

# Evaluation of Triacontanol-Containing Compounds as Anti-Inflammatory Agents Using Guinea Pig Models

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A mixture of the aliphatic alcohol, triacontanol, and other chemically associated naturally occurring alcohols was applied to the denuded dorsal cutaneous surface of guinea pigs to evaluate anti-inflammatory activity. In the setting of a chemical irritation with 2% croton oil and in an allergic dermatitis created with dinitrochlorobenzene sensitization and challenge, the triacontanol-containing preparation was significantly more effective than vehicle alone (DHL skin cream) but not as effective as 0.05% Diprolene® ointment.

Lymphocyte stimulation was studied by tritiated thymidine uptake and morphologic examination for blast transformation. When triacontanol-containing compounds were solubilized in aqueous media, effects on lymphocytes were insignificant. When solubilized in ethanol, there was a marked effect on thymidine uptake but not on blast transformation when compared to parallel controls. *J Invest Dermatol* 89: 380-383, 1987

The need exists for nonsteroid-based anti-inflammatory skin preparations. Potent halogenated steroid compounds commonly used today cannot be applied safely to facial skin, and are occasionally absorbed into areas where they are systemically active. Many naturally occurring preparations have been sought for their reported medicinal values, only to be found ineffective in controlled studies. One such naturally occurring compound, triacontanol, is relatively ubiquitous in plant products and has been known to be effective as a plant growth regulator for many years [1,2]. As these compounds are regularly ingested in our diets and deemed safe by regulatory agencies, limited studies have been conducted in humans. Preliminary studies in humans indicated potential anti-inflammatory and anti-Herpes Simplex properties when applied topically (personal observations, L. L. Clark, 1981-1986), and prompted this animal study to evaluate efficacy in controlled trials.

Chemically, triacontanol is a 30-carbon alcohol, present in significant concentrations in beeswax, and associated with multiple contaminating fatty acid esters and other free acids [3]. It has a solubility of  $<10^{-16}$  molar in aqueous media, is only slightly more soluble in ethanol, but has excellent lipid solubility [4,5]. Previous studies in plants have utilized suspensions of triacontanol to circumvent this problem of relative water insolubility and have been successful. Results of earlier animal studies (unpublished data) suggest that these triacontanol-containing compounds possess moderate anti-inflammatory activity. This study was undertaken to evaluate the effectiveness of a mixture of these compounds in

diminishing inflammation under controlled conditions, and to compare their efficacy with that of established controls.

## MATERIALS AND METHODS

**Experimental Animals** Hartley strain, outbred, female albino guinea pigs, 350-400 g each, were obtained from Charles River Breeding Labs, Wilmington, Massachusetts. The animals were stored 6 per cage with ample access to food and water. To remove dorsal hair, the animals were anesthetized with sodium pentobarbital (Veterinary Laboratories, Lenexa, Kansas) at a dose of 0.25 mg/kg of body weight via i.p. injection. An Oster model A-5 animal clipper (Oster Inc., Milwaukee, Wisconsin) was used to "close clip" each animal [6-9]. This was followed by 2 sequential 10-min applications of the chemical depilatory Nair (Carter-Wallace, New York, New York) to residual dorsal hair after wetting with tap water. The Nair was removed by tap water washing after each application. The denuded skin surface was washed well following the second application and each animal was towel dried. Test areas were demarcated using a black laboratory marker, and the animals were returned to the cage for 24 h before inflammation was induced [10]. Each animal had 5 test areas outlined in the denuded area to allow simultaneous evaluation of 5 pharmacologic treatment regimens.

**Chemicals and Reagents** Dinitrofluorobenzene (DNFB), (1-fluoro-2,4-dinitrobenzene) and dinitrochlorobenzene (DNCB), (1-chloro-2,4-dinitrobenzene) were obtained from Eastman Chemicals, Rochester, New York. Acetone and reagent alcohol were obtained from American Scientific Products, McGaw Park, Illinois. Croton oil was obtained from Sigma Chemical Co., St. Louis, Missouri. Triacontanol-containing compounds and DHL skin cream (both proprietary compounds) were obtained from Royal Pharmaceuticals, Bountiful, Utah, as experimental drugs for evaluation as 1% and 5% (weight triacontanol compound/weight DHL vehicle) concentration suspensions. These triacontanol-containing compounds were prepared by Dr. Sidney A. Williamson (Department of Chemistry, University of Utah, Salt Lake City, Utah); they contained 16.7% 28-carbon alcohol,

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### Abbreviations:

DNCB: dinitrochlorobenzene (1-chloro-2,4-dinitrobenzene)

DNFB: dinitrofluorobenzene (1-fluoro-2,4-dinitrobenzene)

PBML: peripheral blood mononuclear leukocytes

66.6% 30-carbon alcohol, and 16.6% 32-carbon length alcohol components. Mixture purity of >99.9% was verified by gas chromatography prior to use in this study. The DHL vehicle and triacontanol-containing suspensions were prepared by Dr. Robert Petersen (University of Utah College of Pharmacy, Salt Lake City, Utah). Triacontanol was blended into suspension on a proportional weight basis with vehicle. Constituents of the DHL vehicle are listed by percentage of total compound weight: water 54.7%, isopropyl myristate 22.0%, glycerol monostearate 6.0%, isopropyl lanolate 5.0%, triacontanol compound 5.0%, propylene glycol 5.0%, triethanolamine 1.3%, and Germaben-II® 1.0% (Sutton Labs, Inc.). The blending process consisted of a 2-component liquification at 75°C, liquid phase mixing, then continuous mixing while returning to a cream base preparation at room temperature. Diprolene® ointment 0.05% was obtained from Schering Corporation, Kenilworth, New Jersey. [<sup>3</sup>H]Thymidine (6.7 Ci/mmol) was obtained from New England Nuclear, Boston, Massachusetts.

**Treatment Regimen** Each animal was subjected to 5 modes of treatment consisting of: (1) no treatment; (2) vehicle alone (DHL skin cream); (3) 1% triacontanol suspension in DHL vehicle; (4) 5% triacontanol suspension in DHL vehicle; and (5) Diprolene® ointment 0.05%, a very potent topical corticosteroid known to be effective in depressing skin inflammation. Approximately 250 mg of each compound was administered 3 times/day by manually rubbing the drugs onto each of the respective test areas. The animals were isolated one to a cage. The guinea pigs were examined daily by a single-blinded expert observer, and inflammation/irritation were recorded qualitatively utilizing a 1+ (no erythema) to 4+ (severe erythema/untreated control area) scoring system for each test area. Nair was again applied (for 4-min duration only) on day 4 of therapy to return the skin to a denuded condition and expedite reading of erythema.

**Allergic Sensitization** Each of 12 experimental animals had the hair removed from their right flank area via close clipping without anesthesia. Sensitization was accomplished using topical application of 10% DNFB in acetone (vol/vol) onto the clipped right flank area via dripping the solution onto the skin from a graduated 1.0 ml syringe (Pharmaseal Laboratories, Glendale, California) and isolating the animals for 20 min to allow solvent evaporation. At 4 days the animals were challenged with 0.02 ml of 5% DNCB in acetone (wt/vol) applied to the left ear to verify sensitization. A dose-response curve for DNCB in acetone (0.1%–5.0%) was performed on the left ear of 5 sensitized animals to determine the concentration of DNCB yielding the optimal state of contact sensitization on visual inspection 24 h later. The animals were subsequently challenged with a 2% DNCB solution delivered as 0.2 ml aliquots onto each of the 5 outlined test areas

(total volume of 1.0 ml) from a 1.0 ml syringe. Two hours after receiving the challenge dose, the animals were started on the treatment regimen outlined above.

**Skin Inflammation From Chemical Irritation** Each of 12 experimental animals with denuded dorsal areas was treated with a 2% solution of croton oil in acetone/ethanol (70%/30%). The croton oil was delivered to the skin via a 1.0 ml syringe, 0.2 ml to each test area, as described above. Treatment was initiated 2 h after application of the irritant solution, and each animal was examined daily as described above.

**Human Lymphocyte Blast Transformation** Human peripheral blood mononuclear lymphocytes (PBML) were obtained from healthy donors utilizing standard syringe venipuncture technique. A total of 35–40 ml of blood was obtained and heparinized using commercially available sodium heparin (Elkins-Sinn Inc., Cherry Hill, New Jersey). Blood was mixed in a 1:1 ratio with RPMI 1640 tissue culture media (GIBCO Laboratories, Grand Island, New York), layered onto a "cushion" of Ficoll-Paque® (Pharmacia Inc., Piscataway, New Jersey) and centrifuged in a Beckman Model TJ-6R refrigerated centrifuge (Beckman Instruments, Palo Alto, California) for 45 min at 1200 rpm and 4°C [11]. The cell pellet was resuspended in 5.0 ml of RPMI 1640 media containing 10% fetal bovine serum and counted utilizing trypan blue exclusion techniques in a 0.1 ml Neubauer type hemacytometer (American Optical, Buffalo, New York). Cells, 0.1 ml at  $2 \times 10^6$ /ml were transferred to a Falcon Model 3072 96 well flat bottom microtest plate (Becton Dickinson Labware, Oxnard, California). Solutions of pure triacontanol compound were obtained by: (1) serial dilutions of "saturated" stock solution (250 mg of triacontanol preparation stirred in 10 ml of RPMI tissue culture media for 48 h) followed by vigorous centrifugation and filter sterilization; and (2) dissolving 100 mg of triacontanol preparation in 10 ml of absolute ethanol, filter sterilizing, and adding varying volumes to RPMI tissue culture media. Controls and varying concentrations of triacontanol compounds were studied in the presence and absence of PHA (phytohemagglutinin) using standard methodologies. Blast transformation was measured by quantitative lymphocyte counting and correlated with [<sup>3</sup>H]thymidine uptake at 48 h.

## RESULTS

All 24 animals were subjected to the same therapeutic modalities and were evaluated on a daily basis to determine efficacy of experimentally treated vs control areas. Treatments were performed with vehicle and test preparations simultaneously, and experimental treatment areas were varied among the animals between positions on the backs to minimize effects of regional variations on treatment results. All animals in both the croton oil irritation

**Table I.** Significance of DHL Vehicle, 1% Triacontanol, 5% Triacontanol, and 0.05% Diprolene Ointment Treatment Regimens Against Control

Day	Regimen	DHL/Control	1% Triacontanol/Control	5% Triacontanol/Control	Diprolene/Control
1	Croton oil	.0679 <sup>a</sup>	.0431	.0077	.0022
2	Croton oil	.0679	.0180	.0117	.0022
3	Croton oil	.0431	.0117	.0033	.0022
4	Croton oil	.0180	.0077	.0033	.0022
5	Croton oil	.0277	.0077	.0051	.0022
6	Croton oil	.0117	.0051	.0051	.0022
7	Croton oil	.0022	.0022	.0022	.0022
1	DNCB	.0180	.0180	.0117	.0022
2	DNCB	.0117	.0180	.0077	.0022
3	DNCB	.0077	.0180	.0033	.0022
4	DNCB	.0033	.0022	.0022	.0022
5	DNCB	.0277	.0117	.0022	.0022
6	DNCB	.0679	.0117	.0077	.0022
7	DNCB	.0180	.0077	.0033	.0022

DNCB = dinitrochlorobenzene (1-chloro-2,4-dinitrobenzene)

<sup>a</sup>p value: experimental treatment vs control (calculated via Wilcoxon signed-rank analysis).

**Table II.** Significance of 1% Triacortanol, 5% Triacortanol, and 0.05% Diprolene Treatment Regimens Versus DHL Vehicle Alone

Day	Regimen	1% Triacortanol/DHL	5% Triacortanol/DHL	Diprolene/DHL
1	Croton oil	.6858 <sup>a</sup>	.0431	.0022
2	Croton oil	.4630	.2249	.0033
3	Croton oil	.1422	.0180	.0022
4	Croton oil	.0679	.0077	.0022
5	Croton oil	.1422	.0935	.0022
6	Croton oil	.1422	.0630	.0022
7	Croton oil	.1088	.2076	.0022
1	DNCB	.6858	1.0000	.0033
2	DNCB	.6858	1.0000	.0033
3	DNCB	.2076	.7794	.0180
4	DNCB	.3613	.0117	.0022
5	DNCB	.1088	.0117	.0022
6	DNCB	.0679	.1386	.0033
7	DNCB	.1797	.0759	.0033

DNCB = dinitrochlorobenzene (1-chloro-2,4-dinitrobenzene)

<sup>a</sup>*p* value: experimental treatment vs vehicle alone (calculated via Wilcoxon signed-rank analysis).

group and the DNCB sensitization group developed moderate to severe skin responses after application of either irritant preparation. Each experimental treatment regimen began 2 h after skin challenge with the respective sensitizing compound, and continued 3 times/day for 7 days.

All treatment protocols (DHL vehicle, 1% and 5% triacortanol, and Diprolene<sup>®</sup> ointment) were statistically significant with respect to control with *p* < 0.07 for all 7 days tested (Table I). In skin dose-response tolerance trials, DHL vehicle alone showed objective erythema in the absence of previous chemically induced irritation (unpublished data). Despite this fact, DHL vehicle was still significantly better than no treatment in all studies; therefore, all further studies compared experimental results with DHL vehicle alone (Table II). Averaged erythema scores for each treatment regimen on a daily basis are plotted in Figs 1 and 2. Although not apparent from the data, the clinical severity of the skin inflammation on each animal was the major determinant of efficacy in all treatment regimens.

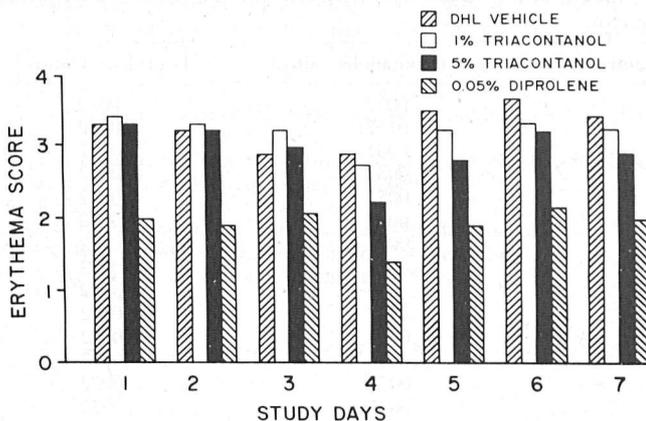
Summary analysis shows that the 1% triacortanol-containing compound was statistically better (*p* < 0.10) than vehicle alone on 1 of 7 test days for both DNCB and croton oil groups. The difference between vehicle and 5% triacortanol compound was significant (*p* < 0.10) for 5 of 7 days in the croton oil arm and 3 of 7 days in the DNCB arm of the study. In a few animals, the 5% triacortanol compound appeared to be almost as effective as the Diprolene<sup>®</sup> ointment in both the croton oil and DNCB groups. Diprolene<sup>®</sup> ointment was clearly the most effective treatment regimen within the statistical limits of this small study (*p* < 0.0022).

Due to the nature of the experimental protocol, as the control areas began to improve spontaneously the absolute differences between test and control areas became less. This made evaluations past 7 days difficult because all treatment area scores began to approach the score of the control area, which had arbitrarily been set at 4+ for each daily observation. Reference to untreated normal skin was difficult to quantitatively separate from Diprolene<sup>®</sup> treated skin, and yielded no additional information.

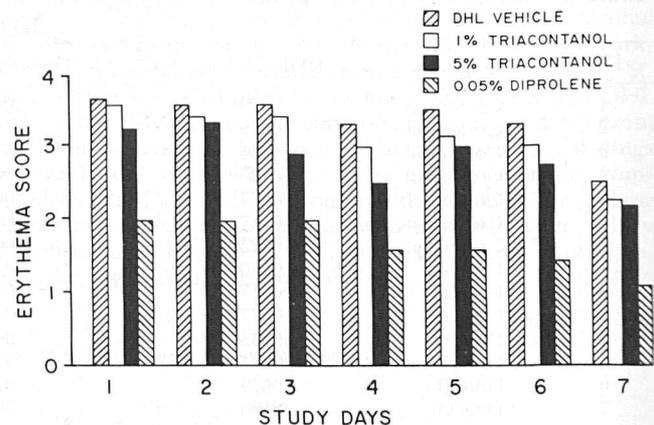
Lymphocyte stimulation was studied by [<sup>3</sup>H]thymidine uptake and morphologic blast transformation. Purified triacortanol compound containing mixed 28-, 30-, and 32-carbon alcohols is only poorly soluble in water (<10<sup>-16</sup>M), and often forms suspensions or crystallizes out of solution when placed in aqueous media. No characterization of these solutions was attempted to define the state of the triacortanol in them, but when dissolved to maximum solubility in RPMI 1640 tissue culture media, there were no demonstrable effects on lymphocytes in tissue culture. Triacortanol compound dissolved in ethanol and then added to lymphocytes in tissue culture had a marked effect on thymidine uptake, but very little effect on blast transformation. It is unclear whether this reflects lymphocyte activation or cellular damage, but ethanol controls had no similar effects when utilized alone (Table III).

## DISCUSSION

The present studies have compared the anti-inflammatory efficacy of 2 triacortanol-containing compounds with 0.05% Diprolene<sup>®</sup> ointment using 2 inflammatory processes on the dorsal skin of guinea pigs as a model. The triacortanol compounds were clearly



**Figure 1.** Graded erythema scores: allergic DNCB model.



**Figure 2.** Graded erythema scores: croton oil irritation.

**Table III.** Lymphocyte Transformation Assay

Regimen	Percent Blast Transformation	[ <sup>3</sup> H]Thymidine Uptake (cpm)
Media	4.0	488
Media/PHA	76.0	1744
Triacantanol media 1:1	6.0	512
Triacantanol media 1:10	5.0	526
Triacantanol media 1:100	6.0	533
Triacantanol media 1:1000	5.0	509
Triacantanol ethanol 1%	7.0	2822
Triacantanol ethanol 0.1%	5.0	1822
Ethanol/media 1%	6.0	552
Ethanol/media, PHA	66.0	1811

PHA = phytohemagglutinin

not as active as Diprolene® in anti-inflammatory potency, but the 5% suspension was statistically superior to DHL vehicle alone. The possibility that DHL vehicle may cause mild erythema is also of interest since use of the DHL vehicle was statistically better than the no treatment control in both allergic and chemical irritation experiments.

There is no way, from these data, to determine the relative potency of these triacantanol compounds as anti-inflammatory agents, but it would appear that they possess anti-inflammatory activity. The method of action is also unknown at this point, but higher concentrations of triacantanol compounds dissolved in ethanol seem to be lymphocyte inducers or cause moderate damage to the cells necessitating DNA repair activity. This lymphocyte activation may be more important in vivo since triacantanol is lipid soluble and could achieve higher concentrations in non-aqueous environments.

Triacantanol and the associated esters, alcohols, and free acids that often contaminate the natural preparations are very difficult to solubilize because of the waxy constituency of the compounds. This produced the major limiting factor in delivery of these chemicals to the skin. The 1% preparation was relatively easy to rub into the skin, but the 5% preparation was not. Application of the 5% preparation always resulted in a paste-like layer on the surface of the skin, and penetration of the entire aliquot was doubtful.

Improvement of the vehicle to allow easier application and less irritation may also increase the relative potency of these compounds.

The need exists for potent anti-inflammatory agents without the side effects of corticosteroids. Results of the current studies suggest that triacantanol-containing mixtures may represent such an alternative class of drugs. Further studies will be necessary in order to define optimal formulations, doses, and application intervals for use of triacantanol-containing mixtures as nonsteroidal anti-inflammatory preparations.

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