

Report Title: Studies on the Potential of Octopirox to Inhibit DNA Synthesis in CD-1 Mouse Epidermis *In Vivo*

Test Type: Genotoxicity Study

Conducting Laboratory and Location: P&G, Miami Valley Laboratories, Biological Testing Facility, Cincinnati, OH

Test Substance(s): G0539.05 – 1% Octopirox in shampoo with and without radiolabeled Octopirox (#BPO-5884 ([6-¹⁴C]-Octopirox)

Species: Female CD-1 mice

of Animals: 4 mice per group for the time course and deposition studies and 10 mice per group for the dose response

Test Conditions: Skin deposition of Octopirox from the radiolabelled shampoo (0.2 or 0.3 ml) was quantified after either 1 or 4 daily dermal applications. Mice were dosed with shampoo, rinsed and dried. After dosing the treated areas were excised, solubilized and radioactivity quantified.

Results: 1) The lowest dose producing a statistically significant inhibition of epidermal DNA synthesis was 0.3 mg or 37.8 ug/cm² which inhibited DNA synthesis.

2) After either 1 or 4 daily applications of OP-containing shampoo, followed by rinse off, the deposition of OP was 1 ug/cm².

3) Consistent with this level of deposition, and the dose response of OP in ethanol, the OP-containing shampoo did not cause detectable inhibition of epidermal DNA synthesis after 1,4 or 8 daily applications.

Study #: B90-0258B, B90-0258C, Bb90-0269A, B90-0269B, B90-0338B, B91-0033, and B91-0044

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RESEARCH AND DEVELOPMENT DEPARTMENT REPORT
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Studies on the Potential of Octopirox to Inhibit DNA Synthesis in
CD-1 Mouse Epidermis *In vivo*

SUMMARY

A series of experiments were conducted to evaluate the potential of Octopirox to inhibit epidermal DNA synthesis, when topically applied to CD-1 mice in ethanol or a shampoo matrix. Epidermal DNA synthesis was determined by measuring the incorporation of [³H]-thymidine into DNA.

In a time-course study, a dose of 1 mg of Octopirox applied in 0.1 ml of ethanol caused a transient decrease in epidermal DNA synthesis. A maximal inhibition of DNA synthesis of about 80% was observed 4 - 6 hr after dosing. This was followed by a recovery and an apparent rebound to ~1.6-times the control level of DNA synthesis by 20 hr after dosing.

A dose-response experiment was conducted in which Octopirox was applied in ethanol and mice were killed 4 hr after treatment. The lowest dose producing a statistically significant inhibition of epidermal DNA synthesis was 0.3 mg (~38 $\mu\text{g}/\text{cm}^2$), which inhibited DNA synthesis 58%. In this experiment, 1 mg of Octopirox (~130 $\mu\text{g}/\text{cm}^2$) caused about a 71% inhibition of DNA synthesis.

In all experiments in which Octopirox was applied topically to mice in a shampoo matrix, the shampoo used contained 1% Octopirox, and was left on the shaved dorsal skin of mice for 5 minutes, then was rinsed off with warm water. The deposition of Octopirox under these conditions was determined using a shampoo spiked with [¹⁴C]-Octopirox. Samples of full thickness skin were collected from the excised treatment site using biopsy punches, and radioactivity was quantified after solubilization of the tissue. This method for measuring deposition was compared to a tape-stripping method, previously used in a human clinical study (1). The tape-stripping method was found to underestimate the deposition of Octopirox on mouse skin by about a factor of 3.

The deposition of Octopirox on mouse skin after a 1 or 4 daily applications of shampoo was ~1 $\mu\text{g}/\text{cm}^2$. Consistent with this level of deposition, and the dose-response for inhibition of DNA synthesis by Octopirox applied in ethanol, the Octopirox-containing shampoo did not inhibit epidermal DNA synthesis after 1, 4 or 8 daily applications.

Procter & Gamble

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QUALITY ASSURANCE STATEMENT

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TEST FACILITY: The Procter & Gamble Company
Miami Valley Laboratories
Cincinnati, Ohio 45239

TYPE OF STUDY: Studies on the Potential of Octopirox to
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Epidermis In vivo

TSIN: G0539.05, BPO-5884

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<u>PORTION(S) OF STUDY AUDITED:</u>	<u>AUDITOR:</u>	<u>DATE AUDITED:</u>	<u>DATE REPORTED TO STUDY DIRECTOR:</u>
Study Data	L. K. Klahr	5/1/92	5/6/92
	M. P. Bauer	5/5/92	5/6/92

This is an investigational program. Only the final study report was audited. No other QA activities occurred. The results presented in this report accurately reflect the raw data of the study.

M. P. Bauer 5/7/92
Quality Assurance Unit - Date

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I. PURPOSE

The purpose of the experiments described here was to evaluate the potential of Octopirox to inhibit epidermal DNA synthesis, when applied to the skin of CD-1 mice in ethanol or a shampoo matrix.

II. METHODS

Materials

Octopirox (TSIN GO539.05) was from Beauty Care Product Development. The shampoo preparations tested were: 1) shampoo matrix without Octopirox (batch M217/3958) and 2) shampoo matrix with 1% Octopirox (batch M217/3957), obtained from Procter & Gamble, Health and Beauty Care Limited, Egham, U.K. (formulations are listed in Appendix I). [6-C¹⁴]-Octopirox, used to prepare radiolabelled shampoo (see below), was Hoechst lot 15075 (P&G radiochemical number BPO-5884, specific activity of 2.3 mCi/g). The radiochemical purity of this material was checked by S. Heitmeyer and found to be essentially 100% (report in Appendix V). Calf thymus DNA was obtained from Sigma Chemical Co. and [(methyl)-³H]-thymidine (5 Ci/mmol) was purchased from Amersham. BTS-450 tissue solubilizer, ReadyOrganic and ReadySafe liquid scintillation cocktails were purchased from Beckman. Other chemicals were of reagent grade or higher quality and their sources are indicated in the study notebooks.

Shampoo containing 1% Octopirox and 0.15% [C¹⁴]-Octopirox for deposition experiments was prepared as follows: 1) 0.85 parts of shampoo with 1% Octopirox was mixed with 0.15 parts of shampoo matrix without Octopirox in a sealed bottle. 2) The mixture was heated to 70° C in a waterbath and stirred for about 20 min. 3) 1.5 mg [C¹⁴]-Octopirox/g shampoo was added to the heated shampoo, which was then stirred for 10 minutes, then allowed to stand at 70° for 5 min to let air bubbles escape. The heated shampoo was a clear solution with no evidence of crystals. Subsequently, the radiolabelled shampoo was allowed to slowly cool to room temperature, at which time it was noted that it was more viscous and grainier in appearance than the original shampoo with 1% Octopirox. The cooled radiolabelled shampoo was found to be homogenous by counting samples from the top and bottom of the bottle, and the measured specific activity was within 1% of the calculated value.

Animals

Groups of female CD-1 mice were received from Charles River Laboratories at 6 weeks of age, and housed 5/shoebox cage on hardwood chip bedding. A 12 hr light/dark cycle (7:00 am to 7:00 pm) was maintained in the animal room (L-42), and Purina Lab Chow and water was available ad libitum. Room temperature and humidity were maintained to Biological Testing Facility (BTF) standards (BTF SOP: ENV 3,4). Mice were carefully shaved during the 7th week of age using a small animal clipper, and only mice in the resting phase of the hair cycle (i.e. animals without obvious hair regrowth within two days of shaving) and without shaving nicks were used (some mice did develop hair regrowth during the course of experiments). Mice were individually housed after shaving, and treatments did not begin until at least 2 days after shaving. In the experiments in which incorporation of [³H]-thymidine into DNA was measured, mice were uniquely identified with coded markings applied to the tail with a permanent ink marker. In the deposition experiments mice were not individually marked, because they were killed immediately after dosing. All mice were killed by CO₂ asphyxiation.

Treatments

Ethanol as the vehicle

Octopirox was applied in 0.1 ml of ethanol to the shaved dorsal skin using a micropipettor, and control mice were treated with ethanol. More specific details on experimental designs are indicated in Tables 1 and 2, and described in the Results section.

To allow dose to be expressed on a per cm^2 basis, the area of treated skin was estimated in the following way. Separate groups of mice were killed with CO_2 , then 0.1 ml of ethanol (group 1) or 1 mg of Octopirox in ethanol (group 2) was applied to shaved skin exactly as described above. The border of the treated area was marked with a permanent ink marker, and then the treated skin was excised and laid flat. The marked area was traced onto a piece of clear plastic film, and the areas were determined by G. M. Ridder using image analysis techniques, as described in Appendix IV.

Octopirox applied in a shampoo matrix

Octopirox was tested at a concentration of 1% in the shampoo matrix, and control animals were treated with the shampoo matrix without Octopirox. In different experiments either 0.2 or 0.3 ml of shampoo (indicated in tables) was applied to the dry, shaved dorsal skin, using a 1 ml disposable Tuberculin syringe. The applied dose was spread over the treated area with a glass rod. After application of shampoo, the treated mice were housed in cages without bedding for 5 min, then the shampoo was rinsed off with a spray of tap water adjusted to about 35°C . The period of rinsing generally was for 15 - 20 sec, then the mice were gently dried by dabbing once with paper towels. Sham-treated mice were similarly handled, but no test substance was applied to the skin.

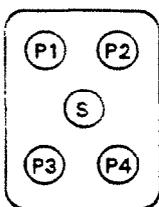
Epidermal DNA Synthesis

Epidermal DNA synthesis *in vivo* was determined by measuring the incorporation of [^3H]-thymidine into DNA by a modification of published methods (2,3), as described in detail in Appendix II. DNA was determined by the method of Burton (4) as described in Appendix III. In experiments in which DNA synthesis was measured, the dosing of mice was staggered so that the times of injection with [^3H]-thymidine and euthanasia were accurate (± 5 min).

Measurement of Skin Deposition of Octopirox from the Shampoo Matrix

Skin deposition of Octopirox from the radiolabelled shampoo described in "Materials" was quantified after either 1 or 4 daily applications. Mice were dosed with shampoo, rinsed and carefully dried off as described above. After the appropriate number of doses, the treated area was excised, and full thickness samples of skin were collected using disposable 6 mm biopsy punches. The skin punches were solubilized and radioactivity quantified by liquid scintillation counting. For comparison, a tape-stripping method used in a human clinical deposition study (1) was also used in the single application experiment. This involved collecting samples by tape-stripping a defined area on the treatment site 4 times, and then quantifying the radioactivity in the strips.

In the single application experiment the pattern and identification of the skin samples were as shown in the following diagram. In the multiple application experiment no tape-stripping was done, otherwise the pattern of collection of punches was the same.



S = tape-stripped site
(samples S1 - S4 in Table 3 were sequential strippings of site S).

PS = punch taken after stripping site S (not shown in diagram).

P1 - P4 = 6 mm punches taken without other treatment of sites.

Excised skin

Full thickness skin samples, collected with the biopsy punches, were placed in 1 ml of BTS-450 tissue solubilizer and allowed to sit at room temperature for 4 days, with occasional mixing. Glacial acetic acid (32 μ l) was added to neutralize the solubilizer, then 10 ml of ReadyOrganic liquid scintillation cocktail was added. All samples were counted using the standard C¹⁴-window in a Beckman LS5801 scintillation counter. The H-number method was used to correct for quenching. In each experiment two biopsies collected from an untreated mouse, were processed as indicated above to determine the counting background.

For tape stripping, 6 mm circles of double-sticky 3M ScotchTM tape were applied to the ends of 1/4" (0.635 cm) plexiglasTM rods. The site on the excised skin to be sampled was marked with an ink circle slightly larger than 6 mm in diameter, and the double-sticky tape was pressed against the skin within the circle, held there for a few seconds, then pulled off. In this way each site was stripped 4 times, and the tape strippings were individually counted for radioactivity. The tape was removed from the rods with a forceps and placed in a scintillation vial with 1 ml of dimethyl sulfoxide, and allowed to sit for 4 days, then 10 ml of ReadyOrganic scintillation cocktail was added. The stripped site was also punched out to allow measurement of any remaining Octopirox. Processing of the punch and liquid scintillation counting was as indicated above. For determination of the counting background, 2 strips were collected from an untreated mouse.

Statistics

For statistical analysis of data, analysis of variance was used, providing that Barlett's test of homogeneity was not significant. Otherwise, Wilcoxon's rank sum test was used (5). Linear regression analysis was performed, using dose as the independent variable (6). This analysis included a check for lack of fit. All statistical tests were conducted at a 5%, two-sided risk level. Significance at the 1% and 0.1% levels are also reported, where appropriate.

III. RESULTS

Topical Application in Ethanol

The levels of epidermal DNA synthesis, expressed as dpm/ μ g of DNA, at various times after the topical application of 1 mg Octopirox in ethanol are shown in Table 1 and Figure 1. It can be seen that under these conditions Octopirox caused a transient inhibition of DNA synthesis, with a maximal inhibition of about 80% occurring 4 - 6 hr after dosing. Subsequently, there was a recovery and the level of DNA synthesis 20 hr after dosing was about 1.6-times the concurrent control level. However, this elevation in DNA synthesis was not statistically significant. Octopirox caused about a 20% increase in acid soluble dpm/mg of epidermis 4 - 6 hr after dosing, which was statistically significant. Because of this small increase in [³H]-thymidine uptake, the level of inhibition of DNA synthesis by Octopirox may have been slightly underestimated.

The dose-response for the inhibition of epidermal DNA synthesis 4 hr after the topical application of Octopirox is shown in Table 2 and Figure 2. In order to allow the dose to be expressed as μ g/cm², the areas of skin covered by 0.1 ml of ethanol or 1 mg of Octopirox in ethanol were determined in

groups of 4 mice, as described in Methods. The measured areas were 8.25 ± 1.56 and 7.63 ± 1.26 cm², for the ethanol and 1 mg groups, respectively (data \pm S.D.). These results indicate that the high dose Octopirox solution spread over essentially the same area as ethanol alone. Therefore, an overall mean value of 7.94 cm² was used to estimate dose on a $\mu\text{g}/\text{cm}^2$ basis for all dose groups.

The lowest dose of Octopirox producing a statistically significant inhibition of epidermal DNA synthesis was 0.3 mg (~ 38 $\mu\text{g}/\text{cm}^2$), which inhibited DNA synthesis 58%. In this experiment 1 mg of Octopirox (~ 130 $\mu\text{g}/\text{cm}^2$) caused about a 71% inhibition of DNA synthesis. Octopirox was without effect on the level of acid soluble dpm/mg of epidermis, except at the 1 mg dose level. At that dose acid soluble dpms were about 20% higher than the control level ($p < 0.01$), consistent with the results of the time-course experiment described in Table 1. The slightly increased uptake of [³H]-thymidine may have been due to irritation and vasodilation; 3/10 mice at the 1 mg dose had slight erythema, and 1/10 had slight edema at the time of killing. No evidence of irritation was noted in mice at the lower doses. Again, because of the increased availability of [³H]-thymidine, the degree of inhibition of DNA synthesis by the high dose of Octopirox may have been slightly underestimated.

Deposition from a Shampoo Matrix

Results of determinations of the deposition of Octopirox from a shampoo matrix containing 1% Octopirox are shown in Tables 3 and 4. Table 3 shows a detailed comparison of the data obtained from individual mice using the tape-stripping and biopsy punch methods. For each mouse the deposition measured in the individual skin punches was very similar, indicating uniform deposition over the treatment area. Averaged over all mice, the level of deposition determined using the punch biopsy method (1.01 $\mu\text{g}/\text{cm}^2$) was about 2.9-times higher than that determined by tape-stripping (0.35 $\mu\text{g}/\text{cm}^2$). From each animal a total of 4 sequential tape-strips were collected from the same site, then the site was cut out with a biopsy punch. In Table 3 it can be seen that with sequential strips there is a general tendency for the amount of [¹⁴C]-Octopirox/cm² to decrease. However, in all cases the last strip still collected substantial Octopirox. Furthermore, the full thickness punches of the stripped sites had levels of Octopirox similar to the punches from sites that were not tape-stripped. For each mouse, the total Octopirox recovered in the 4 tape-strips and the punch of the stripped site, was somewhat higher than the average of the full thickness punches. This was probably a result of the sequential tape-strips and the final punch not sampling exactly the same area. However, the data clearly indicate that the tape-stripping method underestimated the deposition of Octopirox.

In Table 4 the skin deposition of Octopirox after 1 or 4 daily applications of shampoo is shown. (The single application data are from the experiment described in detail in Table 3.) These two experiments were done under similar but not identical conditions. In the single application experiment, 0.2 ml of shampoo was applied, while in the multiple application experiment a volume of 0.3 ml was used, to allow better coverage of the treatment site. These volumes were consistent with those used in subsequent DNA synthesis inhibition experiments. Similar results were obtained in both experiments, with deposition of about 1 μg of Octopirox/cm² of treated skin.

Epidermal DNA Synthesis in Mice Treated with Shampoo Containing Octopirox

Table 5 shows the results of experiments in which the effects on epidermal DNA synthesis of 1 or 4 daily applications of shampoo with 1% Octopirox were evaluated. Mice were killed either 4 or 20 hr after the last treatment. These were the times when a maximal inhibition of DNA synthesis, or an apparent rebound to a higher than control level of DNA synthesis, occurred in mice treated with Octopirox in ethanol. Sham-treated controls were included to determine whether the shampoo treatment alone had any effect. After 1 or 4 applications, neither shampoo with Octopirox nor

shampoo base produced any detectable change in the incorporation of [³H]-thymidine into DNA or in the acid soluble dpm/mg of epidermis.

Table 6 summarizes the results of two experiments in which the effects on epidermal DNA synthesis of 8 daily applications of shampoo containing 1% Octopirox were evaluated. Control mice were included that received only 7 doses, but were killed the same time as the mice receiving 8 doses. These controls were used to allow evaluation of the effects of the last dose on the background of any reponse from the previous dose. For example, if a rebound effect was induced, and the last treatment caused an inhibition of DNA synthesis, it might not be possible to detect an effect without knowing the levels of DNA synthesis induced by the previous treatment. In the first experiment in Table 6, sham-treated mice were also included to allow evaluation of any effect of the shampoo base. Again the the shampoo base and Octopirox-containing shampoo were without effect on either epidermal DNA synthesis or the acid soluble dpm/mg of epidermis. In the case of the Octopirox-containing shampoo, this was true whether the results were compared to those obtained with the shampoo base control, which received 8 treatments, or the Octopirox shampoo control receiving only 7 treatments. The levels of DNA synthesis in the mice receiving 7 treatments of Octopirox and then killed 28 hr later were about 30% lower than those similarly treated with the shampoo base. However, these differences were not statistically significant (p values >0.1), and were likely to have been chance occurrences. This conclusion is supported by the shampoo deposition data (Table 4) and the dose-response for inhibition of DNA synthesis by Octopirox applied in ethanol (Table 2).

IV. DISCUSSION

The data presented here demonstrate that a dose of 1 mg of Octopirox applied to the dorsal skin of CD-1 mice in 0.1 ml of ethanol (~130 $\mu\text{g}/\text{cm}^2$) caused a transient inhibition of epidermal DNA synthesis. At this dose a maximal inhibition of about 70 - 80% occurred 4 - 6 hr after dosing. By 20 hr after dosing incorporation of [³H]-thymidine into DNA had rebounded to 1.6-times the control level. While this effect was not statistically significant, a similar non-significant rebound in epidermal DNA synthesis was observed when a 7.5 mg dose of Octopirox was applied on the skin of mice of the MutaTM Mouse strain (7). The pattern observed after application of Octopirox to mouse skin, with a rapid inhibition followed by a rebound to higher than control levels, is consistent with that seen with other agents that inhibit epidermal DNA synthesis in mice, such as hydroxyurea (8) and acetic acid (9).

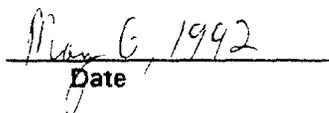
In the dose-response experiment, in which Octopirox was topically applied in ethanol, the lowest dose which caused a statistically significant inhibition of DNA synthesis was 0.3 mg or -37.8 $\mu\text{g}/\text{cm}^2$. After either 1 or 4 daily applications of Octopirox-containing shampoo, followed by rinse-off, the deposition of Octopirox was -1 $\mu\text{g}/\text{cm}^2$. Consistent with this level of deposition, and the dose-response obtained with Octopirox applied in ethanol, the Octopirox-containing shampoo did not cause a detectable inhibition of epidermal DNA synthesis after 1, 4 or 8 daily applications.

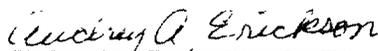
V. ACKNOWLEDGEMENTS

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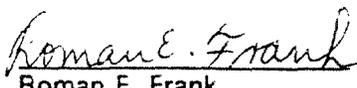


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TABLE 1. TIME-COURSE FOR INHIBITION OF EPIDERMAL DNA SYNTHESIS BY A SINGLE 1 MG DOSE OF OCTOPIROX APPLIED IN 0.1 ML OF ETHANOL^A

Hours After Dosing	dpm/ μ g DNA		Acid Soluble dpm/mg Tissue	
	Control	Octopirox	Control	Octopirox
2	201 \pm 55	60 \pm 30	1208 \pm 46	1148 \pm 57
4	128 \pm 20	27 \pm 6 ^B	1166 \pm 41	1384 \pm 39 ^B
6	173 \pm 19	29 \pm 12 ^C	1208 \pm 58	1479 \pm 91 ^C
8	185 \pm 28	66 \pm 15 ^B	1160 \pm 78	1268 \pm 30
20	182 \pm 30	285 \pm 94	1159 \pm 45	1257 \pm 129

^A N=4; all values \pm S.E. Study B91-0033, Notebook YE-1418, pg. 110.

^B Significantly different from respective control value, $p < 0.05$.

^C Significantly different from respective control value, $p < 0.01$

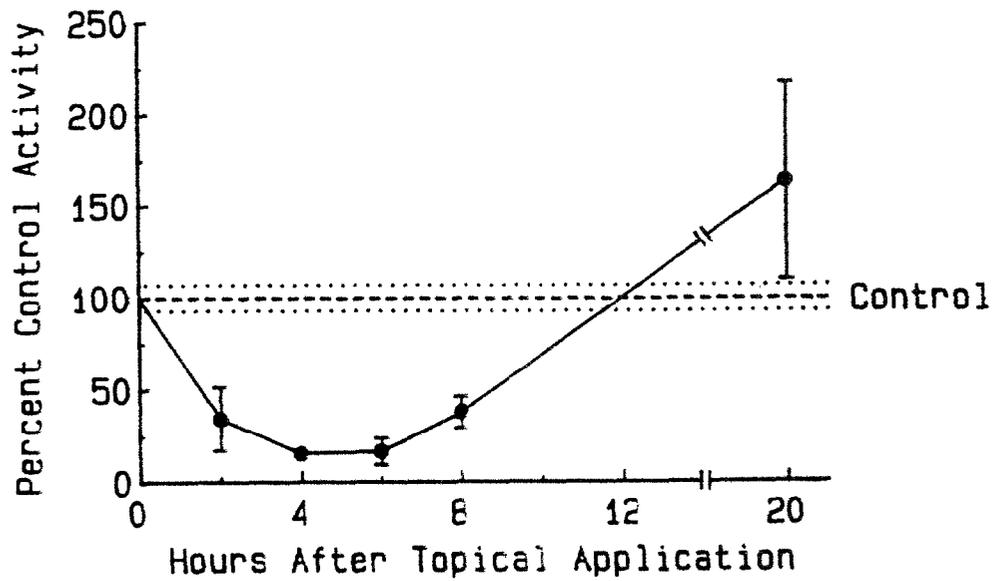


Figure 1. Time-course for inhibition of epidermal DNA synthesis by a single 1 mg dose of Octopirox applied in 0.1 ml of ethanol. At each time point the activity in the Octopirox-treated mice is expressed as percent of the overall mean activity in ethanol control mice. The data are the same as described in Table 1, and the dotted lines represent mean control value \pm S.E. N = 4.

TABLE 2. DOSE-RESPONSE FOR THE INHIBITION OF EPIDERMAL DNA SYNTHESIS BY OCTOPIROX APPLIED IN 0.1 ML OF ETHANOL

Octopirox Dose		dpm/ μ g DNA ^A	% Inhibition ^E	Acid Soluble dpm ^A mg tissue
mg	μ g/cm ²			
0	0	148 \pm 21	0	1014 \pm 37
0.010	1.26	125 \pm 13	15.8	1095 \pm 35
0.030	3.78	135 \pm 17	8.9	1051 \pm 38
0.100	12.6	116 \pm 11	22.1	1067 \pm 24
0.300	37.8	62 \pm 8 ^D	58.1	1064 \pm 39
1.00	126	44 \pm 17 ^D	70.5	1247 \pm 50 ^C

^A N=10; data \pm S.E. Study B91-0044, Notebook YE-1418, pg. 125.

^B Values shown were calculated prior to rounding the dpm/ μ g DNA data.

^C Significantly different than the control group, $p < 0.01$.

^D Significantly different than the control group, $p < 0.001$.

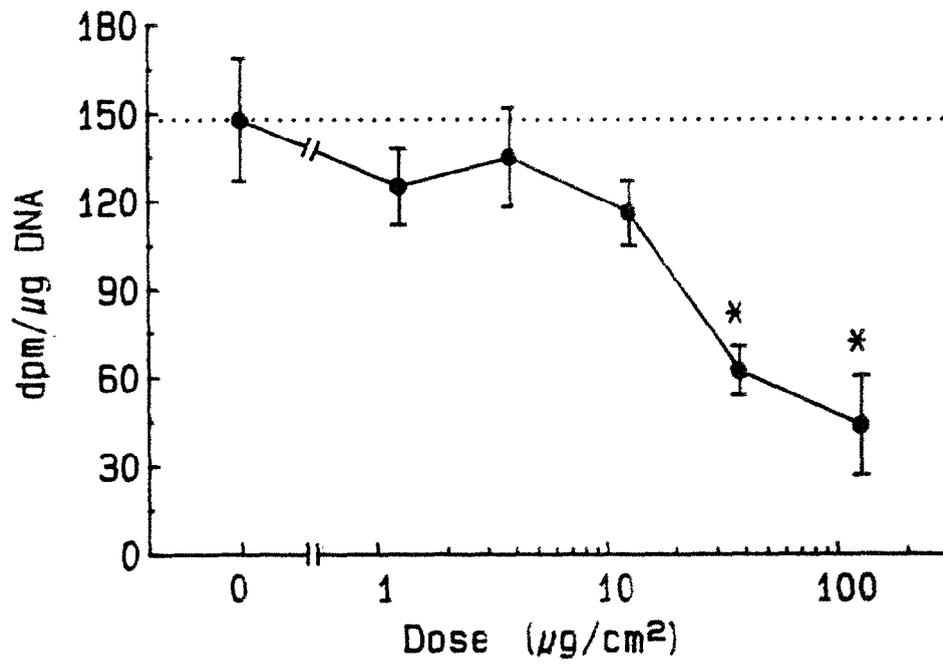


Figure 2. Dose-response for the inhibition of epidermal DNA synthesis by Octopirox in ethanol. $N = 10$; error bars indicate S.E. Data are the same as in Table 2. * Indicates significantly different than the control $p < 0.001$.

TABLE 3. COMPARISON OF TAPE-STRIPPING AND FULL SKIN THICKNESS SAMPLING FOR DETERMINATION OF THE SKIN DEPOSITION OF [^{14}C]-OCTOPIROX FROM A SHAMPOO MATRIX^A

SAMPLE	MOUSE			
	1	2	3	4
	$\mu\text{g } [^{14}\text{C}]\text{-Octopirox/cm}^2$			
Tape Strip 1 (S1)	0.063	0.138	0.347	0.073
Tape Strip 2 (S2)	0.061	0.099	0.095	0.035
Tape Strip 3 (S3)	0.054	0.072	0.076	0.032
<u>Tape Strip 4 (S4)</u>	<u>0.038</u>	<u>0.051</u>	<u>0.087</u>	<u>0.067</u>
Total of Strips	0.216	0.360	0.605	0.207
Punch of Stripped Site (PS)	0.916	1.16	0.732	0.842
Punch 1 (P1)	1.13	1.05	0.912	0.960
Punch 2 (P2)	1.14	1.39	0.963	1.12
Punch 3 (P3)	0.896	1.11	0.712	0.878
<u>Punch 4 (P4)</u>	<u>0.905</u>	<u>1.18</u>	<u>0.975</u>	<u>0.760</u>
Mean of Punch Values \pm S.D.	1.02 \pm 0.14	1.18 \pm 0.15	0.891 \pm 0.122	0.930 \pm 0.151

^A 0.2 ml of a shampoo containing 1% Octopirox was topically applied to mice then rinsed off after 5 minutes. Study B90-0258C, Notebook VE-1418, pg 135.

TABLE 4. DEPOSITION OF [¹⁴C]-OCTOPIROX FROM A SHAMPOO CONTAINING 1% OCTOPIROX AFTER 1 OR 4 DAILY APPLICATIONS.

NUMBER OF APPLICATIONS	TYPE OF SAMPLING	
	FULL THICKNESS PUNCH	TAPE-STRIPPING
	$\mu\text{g } [^{14}\text{C}]\text{-Octopirox/cm}^2$ ^A	
1 ^E	1.01 ± 0.06 ^C	0.35 ± 0.09 ^C
4 ^D	1.31 ± 0.10	ND ^E

^A N = 4, values ± S.E.

^B Study B90-0258C, Notebook VE-1418, pg 135. These are a summary of the data presented in Table 3. A volume of 0.2 ml of shampoo was applied to mice.

^C Significantly different $p < 0.05$

^D Study B90-0269B, Notebook VE-1418, pg 146. A volume of 0.3 ml of shampoo was applied to mice.

^E ND = not determined

TABLE 5. EPIDERMAL DNA SYNTHESIS IN MICE TREATED ONCE OR FOUR TIMES WITH SHAMPOO CONTAINING 1% OCTOPIROX^A

TREATMENT	HOURS AFTER DOSING	dpm/ μ g DNA	ACID SOLUBLE dpm/mg TISSUE
<i>1 Application of Shampoo^B</i>			
Sham	4	232 \pm 47	935 \pm 64
Shampoo Base	4	155 \pm 32	964 \pm 40
Octopirox Base	4	200 \pm 26	1051 \pm 63
Sham	20	153 \pm 23	735 \pm 83
Shampoo Base	20	220 \pm 36	823 \pm 46
Octopirox Shampoo	20	165 \pm 64	898 \pm 103
<i>4 Applications of Shampoo^C</i>			
Sham	4	239 \pm 44	1092 \pm 33
Shampoo Base	4	184 \pm 26	945 \pm 39
Octopirox Shampoo	4	250 \pm 51	982 \pm 46
Sham	20	221 \pm 19	983 \pm 45
Shampoo Base	20	185 \pm 47	1178 \pm 117
Octopirox Shampoo	20	270 \pm 71	1098 \pm 69

A N = 6, except 4 application, sham, 4 hr. where N = 5; all values \pm S.E.

B Study B90-0258B, Notebook YE-1418, pg 1. A volume of 0.2 ml of shampoo was applied to mice.

C Study B90-0269A, Notebook YE-1418, pg 41. A volume of 0.3 ml of shampoo was applied to mice.

TABLE 6. EVALUATION OF THE EFFECT OF 8 DAILY APPLICATIONS OF SHAMPOO CONTAINING 1% OCTOPIROX ON EPIDERMAL DNA SYNTHESIS.

TREATMENT ^A	HOURS BETWEEN LAST TREATMENT AND SAMPLING	dpm/ μ g DNA	ACID SOLUBLE dpm/mg TISSUE
<i>First Experiment^E</i>			
Sham, 7X	28	185 \pm 37	904 \pm 57
Shampoo Base, 7X	28	187 \pm 27	1008 \pm 60
Octopirox Shampoo, 7X	28	130 \pm 20	914 \pm 46
Sham, 8X	4	179 \pm 54	997 \pm 61
Shampoo Base, 8X	4	148 \pm 35	849 \pm 58
Octopirox Shampoo, 8X	4	168 \pm 28	912 \pm 40
<i>Second Experiment^C</i>			
Shampoo Base, 7X	28	224 \pm 28	900 \pm 11
Octopirox Shampoo, 7X	28	171 \pm 16	992 \pm 47
Shampoo Base, 8X	4	149 \pm 19	1072 \pm 53
Octopirox Shampoo, 8X	4	144 \pm 30	1098 \pm 53

A A volume of 0.3 ml of shampoo was applied to mice.

B N = 6, values \pm S.E. Study B90-0314, Notebook YE-1418, pg 63.

C N = 8 except for shampoo base, 7X and Octopirox shampoo, 8X, where N = 7; values \pm S.E. Study B90-0338, Notebook YE-1418, pg 83.

APPENDIX I

FORMULAS OF SHAMPOO BASE AND OCTOPIROX-CONTAINING SHAMPOO

Shampoo base		Shampoo with Octopirox	
Substance	% (w/w)	Substance	% (w/w)
Ammonium lauryl sulfate	52.60	Ammonium lauryl sulfate	52.60
Ammonium laureth sulfate	16.00	Ammonium laureth sulfate	16.00
Ethylene glycol distearate	2.00	Ethylene glycol distearate	2.00
Cocamide monoethanolamide	1.35	Cocamide monoethanolamide	1.35
Citric acid	0.25	Octopirox	1.00
Ammonium xylene sulfonate	0.50	Ammonium xylene sulfonate	1.00
Sodium citrate	0.08	Citric acid	0.25
Dimethicone (60/40)	0.50	Sodium citrate	0.08
Stearyl alcohol	0.13	Dimethicone (60/40)	0.50
Cetyl alcohol	0.32	Stearyl alcohol	0.13
Dye solution	0.10	Cetyl alcohol	0.32
Perfume	0.65	Dye solution	0.10
Kathon CG	0.021	Perfume	0.65
Deionized water	QS to 100	Kathon CG	0.021
		Deionized water	QS to 100

APPENDIX I:

STANDARD PROCEDURE FOR MEASURING EPIDERMAL DNA SYNTHESIS

- 1) All mice will be injected i.p. with 1 $\mu\text{Ci/g}$ body weight of ^3H -thymidine (5 Ci/mmol) 1 hr (± 5 min) before sacrifice. The ^3H -thymidine will be prepared in isotonic saline at 0.8 $\mu\text{Ci}/\mu\text{l}$, and will be injected using 50 μl Hamilton gastight syringes with 25 gauge needles.
- 2) The depilatory, NEET (Whitehall Laboratories), will be applied to the treated skin about 4 min before the time of sacrifice, and the mice will be killed with CO_2 , then the NEET will be removed by washing with deionized water. The skin will be dried with paper towels, and the central portion of the treated area will be excised to ensure that only dosed skin is sampled. Epidermis will be isolated by scraping with a razor blade (Binder *et al.* Carcinogenesis 10: 2351 - 2357, 1989).
- 3) The epidermal scrapings from each mouse will be frozen in liquid nitrogen on the razor blade used for scraping, then weighed in a tared 1.5 ml microcentrifuge tube. The epidermal samples will be stored in a -80°C freezer until analysis.
- 4) The epidermal scrapings from each individual mouse will be homogenized in 2.5 ml of ice-cold 0.4 N perchloric acid (PCA) using a Polytron with a chilled PT10 generator (2 intervals of 15 sec at setting 6) in 16 X 125 mm plastic tubes. The homogenates will be immediately transferred to 12 X 75 mm polypropylene tubes, and kept on ice until the samples from all mice have been homogenized. All homogenates will be allowed to remain on ice for at least 15 min before centrifugation.
- 5) Tubes will be spun at 2500 rpm at 4°C for 10 min in a Sorvall GSA rotor (about 900 X g) to pellet the precipitate.
- 6) The supernatant fractions will be carefully decanted and saved to estimate the soluble ^3H -thymidine pool. During the digestion of the acid precipitable fractions (below), the supernatant fractions will be centrifuged at 7500 rpm for 10 min to eliminate any contamination with acid precipitable material, then one 1.0 ml aliquots of the supernatants will be counted in 10 ml of Beckman Readysafe LSC cocktail. Quantification of ^3H dpm will be as indicated below.
- 7) The pellets will be washed in 2 ml of ice-cold 0.2 N PCA, by vortexing until they are well broken-up. Samples will be centrifuged as in 5) above. The supernatant fluids will be discarded.
- 8) The pellets will then be washed twice in 2 ml of ice-cold absolute ethanol as in 5) above.
- 9) After the final wash, the ethanol will be removed, and 2 ml of 0.5 N PCA will be added to each tube. The pellets will be resuspended by vortexing, taking care not to leave large pieces on the walls of the tubes. The tubes will be tightly capped, then heated in a waterbath at 90°C for 20 minutes, then vortexed again, and placed on ice for 10 min. The hydrolyzed DNA will be separated from protein and RNA by centrifuging for 10 min at 7500 rpm in a GSA rotor, the supernatant fractions will be centrifuged again for 10 min at 7500 rpm.
- 10) About 1.5 ml of supernatant will be carefully decanted from each tube. From each sample a 1.0 ml aliquot will be counted in 10 ml of Readysafe cocktail, and the remainder will be frozen in liquid nitrogen and stored at -80°C for DNA analysis by the Burton method (Appendix III).

- 11) ^3H dpm will be quantified in a Beckman LS5801 liquid scintillation counter set to the standard tritium window (channels 0 - 400). The counter will have been calibrated with Beckman quenched tritium standards, and the H# method will be used to determine counting efficiency.

APPENDIX III:

DIPHENYLAMINE ASSAY FOR DNA

(A modification of the method of Burton [Methods in Enzymology 12: 163-166, 1968])

Solutions

- 1) 1.6% aqueous acetaldehyde
1 ml cooled acetaldehyde + 50 ml deionized water (use pipette chilled in freezer, -20°C)
- 2) Diphenylamine reagent (store at room temperature, stable for 3 - 4 months.)
15 g diphenylamine dissolved in 1000 ml glacial acetic acid, then add 15 ml concentrated sulfuric acid.
- 3) DNA standard (store at 4°C, stable for at least 6 months, check by measuring absorbance at 260 nm)
Calf thymus DNA brought to a final concentration of 200 µg/ml in 5 mM NaOH, based on absorption at 260 nm (1.0 AU = 50 µg/ml) not weight.
- 4) Working DNA standard (stable for at least 3 weeks, store at 4°C)
Mix 5 ml of 200 µg/ml DNA standard + 5 ml 1 N perchloric acid (PCA), then heat in a sealed tube at 90°C in a waterbath for 20 min, then cool on ice.
- 5) 1 N PCA
42.9 ml 70% PCA, QS to 500 ml.
- 6) 0.5 N PCA
Dilute 1 N PCA, 1:2.
- 7) Working diphenylamine reagent, make fresh daily
0.1 ml 1.6% acetaldehyde for every 20 ml diphenylamine reagent.

Assay Procedure

- 1) Standards and unknown samples are prepared in a final volume of 625 µl of 0.5 N PCA in 12 X 75 mm plastic tubes. All tubes should have the same amount of PCA, so 0.5 N PCA is used to adjust the volume.
- 2) The standard curve is constructed using 0, 50, 150, 200, 250 and 300 µg of the working standard DNA.
- 3) For epidermal extracts, 225 µl of extract is adjusted to 625 µl with 0.5 N PCA.
- 4) To each tube 1.25 ml of working diphenylamine reagent is added. Tubes are sealed, vortexed, and then incubated in a waterbath at 30°C overnight.
- 5) Absorbance is measured at 600 nm after ~17 hr. The spectrophotometer flow cell is zeroed with a 2:1 mixture of glacial acetic acid and 0.5 N PCA. (Diphenylamine precipitates in water.)

APPENDIX IV
INTERDEPARTMENTAL CORRESPONDENCE

From: G. M. Ridder

Date: 1/23/91

To: R. L. Binder

Retention Limit:

Subject: Determination of Skin Treatment Areas by Image Analysis

Below are measurements by two image analysis systems of the skin treatment areas that you traced onto overhead transparencies. The two image analysis systems were the IBAS 2000 (Zeiss) and the TCL-Image (Scientific Imaging Solutions) software running on a Macintosh IIfx computer (Apple). The areas in square centimeters were determined by placing the transparencies onto a lighted macrostand and acquiring the images from television cameras (Sony DXC3000P for the IBAS and JVC for the TCL-Image). The IBAS imaging system was calibrated using the centimeter scale of a plastic ruler and the "Scale" command; the results of the sample measurements are listed in column 4. The TCL system results (in cm²; Column 3) were calibrated by measuring the total area of a 20 cm² region of graph paper and ratioing the pixel areas of the samples to the pixel area of the scale (average of three measurement of the scale). The differences between the IBAS and TCL system measurements are listed in the last column and represent less than 2% variation between the two methods. This difference is insignificant compared to the accuracy of tracing the areas onto the transparencies. The TCL-Image results may be more accurate due to a more rigorous calibration scheme. (Notebook Reference: VE-1386 pg 86. This work was performed December 10, 1990)

<u>Sample</u>	<u>TCL(Pixels)</u>	<u>TCL(sq cm)</u>	<u>IBAS(sq cm)</u>	<u>Difference</u>
Scale	204941	20.00		
Scale	204671	20.00		
Scale	204978	20.00		
Skin 1-1	77318	7.55	7.44	0.11
Skin 1-2	78560	7.67	7.58	0.09
Skin 1-3	73767	7.20	7.05	0.15
Skin 1-4	108214	10.56	10.45	0.11
Skin 2-1	84722	8.27	8.06	0.21
Skin 2-2	73180	7.14	7.02	0.13
Skin 2-3	62778	6.13	6.01	0.12
Skin 2-4	92113	8.99	8.78	0.21
Average		7.94	7.80	0.14
			% Difference:	1.8%

G. M. Ridder
G. M. Ridder

INTERDEPARTMENTAL CORRESPONDENCE

FROM: S. A. Heitmeyer

DATE: 7/12/90

TO: Notebook File: YE-1412; Section 3

R/L: Non Discretionary

SUBJECT: Radiochemical Purity of [¹⁴C]-Octopirox (BPO-5884)

The radioactive purity of BPO-5884 evaluated on 7/11/90 using a TLC/radioactivity imaging method. This analysis was conducted at the request of R. Binder (H&ESD).

Method

The working stock solution was made by dissolving 1.0 mg of BPO-5884 ([¹⁴C]-Octopirox·MEA) into 1.0 ml of absolute ethanol. Two subsequent dilutions of the working stock were made (1:2 and 1:5 (v/v)) using absolute ethanol. One sample each of stock solution BPO-5884 ([¹⁴C]-Octopirox·MEA) and each dilution was spotted. Samples (2 μl) were spotted directly onto the silica gel portion of a 10x20 cm Whatman LHP-K silica gel TLC plate (pre-washed in MeOH/0.01M Octopirox·MEA, pH 6.0) which had been turned on its 10 cm end and scored to separate the silica gel portion from the preabsorbent band.

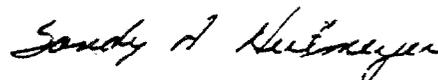
The TLC plate was developed on 7/11/90 at room temperature using a solvent system of MeOH/0.01M Octopirox·MEA, pH 6.0 for approximately 2 hours. Location of the solvent front was made upon removal of TLC plate from solvent; location of radioactivity was made with the aid of an Ambis Radioanalytic Imaging System (Ambis Systems, Inc., San Diego, CA 92123).

Results

Each of the three samples of BPO-5884 ([¹⁴C]-Octopirox·MEA) showed one distinct radioactive spot (PRINT octo-BB). The stock solution demonstrated the strongest radioactive image and therefore was selected as the best representative for analysis. The stock solution (lane L03) analysis showed the resulting peak to contained 100% of the total peak radioactivity and a corresponding R_f value of 0.70 (PRINT octo-BB.L03).

Following comparison to previous analysis (YE-1121; section 18b-c), these results indicate that the R_f value of BPO-5884 ([¹⁴C]-Octopirox·MEA) has remained relatively constant at 0.70 and the radioactive purity has remained at 100% since 8/22/89.

Original radioactivity imaging tracings and data analyses are attached.



S. A. Heitmeyer

Materials Used

<u>Material</u>	<u>Lot Number</u>	<u>Company and Address</u>
TLC Plates (Whatman LHP-K) (20 X 10 cm)	*004384	Whatman Chemical Separation, Inc. 9 Bridewell Place Clifton, NJ 077014
Methanol, absolute	*C50602	J.T. Baker, Inc. Phillipsburg, NJ 08865
Ethanol, absolute	DSP-KY-417 Date: 3/13/90	AAPER Alcohol and Chemical Co. Shelbyville, KY 40065
Hydrochloric Acid (conc. \approx 37%)	*7741 KCRH	Mallinckrodt, Inc. Paris, KY 40361
Octopirox · MEA	*BX288-2	Procter & Gamble Company Cincinnati, OH 45239-8707
[14 C]-Octopirox · MEA (specific activity = 2.3 mCi/g)	BPO-5884	Procter & Gamble Company Cincinnati, OH 45239-8707
pH Meter (Accumet 915)	Serial #3269	Fisher Scientific 711 Forbes Avenue Pittsburgh, PA 15219

Study Notes:

6/29/90

Three TLC plates were placed in methanol/0.01M octopirox (pH 6.0) and allowed wash for \approx 3 hours. After washing, the plates were allowed to dry at room temperature. After drying, the plates were wrapped in clear plastic and stored at room temperature until needed.
Plates in: 1:10 p.m. Plates out: 4:00 p.m.

Sandy A. Hutmyer

7/11/90

One of the pre-washed plates was selected, labeled octo-BB and dated 7/11/90. Following labeling, the plate was spotted with the three samples of BPO-5884, allowed to dry and then developed using methanol/0.01M octopirox for \approx 2 hours.
Plate in: 10:25 a.m. Plate out: 12:30 a.m.

Sandy A. Hutmyer

PRINT

octo-BB

J/S/S/F

11 JUL 90

12:46 PM

ver: 1.81

ser#: 545

volt: 1643

time: 15 min

cnts: 12556

acptd: 64%

hi cnt: 8

CPMcm2: 46

Res plate: 13

(1.6 diameter)

1	1
8	1
1	1
256	504

L01 L02 L03

Bottom of plot
SMI 7/12/90