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Enhanced permeation of theophylline through the skin and its effect on fibroblast proliferation

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Summary

It is believed that theophylline can be helpful in psoriasis treatment by elevating cAMP levels. In the present study, theophylline skin permeation behavior from various enhancing carriers, as well as its efficacy in inhibiting psoriatic fibroblast proliferation in vitro, was studied. The results show that theophylline markedly inhibited normal and psoriatic dermal fibroblast proliferation when added to the cells at 1 mM concentration. Lower theophylline concentrations had no effect on psoriatic fibroblasts. From these results it can be assumed that a high concentration of drug needs to reach the dermis in order to be effective. The effect of oleic acid, propylene glycol and transcutol on the skin permeation fluxes of theophylline from various carrier systems (PEG base, cream, gel and ointment) was measured. The results clearly indicate that PEG base containing oleic acid and transcutol is the best enhancing carrier for theophylline (yielding a 260-times flux increase), among the systems investigated. This enhancing formulation was found to deliver to the dermis radiolabeled theophylline when applied to rat skin in vivo and visualized by autoradiography.

Introduction

Psoriasis is a chronic recurrent inflammatory disease of the skin, associated with an accelerated epidermal cell cycle. This fact has led researchers to focus on the molecular basis of the control of cell proliferation. One of the second messenger systems thought to be involved in the control of cell proliferation is the cyclic AMP cascade. Cyclic AMP has been shown in many cell culture systems to cause differentiation of cells, and a cessation of proliferation (Voorhees et al., 1971, 1982). It has been shown that there is a deficiency of cAMP-dependent protein kinases in psoriatic red blood cells and skin fibroblasts (Evain-Brian et al., 1986; Raynaud et al., 1987). Moreover, Raynaud et al. (1987) found a rationale for the use of retinoids in psoriasis by showing that these compounds elevate protein kinase levels in psoriatic fibroblasts. This elevation of cAMP should be helpful in psoriasis treatment.

Iancu et al. (1979) treated psoriatic patients by oral and topical administration of xanthines, which are inhibitors of phosphodiesterase, the enzyme responsible for breaking down cAMP. They reported limited success by oral administration and no effect after topical application of a xanthine,

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dyphylline, in a hydrophilic base (Iancu, 1979). One problem with oral administration of xanthines is that doses high enough to regulate cAMP would result in blood levels that are very close to systemic toxic levels. By giving the drug directly to the skin, dose-related side effects may be reduced. The ineffectiveness of topical application was explained by the author by poor skin penetration of dyphylline. Therefore, in order to achieve a therapeutic effect with xanthine, its skin delivery must be improved.

Among the approaches which are used to increase the skin permeation of molecules are: chemical enhancers (Touitou and Abed, 1985; Touitou and Fabin, 1988; Hori et al., 1989), prodrugs (Mollgaard et al., 1982), and varied solvent composition (Touitou, 1988). Sloan and Bodor (1982) used the 7-acyloxymethyl derivatives of theophylline to increase effectively the delivery of theophylline through the skin, resulting in a reduction in proliferation of the cells.

The goal of the present work was to improve the delivery of theophylline by selecting the appropriate carrier. With this in mind, permeation of theophylline from various semisolid bases, as well as the effect of enhancers was studied.

Epidermal hyperplasia associated with an inflammatory process is the most prominent indication of psoriasis lesions. Recent studies offer evidence that dermis has a predominant role in the appearance of psoriatic lesions, which combines benign hyperproliferation with abnormal differentiation (Saiag et al., 1985). We therefore used psoriatic fibroblasts as model cells for testing the effect of theophylline on cellular proliferation.

Experimental

Materials

Theophylline and the formulation carriers: polyethylene glycol 400 (PEG 400), PEG 4000, oleic acid (OA), carbopol 934, propylene glycol (PG), ethanol, Polysorbate 80, Sorbitane 80, white beeswax, cetosteryl alcohol and white soft petrolatum, were all analytical grade or conformed to B.P. requirements and were all purchased from Sigma (St. Louis, MO, U.S.A.). Transcutol (DG), the diethylene glycol monoethyl ether, was a gift of Gattfosse (France). [8-^{3}H]Theophylline was purchased from Amersham with a specific activity of 18.6 Ci/mmol and a concentration of 1 mCi/ml.

Dulbecco's modified minimal essential medium (DMEM), fetal calf serum, medium supplements, and trypsin-EDTA were purchased from Beit HaEmek Industries, Israel. Plasticware was purchased from Falcon-Beckton Dickenson (St. Louis, MO, U.S.A.). Trypan blue was purchased from Sigma.

Semi-solid bases for theophylline

The semi-solid bases used for the drug incorporation were:

Ointment base – a mixture of white soft petrolatum and cetostearyl alcohol.

Cream base – a water in oil (w/o) cream containing white soft petrolatum, Polysorbate 80, Sorbitan 80, white beeswax, and water.

PEG base – a mixture of PEG 400 and PEG 4000.

Gel base – a 2% w/w Carbopol 934 hydroalcoholic gel (ethanol : H_2O, 6 : 4).

The concentration w/w of enhancers, when added, was kept constant as follows: oleic acid 10%, propylene glycol 20% and transcutol 20%. The drug concentration in all the formulations was 10 mg/g.

Human dermal fibroblast cultures

Fibroblasts were obtained by culturing skin punch biopsies (3 mm) taken from involved chronic plaques of psoriatic patients and from normal volunteers. After removing most of the fat tissue and reticular dermis, skin biopsies were cut into 0.5–1 mm pieces and grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM non-essential amino acids and 2 mM glutamine (DMEM$^+$) in 35 mm tissue culture plates. Under these conditions, fibroblasts which grew out from the skin were subcultured upon reaching confluency by trypsinisation with trypsin EDTA. For the experiments, fibroblasts matched for subculture number (6th), age and sex were used.

Fibroblast cultures were maintained in a CO_2 incubator at 5% CO_2, 95% humidity, 37°C.

Theophylline solution

Theophylline was sterilized as a powder by heating it in an oven at 140°C for 2 h. A stock solution of theophylline (20 mM in $DMEM^+$) was sonicated continuously for 2 min at 50% duty cycle outputs in a bath sonicator (Heath Systems Ultrasonic) to further ensure sterility. Serial dilutions were prepared in $DMEM^+$ (1 mM to 1 μM). Both stock and serial dilutions were stored at −20°C.

Incubation of fibroblasts with theophylline

Fibroblasts were seeded (1×10^3/200 μl $DMEM^+$) in a 96 multiwell tissue culture plate. 3 days later, at fibroblast subconfluency, medium was removed and 200 μl of $DMEM^+$ containing theophylline (1 mM to 1 μM) were added to the wells, and cultures were incubated in the CO_2 incubator for 3 additional days. Cultures were observed daily under an inverted microscope. On the last day of the experiment, culture media were removed from the wells; the wells were washed twice with phosphate buffered saline (PBS) and fixed with 5% paraformaldehyde in PBS and kept overnight at 4°C for the fibroblast proliferation assay. Duplicate wells were trypsinised, and the fibroblasts were resuspended in 100 μl $DMEM^+$ and 100 μl of 0.4% trypan blue were added to determine viability of cells.

Fibroblast proliferation assay

Fibroblast proliferation assay was carried out by a spectrophotometric test as described by Goldman and Bar Shavit (1979). In this test, the authors found that absorbance values correlate positively and linearly with cell number. Briefly, following fixation, plates were immersed in a bath of 0.01 M borate buffer (pH 8.5) and decanted; the cells were stained (10 min at room temperature) with 1% methylene blue in borate buffer (0.1 ml/well) and washed four times with the same buffer. The cell-bound dye was eluted with 0.2 ml 0.1 N HCl (40 min at 37°C). The eluate was diluted 1 : 6 with water and the absorbance of the dye at 660 nm was determined in the ELISA reader (Sci. Lab. Inst.). The absolute absorbance of each well was used as an index of cell proliferation in the same well. Data are expressed as absorbance (control/theophylline), and are given as the mean of two experiments performed in triplicates.

Skin permeation measurements

Skin permeation was measured in a Franz cell assembly on hairless mouse skin. Full thickness dorsal skin excised from 7 week old male mice was mounted in the cell with a surface area of 1.77 cm^2. The receiver had a volume of about 8 ml and contained 30% ethanol in distilled water, in order to maintain pseudo-sink conditions. The formulation was applied to the stratum corneum of the skin. Samples of 200 μl were withdrawn periodically from the receiver. The experiment was run for 24 h at 37°C.

The theophylline concentration in the samples was assayed by HPLC. A computer program was used for the calculation of kinetic parameters (Touitou, 1986). Each system was tested in triplicates. The results were analyzed using the two-tailed, unpaired or paired (depending on the set of experiments compared) Student's *t*-test. For this analysis, the 'Balance' (IBM) computer program was used.

Measurement of theophylline skin / carrier partition coefficient (K_m)

The test was performed for a number of selected bases, and was carried out in diffusion cells at 37°C, with the receiver compartment empty. Systems containing 10 μg/g drug were applied to the skin in the donor compartment of the cells. K_m was determined by the weight of the skin. The concentration of the donor was measured at 0 time (C_i) and at the end of the 24 h experiment (C). The theophylline concentration was determined by HPLC as described below. The K_m was calculated from $K_m = (C_i - C)/C$.

Assay

The theophylline concentration was determined using a Merck Hitachi HPLC with a 655A variable-wavelength UV monitor. The determination was made at 275 nm on a reverse phase C_{18}

column. The mobile phase was composed of 8% acetonitrile in water and had a flow of 1 ml/min. The standard used was β-hydroxyethyltheophylline. There was no overlap between the peaks of the drug or standard and those of the metabolites and other skin components released into the receiver compartment during the experiment.

Skin autoradiography preparation

700 mg preparation containing 50 μCi [^{3}H]-theophylline was applied to 3.2 cm^2 dorsal skin of hairless rats. The site was covered with a Hill Top cell (Hill Co., USA) and left in place for up to 24 h. The animal was then killed; the skin area of interest was removed by means of a biopsy punch (diameter 3 mm), immersed in Tissue-Tek OCT458 (Miles Laboratories), and frozen immediately at −30 °C. The embedded skin was sectioned transversely at −30°C using a cryostat mircotome model CTI (International Equipment, USA), and the sections were fixed on slides. The slides were prepared for autoradiography by gelatinization and were exposed to Kodak Nuclear Emulsion type NTB-2 (Eastman Kodak) for 3 weeks. The autoradiographs were then developed and stained with haematoxylin and eosin (Touitou et al., 1988).

Results and Discussion

Effect of theophylline on dermal fibroblast proliferation

In the experiments carried out in this work, it was found that three days incubation of subconfluent cultures of psoriatic or normal fibroblasts with increasing concentrations of theophylline (1 μM-1 mM) was not toxic to the cells as demonstrated by their ability to exclude trypan blue (> 95%). In the experiments in which fibroblasts were incubated for an additional 2 days (a total of 5 days incubation) it was similarly found that theophylline does not cause loss of viability of the fibroblasts, and fibroblast morphology at the light microscope level was preserved upon prolonged incubation with the drug (not shown).

In the present study, the efficacy of theophylline in inhibiting psoriatic fibroblast proliferation was tested for the following reasons. It is accepted that the first changes taking place in a psoriatic plaque are dermal and that fibroblasts obtained from psoriatic dermis are functionally and biochemically different from normal fibroblasts. One

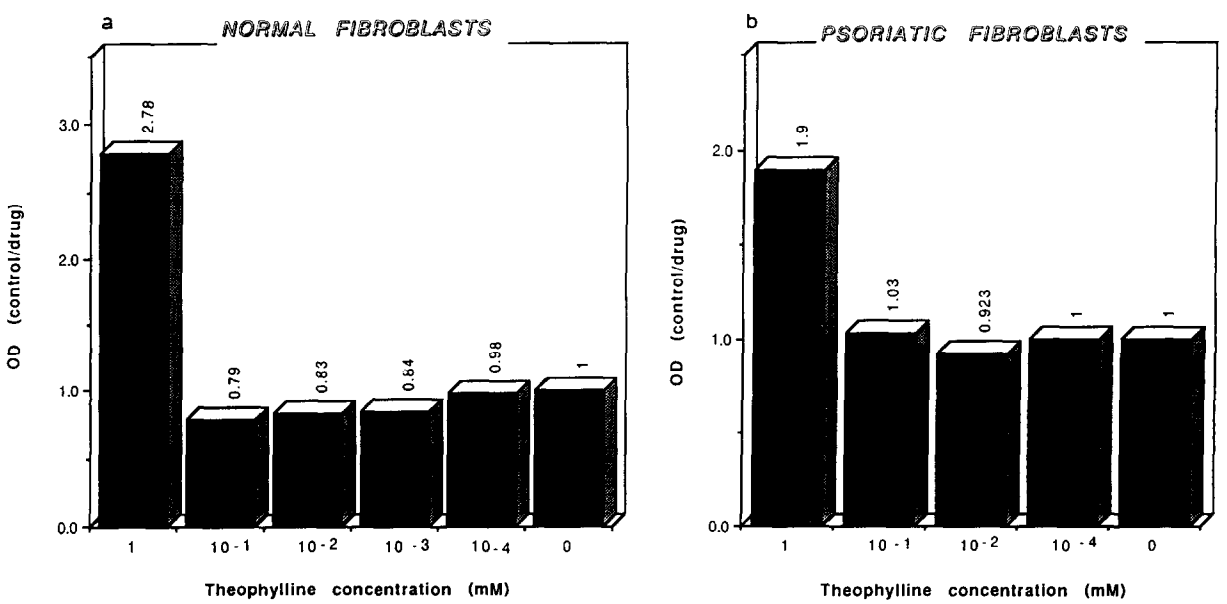

Fig. 1. Effect of various concentrations of theophylline on the proliferation of: (a) normal fibroblasts; (b) psoriatic fibroblasts.

of the altered properties is that psoriatic fibroblasts have an increased proliferation rate. Moreover, Voorhees (Voorhees and Duell, 1971; Voorhees, 1982) has shown that in psoriasis there is a decrease in the adenyl cyclase-cyclic AMP cascade. Therefore, the efficacy of theophylline, a drug known to elevate cAMP levels (Gilman et al., 1985), was measured on fibroblast proliferation.

The effect of theophylline on normal (a) and psoriatic (b) fibroblast proliferation was evaluated, after 3 days of incubation with the drug, spectrophotometrically. Data are expressed as the ratio between absorbance of the control vs absorbance of the drug incubated fibroblasts, and the results are presented in Fig. 1. As can be seen, theophylline markedly inhibited normal human dermal fibroblast proliferation when added to the cells at 1 mM concentration ($P < 0.001$). A similar pattern was detected by incubating theophylline with psoriatic fibroblasts with 1 mM theophylline concentration ($P < 0.001$), although to a somewhat lesser extent than normal human fibroblasts (1.89 vs 2.78). However, lower theophylline concentrations caused a slight increase in proliferation of normal fibroblasts in comparison to the control (the ratio value decreased to below 1). With 0.1 mM theophylline, psoriatic fibroblast proliferation was not inhibited. With other low concentrations, the inhibition was not statistically significant when compared to the control.

This concentration dependent effect of theophylline on fibroblast proliferation is an interesting behavior that we are currently investigating.

Skin partition and permeation of theophylline

Based on the results obtained with fibroblasts in vitro, it can be assumed that a high concentration of theophylline must be supplied to the dermis. One way to enhance skin permeation of drugs is to use chemical substances which may alter the diffusional resistance of the skin or the drug skin/carrier partition. Oleic acid and propylene glycol have been shown to enhance the skin permeation of a number of hydrophobic drugs (Cooper et al., 1985; Touitou and Fabin, 1988; Barry, 1989; Menczel and Touitou, 1989). Transcutol was used in a study on the effect of solvents on griseofulvin penetration in skin, and it was found to be capable of delivering the drug to the skin in amounts greater than reported minimum effective fungistatic levels (Ritschel and Hussain, 1988).

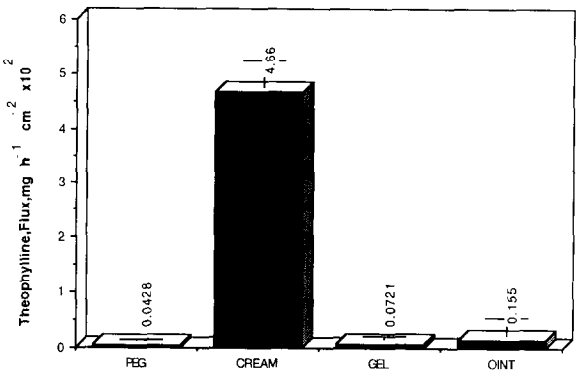

Fig. 2. Fluxes of theophylline through hairless mouse skin from various bases. The composition of the bases is given in Experimental.

Oleic acid, propylene glycol and transcutol have been chosen as additives to the semisolid bases, and their effect on theophylline skin permeation was measured on hairless mouse skin in Franz diffusion cells.

Fig. 2 shows the skin permeation fluxes of theophylline (10 mg/g) incorporated in four bases: PEG base, w/o cream, carbopol hydroalcoholic gel and ointment. The fluxes were obtained from the plot of the cumulative amount of drug accumulated in the receiver vs time, using the Transderm program (Touitou, 1986).

The histograms presented in Fig. 2 show a much greater permeation flux of theophylline from the oily cream than from the other three bases, i.e. 4.7×10^{-2} mg cm^{-2} h^{-1} for cream vs 4.3×10^{-4}, 7.2×10^{-4} and 1.6×10^{-3} mg cm^{-2} h^{-1} for PEG, gel and ointment, respectively. This behavior is sustained by the skin partition values: 0.15, 0.08, 0.06 and 0.05 for the cream, ointment, gel and PEG base, respectively. These data clearly show that the K_m value for the cream system is the highest among the values obtained for the drug incorporated in each of the above four bases.

The addition to PEG base of one enhancer, oleic acid, propylene glycol or transcutol, as well as the addition of two enhancers, oleic acid and transcutol or oleic acid and propylene glycol, increased the theophylline skin permeation flux; the

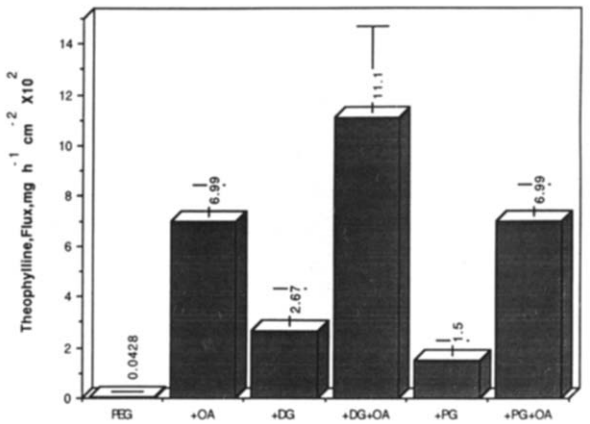

Fig. 3. Effect of enhancers on the flux of theophylline through hairless mouse skin from PEG bases. OA, oleic acid; DG, transcutol; PG, propylene glycol.

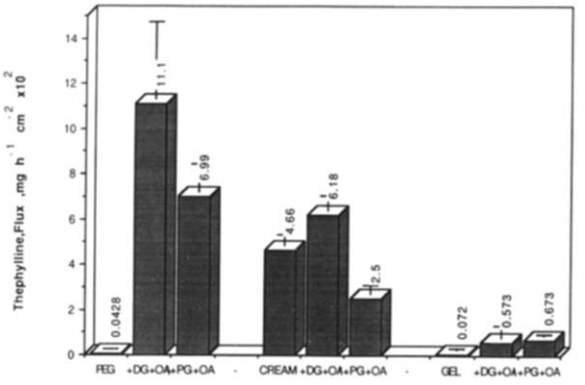

Fig. 4. Effect of propylene glycol (PG) + oleic acid (OA) and transcutol (DG) + oleic acid (OA) on the skin permeation from PEG base, cream and gel base.

best enhancement was 260-times, seen for the system containing oleic acid and transcutol (Fig. 3). It is interesting to note that when only one enhancer was incorporated in this base, the highest enhancing effect was obtained with oleic acid and the lowest with propylene glycol, 163- vs 35-times, respectively.

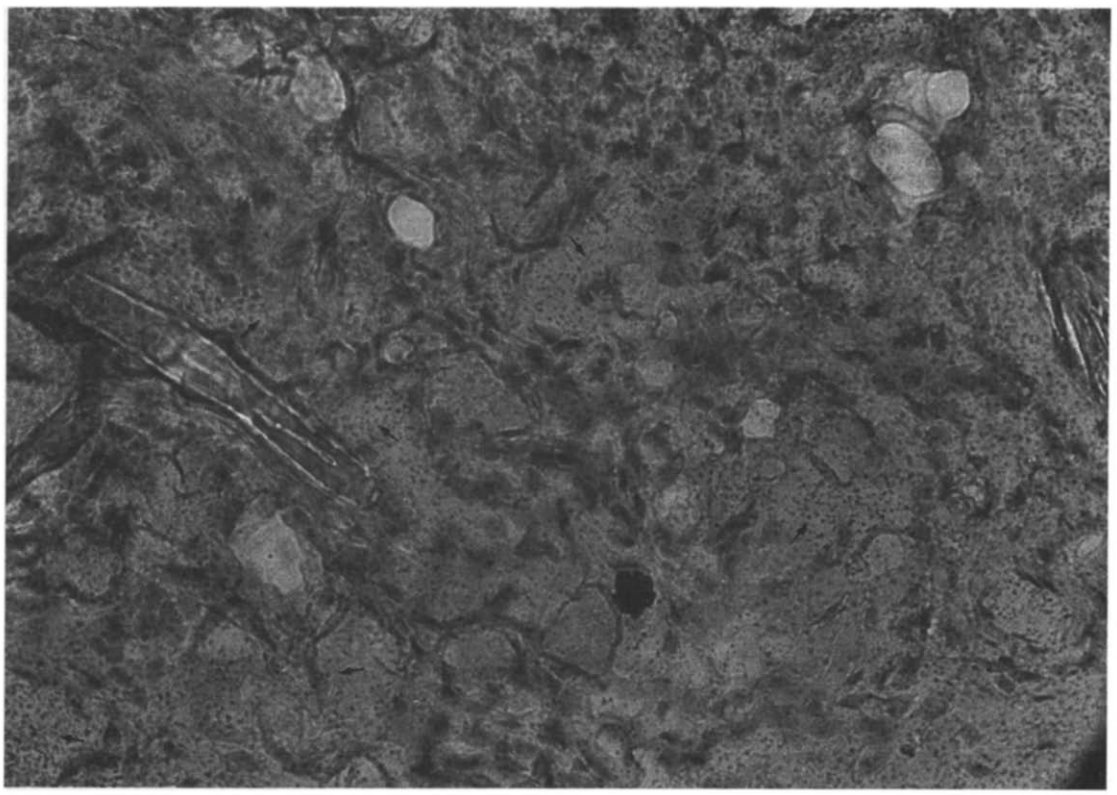

Fig. 5. Autoradiograph (×320) of hairless rat skin after application tritiated theophylline in PEG base containing transcutol and oleic acid.

The addition of propylene glycol to the PEG system containing oleic acid had no effect on the drug permeation flux. On the other hand, when oleic acid and transcutol were incorporated in the PEG base, an additive enhancing effect was observed (Fig. 3).

The effect of oleic acid and transcutol as compared to the effect of oleic acid and propylene glycol was further measured for cream and gel systems. Fig. 4 presents the theophylline fluxes from these enhancing preparations. It is clearly shown that the PEG + DG + OA is the best enhancing carrier among the systems studied in the present work. The combination of oleic acid and transcutol is more effective than oleic acid and propylene glycol for theophylline on the cream base as well. In the case of the gel base, the enhancing effect was smaller (less than one order of magnitude) than for PEG or cream base, and was similar for both combinations, DG + OA or PG + OA.

The PEG enhancing formulation was further tested for its efficacy to deliver theophylline to the dermis in vivo on hairless rats by autoradiography. Fig. 5 presents the autoradiography of the dermis after 24 h application of the formulation containing theophylline on the stratum corneum of the skin of the live animals. The tritiated theophylline was incorporated in the enhancing PEG base containing transcutol and oleic acid. Fig. 5 shows that the silver grains are spread in the bulk of the dermis and concentrated on the collagen filaments and other components of the extracellular matrix, as well as on the fibroblasts. Many fewer grains can be seen in the hair follicles, ecrine sweat glands and sebaceous glands. When the PEG base was used for comparison, no silver grains were detected (not shown). This indicates that no radioactive drug was able to reach the dermis when incorporated in the PEG base without enhancers.

It can be concluded that: (1) theophylline inhibited normal and psoriatic dermal fibroblast proliferation in vitro; (2) high levels of theophylline must be built up in the dermis in order to affect fibroblast proliferation in psoriasis treatment; (3) PEG base containing oleic acid and transcutol was found to be the most effective carrier in delivering theophylline to the dermis among the systems studied.

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