

Report Title: DNA Synthesis In Mouse Epidermis Following Topical Application Of A Test Substance Supplied By Procter And Gamble

Test Type: Genotoxicity Study

Conducting Laboratory and Location: Division of Dermatology, UCLA School of Medicine

Test Substance(s): G0539.04 and Compound B are both solid Octopirox. G0539.04 was dissolved in Tween 80 and water. Compound B was solubilized in Solution A and water.

Species: Female SKH/HR1 hairless mice

of Animals: 6 mice per group

Test Conditions: Part I: Two concentrations of OP (G0539.04) compared to vehicle in 6 mice/group. Mice were dosed topically with 0.05 ml. These mice were dosed with tritiated thymidine 3 hrs after treatment. Three additional mice were dosed with OP and vehicle but were not given tritiated thymidine but processed for histopathology.

Part II: Partial repeat of Part I with reformulated vehicle and vigorous stirring of dose solutions.

Results: Part I: All test preparations induced moderate erythema in all mice by 4 hours after application. The high dose of OP was discarded due to high variance. Low dose of OP decreased epidermal DNA synthesis.

Part II:

Test preparation had no discernible effect on skin color or thickness 4 hours after application. OP in this experiment did not decrease epidermal DNA synthesis.

Study #: B89-5000

**DNA SYNTHESIS IN MOUSE EPIDERMIS FOLLOWING TOPICAL APPLICATION
OF A TEST SUBSTANCE SUPPLIED BY PROCTER AND GAMBLE**

STUDY DIRECTOR: Michael J. Connor, PhD.

STUDY SITE: Division of Dermatology, UCLA School of Medicine.

PURPOSE OF STUDY:

The aim of this study was to measure the impact of a test substance supplied by Procter and Gamble on the incorporation of tritiated thymidine into the epidermal DNA of hairless mice.

ANIMAL SUBJECTS:

Female skh/HR1 (hairless) mice, aged 8-10 weeks, were used throughout. The mice were housed and treated according to institutional ethical guidelines.

TEST SUBSTANCES AND PREPARATIONS:

Four test substances were received from Procter & Gamble:

- G0539.04 - white powder
- HCO479-86B - straw colored viscous liquid
- Solution A - straw colored viscous liquid containing a white powdery suspension that settled out on standing.
- Compound B - white powder

Dose preparations were formulated from these substances as follows:

Part I

- 1) Vehicle - 2.5 g HCO479-86B diluted to 100 ml with water.
- 2) G0539.04 A - 0.5 g G0539.04 + 2.5 g HCO479-86B diluted to 100 ml with water.
- 3) G0539.04 B - 1.0 g G0539.04 + 2.5 g HCO479-86B diluted to 100 ml with water.

Part II

- A) 9 g solution A dissolved in 90 g water.
- B) 9 g solution A and 1 g compound B dissolved in 90 g water.

TREATMENT OF SUBJECTS AND DESCRIPTION OF METHODS:

The study was performed in 2 parts.

In the initial part of the study 3 treatment groups of 6

mice were dosed topically on the dorsal skins with 0.05 ml of preparations 1, 2, or 3. Three hours after treatment they were dosed with tritiated thymidine (30 uCi/mouse). One hour later the mice were sacrificed, the skins were removed, and the epidermis was recovered and processed for determination of the DNA content. Additional groups of 3 mice were treated with 0.05 ml preparations 1, 2, or 3 but were not dosed with tritiated thymidine. These mice were sacrificed 4 hours after treatment. The skins were recovered, were placed in neutral buffered formalin, and were sent to Procter and Gamble for histopathological study.

The second part of the study was a partial repeat of the initial study (using a reformulated vehicle), and was performed because of an unusually high statistical variance in the group treated with preparation 3. Two groups of 6 mice were treated with preparations A and B. Tritiated thymidine incorporation into epidermal DNA was determined in these two groups of mice as described above.

The test materials were stirred vigorously during sampling to reduce potential dosing errors due to settling of the test preparations.

EXTRACTION OF DNA AND DETERMINATION OF THYMIDINE INCORPORATION:

Epidermal DNA was extracted and determined by the Burton reaction as described in Connor and Lowe, Cancer Res., 43: 5174-5177, 1983. Radioactivities associated with the acid soluble fraction and with the extracted DNA were measured to obtain estimates of the thymidine pool size and incorporation rates respectively.

RESULTS:

Test Preparations

Test preparations 1, 2, and 3 had a pH of about 8.
Test preparations A and B had a pH of about 6.

Macroscopic/Gross Effects of Test Preparations

Test preparations 1, 2, and 3 induced moderate erythema in all mice by 4 hours after application.
Test preparations A and B had no discernible effect on skin color or thickness 4 hours after application.

Effects on DNA Synthesis

Individual and mean values for groups 1 and 2, and for groups A

and B are presented in tables 1 and 2 respectively. Incorporation data from the mice treated with test preparation 3 was discarded due to a high variance, suggestive of uneven dosing with the test substance (this was a heavy suspension).

Preparation 2 statistically significantly decreased ($p < 0.05$) thymidine incorporation into epidermal DNA from 5.24 ± 0.58 cpm per ug DNA to 3.00 ± 0.43 cpm per ug DNA, i.e. a 43% suppression.

Preparation B had no statistically significant effect on thymidine incorporation into epidermal DNA compared to preparation A (vehicle) (Means 5.80 ± 1.30 cpm per ug DNA compared to 6.17 ± 1.62 cpm per ug DNA respectively).

Table 1: SUMMARY OF DATA FROM PART I

Mouse #	DNA (mg)	Soluble		Incorporated	
		cpm	Specific Activity	cpm	Specific Activity
Test Preparation 1 (vehicle)					
1	.58	62359	106	2724	4.71
2	.79	76832	97	3967	5.03
3	.79	77180	98	4355	5.51
4	.58	59400	119	3070	5.26
5	.59	57567	98	3657	6.23
6	.73	59687	82	3441	4.70
		Means	100 ± 12		5.24 ± 0.58
Test Preparation 2 (G0539.04A)					
7	.42	39655	95	1256	3.02
8	.42	46205	109	1628	3.85
9	.56	42700	76	1641	2.90
10	.74	47247	64	2112	2.85
11	.52	38550	75	1380	2.67
12	.61	42271	69	1677	2.73
		Means	81 ± 17		3.00 ± 0.43

Specific activities are cpm per microgram DNA

Soluble - refers to acid soluble radioactivity

Incorporated - refers to acid precipitable DNA-incorporated radioactivity

Table 2: SUMMARY OF DATA FROM PART II

Mouse #	DNA (mg)	Soluble		Incorporated	
		cpm	Specific Activity	cpm	Specific Activity
Test Preparation A (vehicle - solution A in water)					
1	.33	31167	94	1528	4.59
2	.56	53841	96	4315	7.65
3	.49	58726	120	3912	8.00
4	.43	44811	105	2990	7.02
5	.38	30745	80	2124	5.53
6	.39	27790	71	1655	4.20
		Means	94 ± 18		6.17 ± 1.62
Test Preparation B (compound B in vehicle)					
7	.44	50826	115	2111	4.75
8	.37	51931	140	2508	6.74
9	.34	34032	99	1535	4.46
10	.44	45525	104	2671	6.10
11	.37	58384	159	2867	7.81
12	.33	39322	120	1658	5.07
		Means	122 ± 23		5.80 ± 1.30

Specific activities are in cpm per microgram DNA

Soluble - refers to acid soluble radioactivity

Incorporated - refers to acid precipitable DNA-incorporated radioactivity

Identification of Test Substances

G0539.04 : Octopirox raw material (solid)

HC0479-86B : Tween 80

Solution A : 2.5% Tween 80
0.59% citric acid solution (50%)
0.71% sodium citrate
water

Compound B : Octopirox raw material (solid)



Attachment 2

PROCTER & GAMBLE PATHOLOGY NUMBER B89-5000

INVESTIGATIVE ACUTE MOUSE PAINTING STUDY

HISTOPATHOLOGY REPORT

Gary R. Johnson

Date: 11/11/70

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Study: B69-5000

HISTOPATHOLOGY REPORT

PROCTER & GAMBLE STUDY NUMBER B89-5000
INVESTIGATIVE ACUTE MOUSE PAINTING STUDY
HISTOPATHOLOGY REPORT

SUMMARY AND CONCLUSIONS

Three groups of eight hairless mice (Skh:HR1) were treated topically with control vehicle or test substance in control vehicle. Five mice per group were injected intraperitoneally with tritiated thymidine three hours later. Three mice per group, not injected with tritiated thymidine, were used to provide skin specimens for histopathologic evaluation. All mice were killed four hours following topical treatment. The purpose of histopathologic evaluation was to determine if the test substance produced any morphologic evidence of an epidermal antiproliferative effect and/or if nontreatment-related lesions were present that might have an effect on the interpretation of data from the tritiated thymidine part of the study.

There was no proliferative, degenerative, inflammatory, or cytopathologic evidence of a test substance-induced effect. Incidental lesions seen focally in mice from all groups were consistent with rubbing or scratching. Inflammation around keratin follicular cysts and aborted hair follicle formation was seen in all mice. This is a normal condition in the hairless mouse. The focal nature of the lesions and their slight to moderate severity suggest they would have only a minor, if any, effect on tritiated thymidine uptake by epidermal cells over a 12.5 sq. cm. treatment area.

PURPOSE OF STUDY

This investigative acute painting study was primarily designed to determine the antiproliferative activity of a test substance supplied by Procter & Gamble by examining the amount of tritiated thymidine incorporated per unit weight of DNA from treated and nontreated skin of the hairless mouse. A second purpose of the study was to look for histopathologic evidence of an epidermal effect and to establish the presence or absence of nontreatment-related lesions.

BASIC STUDY DESIGN

The in-life portion of this study and the tritiated thymidine uptake studies were contracted to Dr. Michael Connor, Dermatologic Research Foundation. Skin from control and test mice not receiving thymidine were sent to Procter & Gamble for histopathologic evaluation. The protocol as prepared for the contract portion of the study appears in the appendices.

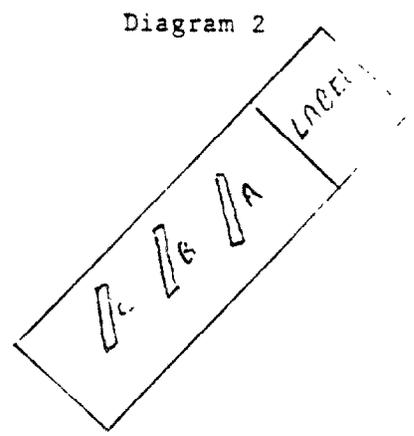
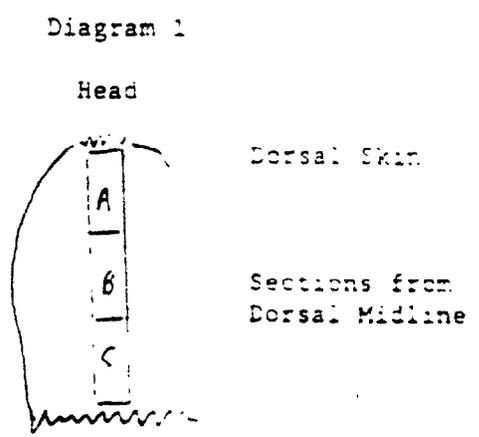
The materials and methods for the histopathologic portion of the study follows:

The skin was surgically removed from the dorsal area of the mice not injected with tritiated thymidine (three mice per group). The skin was placed dermis side down on a piece of photographic paper to prevent curling, and then put in a container of 10% neutral buffered formalin (NBF). There were nine (9) samples of skin received for histopathology. The groups and treatments are:

<u>Group No.</u>	<u>No. of Animals</u>	<u>Treatment</u>
1	6	Vehicle control HCC-79-865 S61
2	6	50 ul of G0539.04 A
3	6	50 ul of G0539.04 B

Three sections from each sample were taken out of the center of the dorsal area and fixed in 10% NBF for at least 24 hours prior to processing (see Diagram 1). The samples were dehydrated through various concentrations of alcohol before embedding.

The samples were embedded in paraffin in cassettes. Following polymerization the samples were sectioned on a microtome at 5-6 um thick. The sections were picked up off a heated water bath onto glass slides and allowed to dry. The sections were stained with hematoxylin and eosin (H&E). Coverslipping the slides was done to permanently seal a mounted, stained tissue and to provide the proper refraction of light for examination of the tissue with a light microscope. The skin sections on the slide were identified as A, B, C (see Diagram 2). Histopathologic evaluation was done using a Leitz orthoplan microscope.



STUDY RESULTS

Results of the histopathologic evaluation are presented in Table 1. Each of the skin sections (A, B, and C) from all three mice in the control and test substance-treated groups had small focal areas of slight to moderate epidermal acanthosis (hyperplasia) with occasional hyperkeratosis. In some of the acanthotic areas there was a slight infiltrate of inflammatory cells with or without slight spongiosis. The majority of epidermis in each section, however, was normal in thickness for this strain of mouse and had no inflammatory cells or edema (spongiosis). Neither the focal lesion areas nor the nonlesion areas showed any evidence of keratinocytic cell degeneration or necrosis. Cytoplasmic and nuclear morphology and tinctorial character were normal.

The dermis contained multifocal areas of slight to moderate increases in cell numbers in the majority of sections (see Table 1). The cells were primarily neutrophils and macrophages and were associated with keratin follicular cysts and/or aborted hair follicle formation.

DISCUSSION

The test substance (G0539.04 A and G0539.04 B) did not result in any epidermal or dermal lesions. No proliferative (hyperproliferative/hypoproliferative), degenerative, or inflammatory lesions were seen in the epidermis or dermis (including subcutis) that could be attributed to acute topical application of test substance. The changes seen histologically occurred in the skin of mice from both vehicle and test material in vehicle treated groups. While a vehicle effect cannot be entirely

discounted as no nonvehicle treated group was present, the types of lesions observed (epidermal acanthosis, hyperkeratosis, and increased dermal cellularity) and their focal or multifocal distribution are consistent with these authors' experience with hairless mice, this strain in particular. Simple rubbing or scratching is sufficient stimulus for focal epidermal acanthosis. Also since tissue collection was four hours post-treatment, the time period was too short for treatment-associated proliferative changes to occur. Inflammation associated with keratin follicular cysts and aborted hair follicle development is common in hairless mice. It also occurred in the vehicle only treated group and was sufficiently mature in its development to preclude its being test related, i.e., vehicle or vehicle and test substance.

It is difficult to precisely determine the effect the lesions observed histologically might have on tritiated thymidine labeling of dividing keratinocytes. Since the lesions, especially epidermal acanthosis, were focal or multifocal, it appears unlikely they would have a significant effect on labeling over the entire 12.5 sq. cm. topical application site.

APPENDIX

HISTOPATHOLOGIC RESULTS

TABLE 1 - INDIVIDUAL ANIMAL SKIN LESIONS

Histopathologic Results

Table 1 - Individual Animal Skin Lesions

Study: B89-5000

	Group 1			Group 2			Group 3		
Group:	1	1	1	2	2	2	3	3	3
Section on Slide:	A	B	C	A	B	C	A	B	C
Skin Lesions:									
Epidermis									
Acanthosis	2*	1	2	2	2	1	1	1	2
Hyperkeratosis	1	1	0	1	0	0	1	0	0
Cellular Degeneration	0	0	0	0	0	0	0	0	0
Cellular Necrosis	0	0	0	0	0	0	0	0	0
Spongiosis	1	0	0	1	0	0	0	0	0
Inflammatory Cell Exocytosis	1	0	0	1	0	0	0	0	1
Dermal Cellularity									
Area Above Cysts	2	1	1	0	0	0	1	0	2
At Cystic Area	1	1	1	1	2	2	1	1	2

*Histologic Grading Scale in the Hairless Mouse

Epidermal Thickening

- 0 = Normal epidermal thickness
- 1 = Slight increase in thickness
- 2 = Moderate increase in thickness
- 3 = Large increase in thickness

Increase in Dermal Cellularity

Area Above Cysts

- 0 = Normal cell numbers
- 1 = Slight increase in cell numbers
- 2 = Moderate increase in cell numbers
- 3 = Large increase in cell numbers

At Cystic Area

Same scale - grade separately

APPENDIX
CONTRACT LAB
PROTOCOL FOR ACUTE MOUSE PAINTING STUDY

CONTRACT OBJECTIVES AND BUDGET

Jane Kelly
B89-5000

This contract is for one experiment using the procedure described below to determine the incorporation of tritiated thymidine into the DNA of skin. The experiment will be conducted, finished and final report submitted by November 6, 1983.

The experiment consists of three groups of eight animals. The objective and description of the experiment that will be conducted under this contract are as follows:

Objective: To determine the antiproliferative activity of a material supplied by Procter by examining the amount of tritiated thymidine incorporated per unit weight of DNA from treated and non-treated skin of the hairless mouse. Hairless mice (SKH:HR1) will be treated topically with control vehicle and test substance in vehicle prior to intraperitoneal injection of tritiated thymidine. Following sacrifice the DNA is extracted from the epidermis and counted in a liquid scintillation spectrometer. Comparison of treated groups to control vehicle will allow for determination of changes in radiolabel incorporation into DNA. Indirect effects that could cause changes in the amount of incorporation will be investigated by determining the amount of radiolabel in the precursor pool and by histopathological examination of treated skin.

Experimental Design:

The procedure used in this study is described in the following reference: Connor, MJ, Lowe, NJ: The induction of ornithine decarboxylase and DNA synthesis in hairless mouse epidermis. Cancer Res. 43, 5174-5177, 1983.

This will specifically include the following steps:

- 1.) Apply 50 ul of the test material (G0539.04 A and G0539.04 B) to 12.5 sq. cm. of the dorsal surface at time-zero.
- 2.) Inject selected mice (five per group) with the tritiated thymidine at time = 3 hours. Do not inject three mice per group.
- 3.) Sacrifice all animals at time = 4 hours.
- 4.) Surgically remove treated skin of mice injected with tritiated thymidine and homogenize in perchloric acid for determination of radioactivity and DNA.
- 5.) Surgically remove the skin from the treatment area from the mice not injected with tritiated thymidine (3 per group). Place the skin dermis side down on a piece of cardboard (such as poster board) to prevent curling and then put in container containing 10 % neutral buffered formalin. The container should be labeled with animal identification number, and treatment group. Send these samples to the sponsor for histopathological examination.

6.) Count radioactivity in the acid soluble extract using the supernatant obtained in the first centrifugation of the homogenized tissue combined with the supernatant from the first washing of the precipitate.

7.) Count the radioactivity in the acid precipitable fraction (DNA).

8.) Report cpm 3H/ug DNA for each treatment group for both the acid soluble (precursor pool) and acid precipitable (DNA) fractions.

DOSE GROUPS

<u>Group</u>	<u>Number of Animals</u>	<u>Treatment</u>
1	8	Vehicle control HCO479-86B solution
2	8	50 ul of G0539.04 A
3	8	50 ul of G0539.04 B

STATISTICAL ANALYSIS: Compare treatment groups to the vehicle control with the level of significance at $p = 0.05$ and $p = 0.01$ using Student's T test. Report individual measurements, group means and group standard deviations for the acid soluble and acid precipitable counts.

RECORDS TO BE MAINTAINED: All records that would be required to reconstruct the study and to demonstrate adherence to the protocol should be maintained.

REPORTS : Upon completion of the study, a summary report shall be prepared. This report shall be prepared in a scientific format and will include all data in a clear and precise, and readily understood style.

Any deviation from the protocol will be discussed with the sponsor and documented in the files of the study.

Test Material for Screen in the Hairless Mouse Thymidine Incorporation Assay.

There are two materials marked as follows.

<u>Test Material Code</u>	<u>Color</u>	<u>Form</u>	<u>Amount Included</u>
G0539.04	White	Solid	14.48 g
HCO479-86B	Yellow	Liquid	180 g

Dosing Instructions

A volume of 50 ul of the following mixtures will be dosed to the dorsal side of the hairless mice:

<u>Treatment</u>	<u>Volume</u>	<u>Dosing Solution</u>
1. Vehicle	50 ul	Dilute 2.5 g of HCO479-86B to 100 ml.
2. G0539.04 A B	50 ul	Add 0.5 g of G0539.04 to 2.5 of HCO479 - 86B and dilute to 100 ml.
3. G0539.04 B B	50 ul	Add 1.0 g of G0539.04 to 2.5 of HCO479-86B and dilute to 100 ml.

Use distilled water for the dilutions. The concentrations indicated are for the treatment of an area of 12.5 sq cm. If a larger or smaller area is treated the doses should be readjusted accordingly. The sponsor must be contacted if this happens to reassure that the concentrations used for dosing are as intended.

All dosing solutions should be prepared on the day of dosing. In addition, the dosing solutions must be stirred continuously during sampling to eliminate settling of particles.

The histological samples should be sent by Federal Express to:

Dr. J.W. Kirley
The Procter and Gamble Co
Sharon Woods Technical Center
11511 Reed Hartman Highway M3 Bldg
Cincinnati, Ohio 45241