±0.06	0.79±0.04
14 Days				
kidney weight (abs.)	1.12±0.07	1.13±0.05	1.21±0.06	1.21±0.08
kidney weight (g/100g)	0.842±0.029	0.840±0.038	0.864±0.032	0.945±0.017**
stomach weight	0.76±0.06	0.75±0.06	0.84±0.08	0.91±0.09**
21 Days				
kidney weight (abs.)	1.25±0.07	1.23±0.11	1.31±0.12	1.40±0.05*
kidney weight (g/100g)	0.784±0.022	0.817±0.030	0.839±0.027*	0.909±0.027**
stomach weight	0.79±0.06	0.74±0.06	0.81±0.13	0.92±0.06*
28 Days				
kidney weight (abs.)	1.34±0.13	1.34±0.04	1.52±0.11**	1.60±0.05**
kidney weight (g/100g)	0.757±0.023	0.771±0.029	0.817±0.028**	0.881±0.024**
stomach weight	0.84±0.05	0.80±0.04	1.00±0.13**	1.01±0.07**

[a]Data from pages 75-82 of the report. * $p < 0.05$; ** $p < 0.01$ vs. control.

As shown, the most significant organ weight effect was that on the kidney, where both absolute and relative weight were consistently elevated at the 15 and 175 mg/kg/day dose levels in treated male rats.

H. **Macroscopic Observations**

The results of gross necropsy examinations (pages 83-86 of the report) showed an increased incidence of white coloration of the stomach, present in all male rats at the 175 mg/kg/day dose level. This lesion was not observed at lower doses.

I. **Microscopic Observations**

A summary of microscopic findings was presented on pages 87-94 of the report. In the stomach, chlorothalonil at 175 mg/kg/day resulted in increased incidence of edema, hemorrhage, erosion, inflammatory infiltration, hyperkeratosis, and squamous cell hyperplasia. In the kidney, increased incidence of vacuolization of the epithelium of the proximal convoluted tubule was observed at all time points at the 175 mg/kg/day dose level. At 15 mg/kg/day, this lesion was observed in one animal only after 28 days of treatment. Findings are summarized below for the 175 mg/kg/day dose:

Table 5
Histopathological Observations in Male Rats Administered Dietary Chlorothalonil at 175 mg/kg/day for 7, 14, 21, or 28 Days[a]

	Day 7	Day 14	Day 21	Day28
Kidney				
vacuolization of proximal tubular epithelium	6/6	6/6	5/6	3/6
Forestomach				
edema	6/6	6/6	6/6	6/6
hemorrhage	2/6	3/6	2/6	2/6
erosion	5/6	4/6	3/6	4/6
inflammatory infiltration	6/6	6/6	6/6	6/6
hyperkeratosis	6/6	6/6	6/6	6/6
squamous cell hyperplasia	6/6	6/6	6/6	6/6

[a]Data from pages 87-94 of the report.

It is noted that in the stomach, lesions were graded as slight for Day 7, but on Days 14, 21, and 28, lesions of inflammatory infiltration, hyperkeratosis,

and squamous cell hyperplasia progressed from a grade of slight to a grade of moderate. This progression is consistent with the irritant properties of chlorothalonil in the forestomach of rats. For Days 7 and 14 of the study, the incidence of forestomach lesions was zero for doses lower than 175 mg/kg/day. On Days 21 and 28, increases in the incidence of edema and inflammatory infiltration were observed at the 15 mg/kg/day dose level which were statistically significant. This is consistent with the irritant properties of chlorothalonil on the forestomach of rats.

J. Immunohistochemistry

Labeling index in PCNA-immunostained epithelial cells of renal proximal tubule cells in treated rats in this study is summarized below. It is noted that a statistically significant increase in the number of PCNA-positive nuclei in cells of the proximal convoluted tubule was observed at 175 mg/kg/day at all sacrifice times. At 15 mg/kg/day, a statistically significant increase in PCNA-positive nuclei was observed on Days 7, 14, and 21. The response at 15 and 175 mg/kg/day was dose but not time dependent. The peak response for the high dose group occurred at Day 7.

Table 6
Labeling Index in PCNA-Immunostained Epithelial Cells of Renal Proximal Tubules of Male Rats Administered Dietary Chlorothalonil[a]

Group	Dose (mg/kg /day)	No. Rats	Day of experiment			
			7	14	21	28
1	0	6	0.58±0.17	0.72±0.19	0.90±0.22	0.94±0.21
2	1.5	6	0.50±0.14	0.84±0.20	1.17±0.32	0.87±0.03
3	15	6	1.21±0.16***	1.98±0.61**	1.35±0.19**	1.10±0.34
4	175	6	6.86±1.92***	4.31±0.85***	3.10±0.81***	2.78±0.39***

[a]Data from page 95 of the report. *p < 0.05; **p < 0,01; ***p < 0.001.

In the stomach, BrdU labeling results in the mucosal epithelium of the anterior stomach (forestomach) showed that, by Day 7, marked and statistically significant increases in labeling were apparent at the 175 mg/kg/day dose level. This increase was evident and relatively constant throughout the study at the 175 mg/kg/day dose level. At the 15 mg/kg/day dose level, a significant decrease in BrdU labeling was evident on Day 14, and a significant increase evident on Day 28. At the 1.5 mg/kg/day dose level, a significant decrease in BrdU labeling was evident on Day 14 of the study. These results are shown below:

Table 7
BrdU-positive Nuclei in Forestomach of Male Rats Administered 175 mg/kg/day Chlorothalonil in Diet[a]

15 15

Chlorothalonil cell proliferation and histopathology [non-guideline]

Group	Dose (mg/kg /day)	No. Rats	Day of experiment			
			7	14	21	28
1	0	6	4±6	7±1	5±6	5±3
2	1.5	6	5±2	5±3*	4±3	9±8
3	15	6	7±3	6±2*	20±19	20±12*
4	175	6	267±90**	319±43**	305±66**	250±79**

[a]Data from page 96 of the report. *p < 0.05; **p < 0.01 vs control.

III. DISCUSSION

The purpose of the present study was to assess the induction of cell injury and cell hypertrophy in the kidney and forestomach of male rats when administered technical chlorothalonil in the diet. Doses of 0, 1.5, 15, and 175 mg/kg/day chlorothalonil were used in this study. Groups of 6 male rats/dose/time point were used, and these groups received dietary chlorothalonil for 7, 14, 21, or 28 days and the degree of chlrorthalonil induced toxicity assessed at these time points. Effects on body weight, food consumption, organ weight, and macroscopic and microscopic organ pathology were assessed. In addition, PCNA immunostaining was performed on epithelial cells of the renal proximal tubules (approximately 2000 cells of the proximal convoluted tubules) and BrdU immunostaining of the forestomach.

There were no significant effects of chlorothalonil on mortality, body weight, or food consumption. Absolute and relative weight of the kidneys were significantly increased at 175 mg/kg/day at all time points measured (Days 7, 14, 21, and 28). At the 15 mg/kg/day dose level, a significant increase in kidney weight was observed for Days 7 and 28 of the study. Microscopically, the forestomach of rats administered 175 mg/kg/day chlrothalonil in the diet showed increased incidence of edema, hemorrhage, erosion, inflammatory infiltration, hyperkeratosis, and squamous cell hyperplasia. These changes were evident at all time points examined. At the 15 mg/kg/day dose level, increased incidence of edema and inflammatory infiltration were observed on Days 21 and 28. In addition, the degree of severity of the stomach lesions at 175 mg/kg/day increased with time. After Day 7, a grade of moderate (vs. slight on Day 7) was observed for the stomach lesions. In the kidney, a statistically significant increase in vacuolization of the proximal convoluted tubules was observed at 175 mg/kg/day at all time points examined. In one rat, this lesion was observed at the 15 mg/kg/day dose level on Day 28 of the study. The progression of the micrposcopic lesions suggests that with repeated administration, chlorothalonil can affect the kidney and stomach of rats at lower dose levels.

Results of immunostaining of the kidney showed a statistically significant

increase in the number of PCNA-positive nuclei in cells of the proximal convoluted tubules at 175 mg/kg/day, consistent with the microscopic changes also observed at this dose. At the 15 mg/kg/day dose level, a significant increase in PCNA-positive nuclei was shown for Days 7, 14, and 21. In the forestomach, the 175 mg/kg/day dose level caused a clear increase in BrdU labeled nuclei at all time points examined, while an increase in BrdU labeled nuclei was observed on Day 28 at 15 mg/kg/day.

The results of these studies show histopathologic alterations of the stomach and kidney at doses of 15 and 175 mg/kg/day chlorothalonil. Histopathologic alterations are accompanied by increases in labeling indices at these doses. Based on the data in this study, a No Observed Effect Level of 1.5 mg/kg/day can be identified for cell proliferation and toxicity, with a Lowest Observed Effect Level of 15 mg/kg/day. However, this must be interpreted with some caution. The data represent only a 28 day repeated dose scenario. Longer term administration could conceivably result in adverse effects at lower doses. Carcinogenicity data do not, however, show tumors of the kidney at doses lower than 15 mg/kg/day after chronic administration to the rat. Thus, although a non-neoplastic effect could be identified at a dose lower than 15 mg/kg/day, the tumorigenic effect appears to occur at 15 mg/kg/day and above in rats. This raises the question of whether the data support a "threshold" effect for toxicity and/or carcinogenicity. Although it is accepted that chlorothalonil does not appear to act by a genotoxic mechanism, a tumorigenic and toxic response appears at 15 mg/kg/day. A much stronger response appears at 175 mg/kg/day. If the mechanism of toxicity is directly related to the production of tumors, then the issue appears to be what degree of toxic insult is necessary for tumorigenicity. In this case, differences in degree of toxicity are evident at 15 and 175 mg/kg/day, as are differences in tumorigenicity (from the chronic rat study, MRID # 41250502). A threshold mechanism for toxicity and/or carcinogenicity may or may not be supported from these data, but this issue requires further discussion.

IV. Classification

This study is classified **acceptable** (non-guideline). This study does not satisfy a specific guideline requirement, but provides scientific data demonstrating a toxic response in the kidney and forestomach after repeated dietary administration of 15 and 175 mg/kg/day technical chlorothalonil.

Reviewed by: Timothy F. McMahon, Ph.D. Date: 5/22/97
Section I, Toxicology Branch II (7509C)
Secondary Reviewer: Nancy McCarroll Date: 5/22/97
Section II, Toxicology Branch II (7509C)

Data Evaluation Record

Study type: Cell Proliferation - rats
Guideline: non-guideline

EPA ID Numbers: MRID number: 44223002 DP Barcode: D235134
P.C. Code: 081901 Submission: S521281

Test material: Chlorothalonil, 97.9% a.i.

Study number(s): not stated; document no. 6704-96-0010-TX-003

Sponsor: ISK Biosciences Corporation

Citation: Mizens, M. (1996): A 90-Day Pilot Study for the Evaluation of Cell Proliferation in the Kidneys of Male Rats Following the Oral Administration of Technical Chlorothalonil. Study performed by Ricerca, Inc. MRID # 44223002. Unpublished.

Executive Summary:
In a cell proliferation study, 28 male Fischer 344 rats received technical chlorothalonil (97.9% a.I.) in the diet at 175 mg/kg/day for up to 91 days. Scheduled sacrifices occurred on Days 7 (14 rats), 28 (7 rats), and 91 (7 rats) for the purpose of assessing the effect of chlorothalonil administration on cell proliferation in the kidney. Rats were implanted with Alzet minipumps containing bromodeoxyuridine 3.5 and 6.5 days prior to sacrifice (Day 7), or 3.5 days prior to sacrifice (Days 28 and 91). Mean labelling index was statistically increased in the kidneys of male rats treated with 175 mg/kg/day chlorothalonil at all scheduled sacrifice times. From Day 7 to Day 28, the fold increase in labeling index was relatively stable (approximately 10-fold over control), with a decrease to approximately 3.5-fold over control on Day 91. Increased cell proliferation correlated with histopathological lesions of degeneration of the proximal convoluted tubules and epithelial hyperplasia. The results of this study demonstrate a sustained cell proliferative response as a result of dietary administration of technical chlorothalonil at a dose of 175 mg/kg/day. The apparent lack of cytotoxicity compared to the hypertrophic response in this study is not readily explained by the available data .

This study is classified as **acceptable** (non-guideline). The study does not satisfy a particular guideline requirement, but demonstrates a cell proliferative effect of chlorothalonil on the kidney at a dose which also produces kidney tumors.

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Compliance

Signed and dated statements of GLP, Quality Assurance, and Data Confidentiality were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test Material

Technical chlorothalonil
Purity: 97.9% lot no: 1002
Description: gray powder
CAS # 1897-45-6
Storage: in the dark at room temperature.
Stability: range of 97.6-99.0% a.i. demonstrated over 3 year period.

2. Vehicle: dietary preparation.

3. Test Animals: Male Fischer 344 rats. Source: Charles River Breeding Laboratories, Raleigh, N.C. Age: 71-73 days old at receipt and 84-86 days old at study initiation. Rats were housed in a separate room individually in stainless steel cages. Food (PMI Feeds Inc. Lab Diet Certified Rodent Diet #5002) and water were available *ad libitum* except for an overnight fast. Rats were acclimated for 13 days prior to start of treatment. Temperature and humidity were maintained at 71 _ 7 °C, and 40-70%, respectively with at least 10 air changes per hour and a 12 hour light/dark cycle. The dose groups are summarized as follows :

B. STUDY DESIGN

1. Animal Assignment:

Test Group	Treatment	Dose level	No. animals
control	--	0 mg/kg/day	28
treated	chlorothalonil	175 mg/kg/day	28

2. Dietary Mixtures:

Dietary mixtures in this study were not adjusted for purity. From page 19 of the report: "Appropriate amounts of test material were mixed fresh weekly with the feed. A premix was prepared using a mortar and pestle. The test material and rodent feed were ground together until the test material was uniformly dispersed in the mixture (10 minutes). The final mix was prepared by mixing the premix with the balance of the feed in a Patterson-Kelly 1 cubic foot blender for ten minutes. Seven-kilogram batches were prepared Weeks 1 and 2. For all subsequent weeks, 4-kg batches were prepared.

Concentrations of the test material were adjusted weekly to achieve desired dietary intake of test material. The adjustment was based on the most recent body weight and food consumption data.
Homogeneity and stability analyses were performed on a test batch of 3500 ppm chlorothalonil mixture prepared using the same procedures as for test diets. A second batch of 4000 ppm chlorothalonil diet was analyzed for homogeneity on Week 1, based on the observation that a dietary concentration in excess of 3500 ppm might be needed. Five subsamples from each concentration were used in homogeneity analysis. Verification of dietary concentration was performed by collecting duplicate samples of test diet in amber glass bottles. No samples were obtained during Week 10, due to error. One duplicate sample was analyzed for Weeks 1,2,7, and 12.
Results of homogeneity analysis are shown below:

Table 1
Homogeneity of Chlorothalonil in Rodent Feed[a]

First Homogeneity Analysis (3500ppm)		Second Homogeneity Analysis (4000p	
Sample ID	µg/g in feed	Sample ID	µg/g in feed
1	3375	20	3714
2	3383	21	3717
3	3392	22	3692
4	3381	23	3703
5	3380	24	3684
Average	3382	Average	3702
Std. Deviation	6.45	Std. Deviation	14.2
% RSD	0.19	% RSD	0.38
Nominal Recovery	97.9%	Nominal Recovery	96.3%

[a]Data from page 121 of the report.

Stability data are summarized below:

Table 2
Stability of Chlorothalonil in Rodent Feed[a]

Week of Study	Nominal Conc.ppm	Corrected mean ppm	% of Nominal
1	2782	2712	97.5%
2	3411	3315	97.2%
7	3259	3198	98.1%
12	3327	3208	96.4%
Mean =	3195	3108	97.3%

[a]Data from page 122 of the report.

The above data show that dietary mixtures of chlorothalonil were homogenous and stable for the period of this study.

3. Statistical Analysis:

Body weight, organ weight, and food consumption data were statistically analyzed to compare the treatment group mean to the control group mean. Bartlett's test was used to determine homogeneity of variance. If significance was observed at the 1% level, non-parametric procedures (Dunn's summed-rank test) were used; otherwise, parametric procedures (t-test) were used. Labeling index data were transformed using a square root transformation and analyzed by Bartlett's test.

C. METHODS

1. Observations:

Rats in this study were observed twice daily for viability. Cageside observations were made once daily after the start of the study. Type of clinical sign, time of onset, duration, and time of recovery were recorded.

2. Body Weight

Individual body weights were taken weekly from one week prior to start of the study to study termination.

3. Food consumption and compound intake

As with body weight, food consumption was measured weekly. Compound consumption was also recorded.

4. Cell Proliferation Labeling

Cell proliferation was evaluated on Days 7, 28, and 91 of the study. ALZET minipumps containing bromodeoxyuridine (BrdU, 16 mg/ml, dissolved in Dulbecco's phosphate-buffered saline) were implanted subcutaneously 3.5 or 6.5 days before sacrifice. The time of pump implantation for Days 28 and 91 was determined from the results of Day 7. The BrdU solutions were prepared fresh for each implantation interval. All procedures were performed aseptically.

II. OBSERVATIONS AND RESULTS

A. **Mortality and Clinical Observations**

Rats were observed twice daily for viability. Cageside observations were made once daily after test material administration. There were no treatment-related deaths reported in this study. In treated rats (but not controls), dark yellow urine was observed on the cage boards beginning on Day 54 for two of the rats. By Day 55, all treated rats were observed with this sign. The only other observation noted was that of decreased feces on Day 0 in one treated rat.

B. **Body Weights**

Individual body weight was recorded weekly, from one week prior to initiation of test material administration to study termination and again at terminal necropsy. Group mean body weights, as summarized on page 29 of the report, were not significantly changed in treated rats vs. control except for Week 1, where group mean body weight in treated rats was decreased by 5%. Although statistically significant, this decrease is not considered toxicologically meaningful, especially in light of the lack of changes in body weight at subsequent intervals.
Cumulative body weight gain for Weeks 1 and 2 of the study was decreased significantly in treated rats vs control (43% decrease for Week 2); rats lost weight during the first week of treatment). At Week 7, cumulative body weight gain was decreased from 67 grams in control to 49 grams in treated rats (27% decrease). Overall weight gain for Weeks 0-13 was decreased from 80.7 grams

in control to 62.4 grams in treated rats (23% decrease). Although not statistically significant, body weight gain decrements of this magnitude could be considered toxicologically significant.

Group mean body weights at selected times are presented in **Table 3.**

TABLE 3

Group Mean Cumulative Body Weight Gain (grams) in Male Rats Given Chlorothalonil in the Diet for 13 Weeks[a]

	Control (0 mg/kg/day)	Treated (175 mg/kg/day)
Week 0-1	10.5±5.7	-3.4±7.8**
Week 0-7	67.0±10.0	49.0±21.8
Week 0-13	80.7±17.6	62.4±20.3

[a]Data from page 30 of the report. **p < 0.01.

C. Food Consumption and Efficiency

As for body weight, food consumption was measured weekly beginning 1 week prior to study initiation until study termination. Results of food consumption measurements showed no significant changes in food consumption except for Week 1 of the study, where consumption was decreased 21% in treated rats vs. control. At all subsequent time points, food consumption was equivalent or slightly higher in treated vs control rats.

Mean relative food consumption (as g/kg/day) data showed that in treated male rats, increased relative consumption was evident throughout the study except during Week 1. That is, treated male rats consumed more food on a per kg basis than controls, yet demonstrated decreased body weight gain. This supports the conclusion of a decrease in efficiency of food utilization.

Group mean relative food consumption data are presented in **Table 4** below:

TABLE 4

Group Mean Relative Food Consumption in Male Rats Given Chlorothalonil in the Diet for 13 Weeks [a]

Week of Study	Food consumption (grams / kg b.w. / day) Controls (0 mg/kg/day)	Treated (175 mg/kg/day)
0	62.1±3.8	62.9±3.9
1	62.7±3.2	51.3±7.2*
2	57.5±3.1	60.1±2.3*
3	55.9±2.8	58.6±3.4*
4	55.1±3.1	58.9±2.8**
5	53.4±1.6	57.0±2.2**
6	50.6±1.0	53.7±3.0*
7	50.1±0.9	55.1±0.9**
8	49.3±1.6	54.9±3.1**
9	48.0±1.6	53.1±2.3**
10	45.7±1.1	49.6±1.5**
11	47.4±1.3	52.6±1.6**
12	46.1±1.6	50.0±1.2**
13	48.9±1.6	54.0±1.6**

[a]Data from page 32 of the report.

D. Intake of Chlorothalonil

Available data on the intake of chlorothalonil throughout the study were provided in Table 5 of the study. Average intake for the 13 week test period is summarized as follows (Table 5):

Table 5
Compound Consumption in Male Rats Administered Chlorothalonil in the Diet for 13 Weeks

Study Week	Mean Intake (mg/kg/day)
1	142.9±20.0
2	205.1±7.3
3	170.6±9.5
4	176.1±8.3
5	169.1±7.4
6	164.6±9.0
7	179.3±3.1
8	173.7±10.5
9	169.3±7.3
10	163.0±4.9
11	184.7±5.2
12	166.7±4.1
13	188.6±5.2

[a]Data from page 33 of the report.

Mean compound consumption for the 13 week period was calculated to be 173.3 mg/kg/day, which is within an acceptable range of nominal.

E. Ophthalmoscopic Examination

Ophthalmoscopic examination was not performed in this study.

F. Clinical Pathology

This study did not specifically examine clinical pathology, as this was not the purpose of the study.

G. Organ Weights

Organ weight data were shown on pages 37-38 of the report. Representative data are shown below.

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Table 6
Organ Weights (in GRAMS) in Chlorothalonil Treated Male Rats[a]

	Males (mg/kg/day)	
7 Days (N = 14)	**0**	**175**
Terminal b.w.	230.2±8.0	217.4±8.0**
kidney weight (abs.)	1.555±0.07	1.698±0.102**
kidney weight (g/100g)	0.675±0.018	0.781±0.039**
brain weight	1.764±0.038	1.741±0.045
28 Days		
Terminal b.w.	251.9±12.7	250.9±10.3
kidney weight (abs.)	1.607±0.107	1.930±0.074**
kidney weight (g/100g)	0.638±0.026	0.770±0.025**
brain weight	1.811±0.023	1.751±0.032**
91 Days		
Terminal b.w.	303.1±20.8	290.1±21.2
kidney weight (abs.)	1.947±0.121	2.373±0.217**
kidney weight (g/100g)	0.643±0.026	0.818±0.050
brain weight	1.851±0.046	1.844±0.037

[a]Data from pages 37-38 of the report.

As shown, significant organ weight effects were noted on the kidney, where both absolute and relative (to body and brain) weight were consistently elevated in treated male rats over the course of the study. This is an effect observed previously with chlorothalonil in long term studies at the 175 mg/kg/day dose.

H. Macroscopic Observations

The results of gross necropsy examinations (pages 34-36 of the report) showed that in treated rats, thickened forestomach and/or pinpoint erosions were present in all rats at all time points of sacrifice.

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H. **Microscopic Observations**

A summary of microscopic findings was presented on pages 139-141 of the report, and individual animal data were presented on pages 147-170 of the report.

The report provided the following information regarding microscopic lesions in the kidney induced by chlorothalonil:

Day 7: "...moderate to severe degeneration of the proximal convoluted tubules and a slight to moderate epithelial hyperplasia in the proximal convoluted tubules. The degeneration was characterized by cytoplasmic vacuolation, nuclear pyknosis, karyolysis and cell swelling. Some of the epithelial cells appeared necrotic. Gross lesions were seen in the forestomach from all of the animals receiving 175 mg/kg/day technical chlorothalonil. Microscopically, submucosal edema, hyperkeratosis, and squamous epithelial hyperplasia were seen from a moderate to moderately severe degree in the forestomach of all treated rats." It is noted that the kidney lesions were observed in all treated rats examined on Day 7 (14 animals), and slight to mild regenerative epithelium of the kidney was noted in 2 of 14 treated rats examined.

Day 28: "...similar changes [in kidneys] to that seen following seven days of chlorothalonil administration but to a slightly less degree of severity. These microscopic alterations were characterized by degeneration and hyperplasia of the epithelial cells in the proximal convoluted tubules. In four of 7 rats examined, an increase in size of the proximal convoluted tubules was seen. Microscopic lesions in the stomach included submucosal edema, hyperkeratosis, and squamous epithelial hyperplasia.

At 91 days, all treated rats were observed with microscopic kidney lesions. These were characterized by degeneration and hyperplasia of the epithelial cells of the proximal convoluted tubules. Tubular hypertrophy was also observed in 5 of 7 rats examined at this time point.

The report noted that labelled proliferating cells were observed in the same region of the kidney in which microscopic lesions were observed.

I. Cell Proliferation Evaluation

Cell proliferation data in the report showed that in treated rats, the mean labeling index (LI) was statistically increased at all sacrifice times (Days 7, 28, and 91). The LI was observed to be relatively stable for the first 28 days of the study (10.8-fold increase over control on Day 7; 10.1-fold increase over control on Day 28), with a decline observed on Day 91 (3.5-fold increase over control).

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Table 7
Cell Proliferation in Kidneys of Male Rats
Administered 175 mg/kg/day Chlorothalonil in Diet[a]

Day of Sacrifice	0 mg/kg/day	175 mg/kg/day
Day 7 (3.5 day pump)	2.67_0.88	28.84±8.99** (10.
Day 7 (6.5 day pump)	4.44±1.21	20.49±5.36** (4.6
Day 28	1.24±0.85	12.51±4.18** (10.
Day 91	1.42±0.60	4.95±2.32** (3.5)

a- Data (from page 26 of the report) represent the mean labeling index, calculated for each animal by dividing the number of labeled nuclei by the total number of nuclei counted (labeled and unlabeled) and the result expressed as a percentage. **p < 0.01.

b- Fold increase = labeling index (treated)
labeling index (control)

As shown, the mean LI was significantly increased at all time points in treated rats. For the Day 7 and Day 28 measurements, the fold increase was relatively stable (10.8 and 10.1, respectively). The implantation of a minipump for 6.5 days appeared to result in reduced labeling index as opposed to implantation of a minipump for 3.5 days. The report attributed this decrease to possible toxicity of chlorothalonil on previously labeled cells. This theory was not tested in this report. In general, labeling index should increase with increasing duration of exposure. There is no definitive explanation for the decrease in labeling after 6.5 days of exposure as compared to 3.5 days of exposure other than the theory advanced in the report. Nonetheless, a 3.5 day implantation time was chosen for use in this study based on these results.

III. DISCUSSION

The purpose of the present study was to examine whether cell proliferation could be detected in the kidneys of male rats administered technical chlorothalonil at a dose (175 mg/kg/day) which is also known to produce significant renal toxicity and tumorigenicity. The results of this study do show significant cell proliferative activity in male rat kidney following a repeated oral dose of 175 mg/kg/day chlorothalonil. The study also demonstrates that significant toxicity occurs in the same area of the kidney in which cell proliferation occurred, thus lending support to the idea that cell proliferation may be of predictive value.

Although body weight was not significantly affected, body weight gain was decreased significantly during the study in treated rats. The decrease in weight gain could have been based upon a decreased food utilization ability.

Kidney weights were significantly increased (both absolute and relative) in treated rats at all time points examined, which could correlate with the hypertrophic response observed in the kidney proximal tubular epithelium. Cytotoxicity, which would not lead to significant increases in kidney weight, should have been apparent, but was not (although cell proliferation, which would be expected to follow from cytotoxicity, was apparent). This apparent discrepancy requires further explanation. It would seem that in this case, the hypertrophic response outweighed the cytotoxic response, or, sacrifice occurred subsequent to observation of any significant cytotoxicity (i.e. cytotoxicity may have occurred prior to 7 days post-dose).

IV. Classification

This study is classified acceptable (non-guideline). This study does not satisfy a specific guideline requirement, but provides scientific information on the potential mechanism of action of chlorothalonil in production of renal tumors.