5-8-96

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

MAY - 8 1996

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

MEMORANDUM

SUBJECT: Iprodione - In Vitro Study in Leydig Cells

TO: Bill Wooge
PM Team Reviewer (52)
Reregistration Branch, SRRD (7508C)

FROM: Linda L. Taylor, Ph.D. Linda Lee Taylor 4/30/96
Toxicology Branch II, Section II,
Health Effects Division (7509C)

THRU: K. Clark Swentzel K. Clark Swentzel 4/30/96
Section II Head, Toxicology Branch II
Health Effects Division (7509C)

and

Stephanie R. Irene, Ph.D. Stephanie R. Irene 5/3/96
Acting Chief, Toxicology Branch II/HED (7509C)

Registrant: Rhone-Poulenc Ag Company
Chemical: [3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolidinecarboxamide]; [3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide]
Synonym: Iprodione; 26019 RP; Rovral®; Glycophene
Case No.: 816345
Caswell No.: 470A
Submission No.: S501291
P.C.Code: 109801
DP Barcode: D223687
MRID No.: 43830601
Action Requested: Please review and provide a report of your findings.

Comment: The Registrant submitted a mechanistic study, which was performed to monitor the steroidogenic activity of cultured porcine Leydig cells exposed to Iprodione and its metabolites. This study has been reviewed, and the DER is attached.

In an in vitro study [MRID 43830601] using porcine cultured Leydig cells, Iprodione [99.7%] and two of its metabolites [RP36112 (99.2%) and RP36115 (96.7%)] were shown to inhibit gonadotropin-stimulated testosterone secretion in a concentration range of 1-10

µg/mL. Inhibition by Iprodione was observed after short-term exposure [3 hours], and the inhibitory effects were similar to those observed with the fungicide Ketoconazole. The inhibitory effects do not appear to be related to Leydig cell damage because the removal of Iprodione from the culture medium for 72 hours resulted in the recovery of the cells ability to secrete testosterone following hCG stimulation. There was no discussion as to how the concentrations of Iprodione exhibiting inhibition [1-10 µg/mL] in the Leydig cell cultures relate to the levels attained within testicular cells following oral dosing in the rat carcinogenic study.

This study is classified Acceptable, nonguideline.

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[**IPRODIONE**] Mechanistic Study/Testosterone Secretion/Leydig Cells

EPA Reviewer: Linda L. Taylor, Ph.D. Date 4/23/96
Review Section II, Toxicology Branch II (7509C)
EPA Secondary Reviewer: K. Clark Swentzel Date 4/30/96
Review Section II, Toxicology Branch II (7509C)

DATA EVALUATION RECORD

STUDY TYPE: Mechanistic Study [porcine isolated Leydig cells from testes]; OPPTS/§ none

DP BARCODE: 223687 SUBMISSION CODE: S501291

P.C. CODE: 109801 TOX. CHEM. NO.:

TEST MATERIAL (PURITY): Iprodione [99.7%]

CHEMICAL: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide

SYNONYMS: RPA095207, RPA590611, RP26019

CITATION: Author [Benahmed, M.] (July 13, 1995) Effects of Iprodione and Its Metabolites on Testosterone Secretion in Cultured Leydig Cells. INSERM U. 407; Communication Cellulaire en Biologie de la Reproduction. Laboratoire de Biochimie, Bat. 3 B, Centre Hospitalier Lyon-Sud, France. Report INSERM/U407/95001, [study dates not provided]. MRID 43830601. Unpublished.

SPONSOR: Rhone-Poulenc Secteur Agro/France

EXECUTIVE SUMMARY: In an *in vitro* study [MRID 43830601] using porcine cultured Leydig cells, Iprodione [99.7%] and two of its metabolites [RP36112 (99.2%) and RP36115 (96.7%)] were shown to inhibit gonadotropin-stimulated testosterone secretion in a concentration range of 1-10 µg/mL. Inhibition by Iprodione was observed after short-term exposure [3 hours], and the inhibitory effects were similar to those observed with the fungicide Ketoconazole. The inhibitory effects do not appear to be related to Leydig cell damage because the removal of Iprodione from the culture medium for 72 hours resulted in the recovery of the cells ability to secrete testosterone following hCG stimulation. There was no discussion as to how the concentrations of Iprodione exhibiting inhibition [1-10 µg/mL] in the Leydig cell cultures relate to the levels attained within testicular cells following oral dosing in the rat carcinogenic study.

This study is classified Acceptable, but it does not satisfy any guideline requirement.

COMPLIANCE: Signed and dated GLP and Data Confidentiality statements were provided. There was no Quality Assurance statement *per se*. The GLP statement indicated that the mechanistic study was not conducted in compliance with the Good Laboratory Practices Regulations, but the Sponsor's Quality Assurance Unit inspected the testing facility and audited the raw data. No flagging statement was provided.

[IPRODIONE] Mechanistic Study/Testosterone Secretion/Leydig Cells

I. **MATERIALS AND METHODS**

A. MATERIALS

1. Test Material: (1) Iprodione, (2) RP36112 (metabolite), (3) RP36115 (metabolite); Description: white powders; Batch #: (1) TV3015C, (2) BES1526, (3) BESS129; Purity: (1) 99.7%, (2) 99.2%, (3) 96.7%; Stability of compound: information not provided; CAS #: (1) 36734-19-7. NOTE: Three other metabolites were tested [RP 25040, RP 32490, and RP 36118], but since no effects were observed with these, those results are not enumerated in this DER.

Structure:

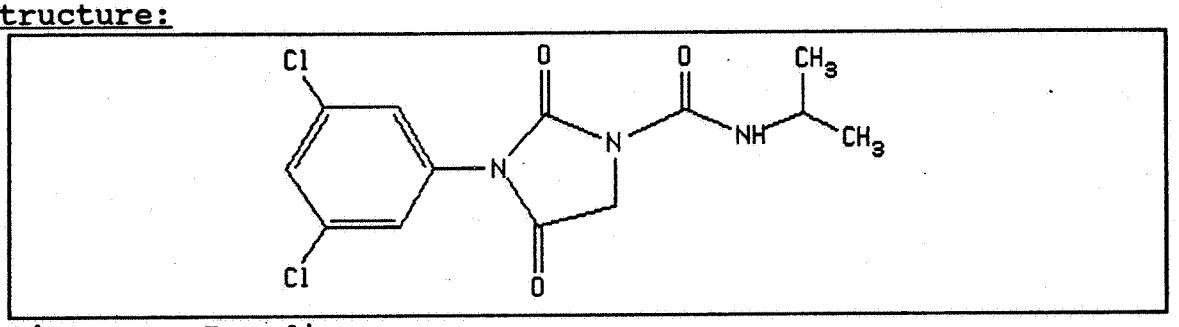

Figure 1 Iprodione

Formula: (1) $C_{13}H_{13}Cl_2N_3O_3$;
Source: Rhone-Poulenc Secteur Agro/France

2. Vehicle: absolute ethanol; Batch #: not provided.
Positive control: Ketoconazole; Batch #: not provided; Source: Biolmol Laboratories distributed through TEBU France.

3. Test animals: Species: isolated Leydig cells from immature porcine testes; Strain: not provided; Age: 3 weeks old; Source: not provided.

B. STUDY DESIGN and METHODS

1. In life dates - not provided. It was stated that all experiments in this study were performed within a weeks' time.

2. Preparation of cultures - Isolated Leydig cells were prepared according to the methods described by Mather and Phillips [1984], as modified by Benahmed, et al. [1987]. As described in the Methods' section of the report, decapsulated testes were minced, washed twice in DME-F12 medium, and after collagenase dissociation, the cells were washed by centrifugation [200 x g for 10 minutes]. The pellets were resuspended and submitted to two successive sedimentations of 5 and 15 minutes. The crude interstitial cells were recovered from the supernatants, and Leydig cells were prepared from this fraction by Percoll density gradient centrifugation. Leydig cells were recovered from this gradient and characterized by their ability to bind LH/hCG

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and to produce testosterone in response to this hormone. The report stated that the percentage of Leydig cells in this final preparation [as established by staining for 3β-hydroxysteroid dehydrogenase activity] was always greater than 90%.

Leydig cells were plated in Falcon 24-multiwell plates and cultured at 32° C in a humidified atmosphere of 5% CO_2-95% air [Heraeus incubator] in DME-F12 medium containing sodium bicarbonate, HEOES, and gentamicin. This medium was supplemented with insulin, transferrin, and vitamin E. It was stated that differentiated [steroidogenic] activity of cultured porcine Leydig cells had been shown previously to remain stable for at least one week.

3. Dosing and procedures - After seeding, the medium was changed from each culture every day for the first 2 days. Iprodione, RP36112, or RP36115 were then dissolved in ethanol and added to fresh culture medium and incubated with cultured Leydig cells for various periods of time.

a. Effects on hCG-stimulated testosterone secretion: Leydig cells were incubated for 3 days with Iprodione or its metabolites at concentrations of 0.03, 0.1, 0.3, 1, 3, and 10 µg/mL for each substance. Following 3 days incubation, untreated [control] and treated cells were stimulated with maximal efficient concentration of hCG [3 ng/mL, 3 hours].

b. Leydig cells were incubated with maximum inhibitory concentrations [10 µg/mL] of the test materials for 2 days and were then stimulated for 3 hours with increasing concentrations of hCG [0.01, 0.03, 0.1, 0.3, 1, and 3 ng/mL].

c. Leydig cells were treated with Iprodione [0.03, 0.1, 0.3, 1, 3, and 10 µg/mL] for 24 hours and then Iprodione was withdrawn from the Leydig cell cultures for 72 hours before evaluating hCG-stimulated testosterone secretion. For this purpose, the 24-hour Iprodione-treated Leydig cells were washed several times with fresh medium and then incubated in the presence of control [Iprodione-free] medium for 72 hours. At the end of the incubation period, the cells were tested for their steroidogenic capacity after acute [3 hour] stimulation with maximum concentration of hCG [3 ng/mL].

d. Leydig cells were incubated with increasing concentrations of Iprodione [same dose as above] for 3, 24, 48, or 72 hours. The Leydig cells were then stimulated with hCG [3 ng/mL for 3 hours] to test their steroidogenic capacity.

e. Leydig cells were treated for 2 days with increasing concentrations of Iprodione or Ketoconazole [same concentrations as above for both]. The cells were then stimulated with hCG as before to test their steroidogenic capacity.

4. Leydig cell testosterone secretion - Leydig cells from each control and test material group were cultured in 3 separate wells. Using three samples of different volumes [50, 100, and 200 µL], testosterone concentration in each well was determined by specific Radioimmunoassay [RIA] in triplicate. Testosterone concentration [ng/mL] in each well was then calculated from the concentrations of these 3 samples, and the results were expressed as the mean of testosterone concentrations from the 3 different wells of each control and test material group.

testosterone control values: testosterone concentrations in untreated cultures that have been challenged by an hCG stimulation for 3 hours
testosterone basal values: testosterone concentrations in untreated or treated cultures that have not been challenged by an hCG stimulation

II. RESULTS

A. Effects on hCG-stimulated testosterone secretion: Testosterone secretion was inhibited by Iprodione [Table 1], RP 36112 [Table 2], and RP 36115 [Table 3] at dose levels from 1 µg/mL and greater, with the maximum inhibitory effects being observed between 3 and 10 µg/mL for all three compounds.

Table 1. Inhibition of Testosterone Secretion by Iprodione

Dose [µg/mL]	mean pg T♦/sample	mean T pg/mL	T conc. [ng/10^6 cells]
0 [control]	235	2137	4.27
0.03	199	1834 [85]♥	3.67
0.1	189	1726 [80]	3.45
0.3	157	1418 [66]	2.84
1	116	1021 [47]	2.04
3	56	483 [22]	0.97
10	26	220 [10]	0.44
0 [control]	243	2189	4.38

♦ #s in this column calculated using data in table on page 21 of the report;
#s in other columns from data in that table; T = testosterone; ♥ [% of mean control values]

Table 2. Inhibition of Testosterone Secretion by RP 36112

Dose [µg/mL]	mean pg T♦/sample	mean T pg/mL	T conc. [ng/10^6 cells]
0 [control]	244	2182	4.36
0.03	286	2642	5.28
0.1	287	2683	5.37
0.3	218	2028	4.06
1	144	1287	2.57
3	59	527	1.05
10	260♪	2451♪	4.90♪
[control]	356	3158	6.32

♦ #s in this column calculated using data in table on page 22 of the report;
#s in other columns from data in that table; T = testosterone; ♥ [% of mean control values];
♪ results listed as abnormal

Table 3. Inhibition of Testosterone Secretion by RP 36115			
Dose [µg/mL]	mean pg T♦/sample	mean T pg/mL	T conc. [ng/10^6 cells]
0 [control]	222	1920	3.84
0.03	220	1874	3.75
0.1	212	1820	3.64
0.3	203	1753	3.51
1	116	1004	2.01
3	36	337	0.67
10	35	320	0.64
[control]	274	2401	4.80

♦ #s in this column calculated using data in table on page 23 of the report;
#s in other columns from data in that table; T = testosterone; ♥ [% of mean control values]

B. Effects on testosterone secretion at various concentrations of hCG: Nearly complete inhibition of testosterone secretion by 10 µg/mL of Iprodione, RP 36112, and RP 36115 was observed at all concentrations of hCG [Table 4].

Table 4. Effects on Testosterone Secretion w/ Increasing [hCG] - (testosterone ng/10^6 cells)				
+hCG [ng/mL]	Control	Iprodione	RP 36112	RP 36115
[0 ng/mL]	0.95	0.85	1.57	1.04
[0.01 ng/mL]	1.85	0.85	1.41	1.01
[0.03 ng/mL]	2.87	0.80	1.47	1.09
[0.1 ng/mL]	3.47	1.09	1.53	1.20
[0.3 ng/mL]	4.29	1.44	1.54	1.28
[1 ng/mL]	4.32	1.37	1.43	1.30
[3 ng/mL]	3.98	1.35	1.47	1.21
[0 ng/mL]	0.90	1.37	1.50	0.93

data from tables on pages 27-30 of report

C. Reversibility of the steroidogenic inhibitory effects: Compared to the results after 24 hours of treatment, where Iprodione was shown to inhibit hCG-stimulated testosterone secretion in a dose-related manner, Iprodione removal from the cell cultures for 72 hours resulted in a recovery of the Leydig cells of their steroidogenic functions [Table 5].

Table 5. Reversibility of Inhibition of Testosterone Secretion by Iprodione				
Dose [µg/mL]	24-hour treatment		72-hour withdrawal	
	mean T pg/mL	T conc. [ng/10^6 cells]	mean T pg/mL	T conc. [ng/10^6 cells]
0 [control]	2229	8.92	1709	7.43
0.03	2604	10.42	1775	7.72
0.1	2368	9.47	1726	7.50
0.3	2209	8.84	2062	8.96
1	1686	6.74	2158	9.38
3	916	3.66	2102	9.14
10	476	1.90	2074	9.02
[control]	2248	8.99	2236	9.72

data from tables on pages 31 and 32 of report; T = testosterone

D. Time course of effects on hCG-stimulated-testosterone secretion: The inhibitory effects of Iprodione [from 1 µg/mL and above] on hCG-stimulated testosterone secretion were observed at all times tested [3 hours to 72 hours], which demonstrates, according to the author, that the inhibitory effect is a rapid phenomenon [Table 6].

Table 6. Time Course of Inhibition of Testosterone Secretion by Iprodione [T conc. (ng/10^6 cells)]				
Dose [µg/mL]	3 hours	24 hours	48 hours	72 hours
0 [control]	5.08	6.16	6.36	5.27
0.03	4.67	5.58	6.48	7.11
0.1	4.58	6.32	6.69	8.34
0.3	4.32	5.67	5.43	6.92
1	3.83	4.76	2.97	5.83
3	2.45	3.14	1.66	3.93
10	1.72	2.34	1.14	2.19
[control]	4.78	6.88	5.52	8.08

data from tables on pages 33-36; T = testosterone

E. Inhibitory effects on hCG-stimulated-testosterone secretion: Both Iprodione and Ketoconazole were shown to inhibit hCG-stimulated testosterone secretion at dose levels of 1 µg/mL and above. Maximum inhibition was observed at 10 µg/mL [highest concentration tested] for both compounds [Table 7].

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Table 7. Inhibition of Testosterone Secretion by Iprodione and Ketoconazole [T conc. (ng/10^6 cells)]		
Dose [µg/mL]	Iprodione	Ketoconazole
0 [control]	4.47	6.66
0.03	4.70	5.96
0.1	4.43	6.53
0.3	3.89	6.40
1	2.43 (54)♦	5.34 (80)
3	1.88 (42)	2.79 (42)
10	0.94 (21)	1.12 (17)
[control]	5.30	6.94

data from tables on pages 37 & 38 of report; T = testosterone;
♦ (% of first control listed for each compound)

III. DISCUSSION

A. The objective of this study was to assess the potential inhibitory effect of Iprodione and its metabolites on Leydig cell steroidogenesis using an in vitro system [porcine Leydig cells cultured in serum-free, defined medium]. There were 3 specific objectives listed: (1) to detect a potential inhibitory effect on hCG-stimulated testosterone secretion in cultured Leydig cells; (2) to characterize the inhibition; and (3) to compare the inhibitory potential of Iprodione to that of Ketoconazole, a well characterized inhibitor of steroidogenesis [Feldman, D. (1986) Endocrine Reviews 7, 409-420]. Iprodione was shown to inhibit testosterone secretion at concentration levels as low as 1 µg/mL, and the inhibition was shown to be rapid and reversible. Additionally, Iprodione displayed similar inhibitory effects to those of Ketoconazole. The author concluded that the absence of cytotoxicity [Leydig cell damage] following Iprodione exposure and the recovery of Leydig cell steroidogenesis following removal of Iprodione from the cells strongly suggests an interference of Iprodione with some biochemical step(s) involved in testosterone secretion. TB II points out that although Iprodione was found to inhibit hCG-stimulated testosterone secretion in this in vitro system at concentrations of 1 µg/mL and above using Leydig cells from a pig, similar studies with rat Leydig cells have not been submitted. Additionally, there was no discussion of the relationship of the concentration used [attained in the cells] in these in vitro studies to the levels attained in the cells [in vivo] following oral exposure. It is stated that studies are underway to determine the precise location of the biochemical lesion(s) involved in the inhibition of testosterone secretion. Although Iprodione may be found to interfere with a biological step involved in testosterone secretion, if the concentration necessary for this interference is not attained within the cells of the rat in vivo, the findings would be irrelevant. TB II suggests that similar studies to the one reported here be performed in the rat Leydig cells, and an investigation into the serum/tissue levels of Iprodione attained in the rat at the dose levels tested in the chronic toxicity/carcinogenicity study would be most useful in the interpretation of the mechanistic data being generated.

B. Study deficiencies None that would affect interpretation of the study.

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[IPRODIONE] Mechanistic Study/Testosterone Secretion/Leydig Cells

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Study Type: Guideline: OPP § none
mechanistic [Cultured Leydig Cell from Porcine Testes]

Test Material: Iprodione [99.7%]

Chemical: 3-(3,5-dichlorophenyl)-N-isopropyl-2,4-dioxoimidazolidine-1-carboxamide

EPA MRID No.: 43830601

Testing Facility: Communication Cellulaire en Biologie de la Reproduction. Laboratoire de Biochimie, Bat. 3 B, Centre Hospitalier Lyon-Sud, France

Study Number: Report INSERM/U407/95001

Report Issued: July 13, 1995

EXECUTIVE SUMMARY: In an in vitro study [MRID 43830601] using porcine cultured Leydig cells, Iprodione [99.7%] and two of its metabolites [RP36112 (99.2%) and RP36115 (96.7%)] were shown to inhibit gonadotropin-stimulated testosterone secretion in a concentration range of 1-10 µg/mL. Inhibition by Iprodione was observed after short-term exposure [3 hours], and the inhibitory effects were similar to those observed with the fungicide Ketoconazole. The inhibitory effects do not appear to be related to Leydig cell damage because the removal of Iprodione from the culture medium for 72 hours resulted in the recovery of the cells ability to secrete testosterone following hCG stimulation.

This study is classified Acceptable, but it does not satisfy any guideline requirement.

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