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(54) Title: A TOPICAL COMPOSITION CONTAINING A VITAMIN D DERIVATIVE

(57) Abstract: The invention provides a topical composition comprising a conjugate of a vitamin D derivatiave and a fatty acid.

A TOPICAL COMPOSITION CONTAINING A VITAMIN D DERIVATIVE

The present invention relates to a topical composition containing a Vitamin D derivative. More particularly the present invention relates to a topical composition comprising a conjugate of a Vitamin D derivative and a fatty acid and especially to such compositions for topical treatment of hyperproliferative skin diseases such as psoriasis vulgaris.

Psoriasis, a chronic inflammatory dermatosis characterized by scaling, infiltration and erythema, affects approximately 2% of the population [1]. The cause of psoriasis is unknown, but it is considered that disordered arachidonic acid metabolism may play a role in the pathogenesis of the disease through the chemotactic effect of the metabolites in human skin [2, 3]. It is well known that vitamin D_3 analogs, 1α,25-dihydroxyvitamin-D_3 (1,25$(OH)_2D_3$) and calcipotriol, are useful for the treatment of hyperproliferative skin diseases, such as psoriasis vulgaris [4-7]. Vitamin D_3 analogs exert their effects through interaction with vitamin D_3 receptor (VDR) located in epidermal keratinocytes [8]. The efficacy of these compounds in inhibition of proliferation in a variety of cell types, and their therapeutic potential in the topical treatment of psoriasis has been established in a large number of clinical trials [9, 10]. However, apart from their established beneficial activity, vitamin D_3 analogs have additional side effects. It is recognized that 1,25$(OH)_2D_3$ induces several biological effects influencing a number of signal transduction pathways, such as intracellular calcium increase and protein kinase C activation [11] and calcipotriol may lead to cutaneous irritant reactions in approximately 20% of the patients [12].

Polyunsaturated fatty acids (n-3 PUFAs) have been used for many years as a dietary supplement (mainly in fish oil) and have also been presumed to have beneficial effects in psoriasis. Although not completely established yet, there were still several indications that such PUFAs may possess a therapeutic potential for psoriasis. It was shown by Kew et al [13] that n-3 PUFA can reduce markers of immune cell function. Some other studies reported on moderate beneficial effects of orally-administered fish oil (in capsules) on psoriasis [14, 15]. However, the intrinsic effectiveness of the n-3 PUFAs in clinical trials could not have been reflected by such protocols. Clinical trials using n-3 PUFAs given to patients by intravenous administration have shown to have beneficial and significant effect in psoriasis [16].

In view of these results, it has been postulated that localized delivery of n-3 PUFAs into the skin by topical preparations would be more advantageously acceptable. The development of an efficient means of topical delivery can increase local soft-tissue and joint-drug concentration while reducing the systemic distribution of a drug [17, 18].

Calcipotriol (and $1,25(OH)_2D_3$) has poor penetration through the stratum corneum into the epidermis (internal report) which may explain its limited efficacy and frequently its treatment failures [19-21].

With this state of the art in mind, there is now provided, according to the present invention a topical composition comprising a conjugate of a vitamin D derivative and a fatty acid.

In preferred embodiments of the present invention said vitamin D derivative is a vitamin D_3 analog.

Preferably said vitamin D_3 analog is calcipotriol.

As will be understood from the description above, in preferred embodiments of the present invention said fatty acid is a polyunsaturated fatty acid.

Especially preferred are embodiments wherein said polyunsaturated fatty acid is an n-3 polyunsaturated fatty acid.

Preferably said polyunsaturated fatty acid is linolenic acid or γ-linolenic acid..

The topical compositions of the present invention are especially preferred for the treatment of a hyperproliferative skin disease such as psoriasis.

In another aspect the present invention is directed to the use of a conjugate of a vitamin D derivative and a fatty acid for the manufacture of a topical pharmaceutical composition for the treatment of a hyperproliferative skin disease.

Topical compositions according to the present invention are prepared by methods known per se in the art using the standard adjuvants and carriers.

Thus according to the present invention, it has now been found that in order to enhance drug-skin permeation of vitamin D_3 analogs there is a need to modify the lipophilicity and to optimize partitioning into the skin and to maximize skin permeation. On a basis of "mutual prodrug" in which each part (i.e. PUFA and calcipotriol) functions as a co-drug or as the promoiety bound to the drug, new molecules that combine calcipotriol and several PUFAs through an ester bond were synthesized and evaluated. As will be seen in the examples hereinafter, the ester

bond is partially and gradually hydrolyzed by skin esterases, releasing the free active co-drugs at high levels in the deep skin layers, leading to sustained drug delivery followed by prolonged activity.

While the invention will now be described in connection with certain preferred embodiments in the following examples and with reference to the accompanying figures so that aspects thereof may be more fully understood and appreciated, it is not intended to limit the invention to these particular embodiments. On the contrary, it is intended to cover all alternatives, modifications and equivalents as may be included within the scope of the invention as defined by the appended claims. Thus, the following examples which include preferred embodiments will serve to illustrate the practice of this invention, it being understood that the particulars shown are by way of example and for purposes of illustrative discussion of preferred embodiments of the present invention only and are presented in the cause of providing what is believed to be the most useful and readily understood description of formulation procedures as well as of the principles and conceptual aspects of the invention.

In the drawings:

Figure 1 is a comparative graphical representation of the formation of the free calcipotriol moiety generated in porcine skin 12 hours after application of 12μM solutions of its monoesters with linolenic acid (18:3n-3) or γ-linolenic acid (18:3n-6).

Figure 2 is a comparative graphical representation of skin biotransformation into bioforms (1) and (3) of mono-PUFA 18:3n-3 and mono-PUFA 18:3n-6 after 12-hour *in-vitro* treatment with mono-PUFA 18:3n-3 (2) and mono-PUFA 18:3n-6 (2) respectively.

Figure 3 is a comparative graphical representation of measured skin calcipotriol in pmoles/cm2 as a result of use of different mono derivatives of PUFA; and

Figure 4 is a comparative graphical representation of the antiproliferative activity of calcipotriol and calcipotriol/ PUFA (1:1 mole equivalent) mixtures, simulating full hydrolysis, in cultures of grown keratinocytes.

EXAMPLES

Materials and methods

Materials

Calcipotriol (Calcipotriene) was a gift from Teva Pharmaceutical Industries Ltd. (Kfar Saba, Israel). Linolenic acid, γ-linolenic acid, dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP) were obtained from Sigma (Rehovot, Israel). High-performance liquid chromatography (HPLC) grade solvents were obtained from Merck (Darmstadt, Germany).

Synthesis of conjugates

Dicyclohexylcarbodiimide (DCC) (33.3 mg; 0.16 mmol) and 4-(dimethylamino)-pyridine (DMAP) (1.975 mg; 0.016mmol) were added into a solution of calcipotriol (100 mg; 0.24 mmol) in dry CH_2Cl_2 (20 ml). The mixture was stirred at 0^0C and a solution of linolenic acid (45 mg; 0.1615mmol) or γ-linolenic acid (45 mg; 0.1615mmol) in dry CH_2Cl_2 (2 ml) was added dropwise under nitrogen atmosphere. The mixture was stirred at room temperature for 3 hours. The reaction mixture was washed with 0.5N HCl, saturated $NaHCO_3$ solution, water and then dried (with $MgSO_4$). The solvent was evaporated under reduced pressure, and the residue was chromatographed on silica gel (eluting with 1% chloroform in methanol).

^{1}H NMR spectra were recorded on a Bruker DMX-500 operating at 500.1 MHz, and chemical shifts are reported in parts per million (δ) using TMS as the internal standard. ^{1}H-NMR ($CDCl_3$): δ =5.33-5.40 (m, 6H), 5.00 (s, 1H), 4.65-4.75 (m, 1H), 4.45 (bs, 1H), 4.25 (bs, 1H), 3.42-3.55 (m, 4), 2.70-2.90 (m, 4H). TLC (5% MeOH in $CHCl_3$): Rf =0.33. LC-ESI-MS: *m/z* =672.2.

In vitro skin penetration study

The permeability of calcipotriol-PUFA conjugates through pig skin was measured *in vitro* with a Franz diffusion cell system (Crown Bioscientific, Inc., Clinton, NJ, USA). The diffusion area was 1.767 cm^2 (15 mm diameter orifice), and the receptor compartment volumes varied from 11 to 12 ml. The solutions in the receiver side were stirred by externally driven, Teflon-coated magnetic bars. The testing was basically performed as previously described [22, 23]. Each set of experiments was performed with at least 4 diffusion cells (n≥4). Full-thickness porcine skin was excised from fresh ears of slaughtered white pigs (breeding of Landres and Large White, locally grown in Kibbutz Lahav, Israel). Skin sections (about 2X2 cm) were cut and subcutaneous fat was removed from the skin sections with a scalpel. Transepidermal water loss measurements (TEWL, Dermalab® Cortex

Technology, Hadsund, Denmark) were performed and only those pieces that the TEWL levels were within specification (<10 g/m^{2}h) were mounted in the diffusion cells, ready for testing. The skin pieces were used for penetration studies within 2 weeks of preservation at -20°C, from the time of slaughtering. Each skin section was placed with the stratum corneum facing up on the receiver chambers, and then the donor chambers were clamped in place. The receiver chamber, defined as the side facing the dermis, was filled with phosphate buffer (4mM, pH 7.4) - ethyl alcohol (analytical grade) (7:3). After 15 minutes of skin washing at 37°C, the buffer was removed from the cells and the receiver chambers were refilled with phosphate buffer (4mM, pH=7.4) - ethyl alcohol (analytical grade) (7:3). Calcipotriol (0.005%, 12 μM) and Calcipotriol-PUFA conjugates at an equimolar concentration in isopropanol-propylene glycol (1:1 v/v) solutions, were applied on the skin (50 μl in each cell). After 12 hours, samples (1 ml) were taken from the receiver chambers into 2-ml vials, and the exposed skin pieces were extracted with ethanol containing 0.005% BHT (as described below). The receiver and the skin extract solutions were transferred quantitatively into vials and dried under vacuum using DNA-mini concentrator apparatus (Heto Lab Equipment, Denmark). The dry samples were kept at -20°C until analyzed by HPLC.

Skin extraction

At the end of the diffusion process, the exposed skin was wiped off carefully, followed by 10 consecutive measures of tape stripping. The skin pieces were cut to small pieces and inserted in 2-ml vials. The pieces in each vial were extracted by 1-ml ethyl alcohol containing 0.005% BHT. Each extraction was performed by incubation in a 40°C shaking water bath (150 rpm) for 1 hour. Aliquots of 0.8 ml of the skin extracts were evaporated to dryness (as described above), and just before analysis the residue was reconstituted with 200 μl of methanol followed by injection into the HPLC system. The total recovery of known quantities of calcipotriol impregnated in skin pieces and processed as above was 98.9±1.9% (n=4).

HPLC analysis of samples from receiver solutions and skin extracts

Aliquots of 20 μl from each sample were injected into HPLC system (Shimadzu VP series including SPD-M10Avp photodiode array detector), equipped with a prepacked C_{18} column (Betasil C_{18}, 5μm, 250X4.6mm, ThermoHypersil, UK).

The quantitation of calcipotriol and its PUFA conjugates was performed by integration of peaks detected at 265 nm. The samples were chromatographed using an isocratic mobile phase consisting of water-isopropyl alcohol-methanol (10:9:81) for calcipotriol and methanol only for its PUFA conjugates. A flow rate of 1 ml/min was used. A calibration curve (peak area versus drug concentration) was constructed by running standard calcipotriol solutions in methanol containing 0.005% BHT for every series of chromatographed samples. Identification of unknown calcipotriol and calcipotriol- PUFA conjugates was performed by analyzing their UV spectra. Calibration curves were linear over the range 0.1-5 μg/ml (0.1, 0.2, 0.5, 1, 2, 3, 5 μg/ml). Data were expressed as the permeating drug quantity per unit of skin surface area, Q_t/S ($S = 1.767\ cm^2$).

Determination of growth arrest and differentiation of keratinocytes

The direct effect of the new substances as compared to free PUFA and free calcipotriol (controls) on the proliferation level of keratinocytes was assessed *in vitro* using human keratinocyte cell lines. Cultures of normal human keratinocytes ((HaCaT)) were used as previously described [24]. Cell growth was evaluated by vital dye assay (alamara blue and neutral red tests).

Statistical analysis

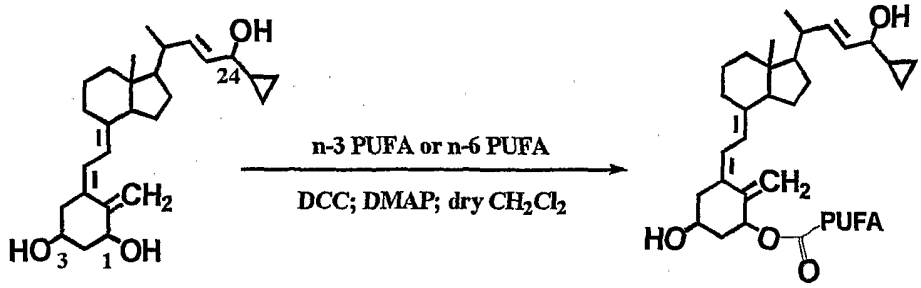
The statistical difference between the skin penetration levels of the various conjugates was analyzed. The two-way unweighted means analysis of variance (ANOVA) test for the differences among group means was first run. However, this test required a normal distribution of data. If normality of data analyzed by the ANOVA procedure failed while using the Kolmogorov-Smirnov test, the nonparametric Mann-Whitney rank sum test for unpaired data was applied at a significant level of 0.05.

Results and Discussion

New complexes on the basis of "mutual prodrugs" built of calcipotriol and polyunsaturated fatty acids (PUFAs) were prepared. The corresponding fatty acid, linolenic acid (18:3n-3 PUFA) or γ-linolenic acid (18:3n-6 PUFA), was condensed with calcipotriol in the presence of dicyclohexylcarbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP) in dry dichloromethane (Scheme 1). Although the calcipotriol structure includes three hydroxyl groups (1α, 3β, 24S), the preferable

isomer was found to be an esterification on the hydroxyl in position 1α (Scheme 1). The structures of the monoester derivatives were determined by NMR and mass spectrometric analysis.

Scheme 1



Calcipotriol (calcipotriene) **acyl ester of calcipotrio**

The synthetic conjugate, its bioforms and the free calcipotriol moiety (after skin hydrolysis) were identified and quantified by HPLC-UV from skin extracts 12 hours after the conjugate solution (12 μM) was applied. This quantification analysis was compared with the drug accumulated in skin after application of calcipotriol solution at the same molar concentration (12 μM) as the conjugates' solutions. Figure 1 presents the formation of the calcipotriol moiety after conjugates' hydrolysis by porcine skin, or the cutaneous penetration of applied calcipotriol in solution. The free calcipotriol generated in porcine skin was detected by HPLC twelve hours after application of its monoesters with two different fatty acids, 18:3n-3 and 18:3n-6 PUFAs. As shown in Figure 1, the accumulation of the free calcipotriol generated from the mono conjugates was about 35-50 ng/cm^2, while no calcipotriol penetration was detected after calcipotriol solution had been applied to skin. It can be obviously deduced that the depot formation of free calcipotriol is due to the enhanced penetration process exerted by the lipophilic nature of the mono PUFA-calcipotriol derivatives. The difference in the extent of hydrolysis between the conjugates containing the 18:3n-3 and 18:3n-6 PUFA represents a preference of the n-3 fatty acid (Figure 1), which is related to a difference in penetration of these conjugates into the skin (Figure 2).

Referring to Figure 1 it is to be noted that no detected calcipotriol was found in the skin after calcipotriol 12μM solution was applied for 12 hours.

As shown in Figure 2, an interesting biotransformation activity occurred during and after penetration of n-3 and n-6 PUFA conjugates into the skin. It was found that each one of the penetrating conjugates was converted into two other isomer forms (bioforms 1 and 3, in which the acyl group is bound to carbon positions 3 and 24), presumably by trans-esterification of the synthetic form 2 (in which the acyl group is bound to carbon position 1) with nonspecific acyl-transferases. The three forms which were accumulated and detected in the skin were then undergone enzymatic hydrolysis to release both free calcipotriol and PUFA. The three different calcipotriol-PUFA ester derivatives were numbered according to their appearance in the reverse-phase chromatography: the penetrating synthetic conjugate (the one that was applied on the porcine skin) was called form 2, a more hydrophilic compound was called bioform 1, and a less hydrophilic compound found in relatively large quantities was named as bioform 3 (Figure 2). These compounds were identified and confirmed by UV spectra and by LC-ESI-MS.

The concept of specific skin transformation of the synthetic compounds to a preferred mono derivative was supported by a penetration study performed with *di*-PUFA ester of calcipotriol. As already demonstrated by the previous 12-hour experiments, the synthetic mono-PUFA derivative of form 2 was found after 8 hours to be converted preferably into the mono derivative of bioform 3. However, the treatment with the corresponding *di*-PUFA derivative (in which two acyl groups were attached to two carbon positions - 1 and 24) led to an exclusive hydrolysis into the mono derivative of bioform 3. This indicates that the formation of PUFA ester on the hydroxyl group of carbon position 24 of calcipotriol is the dominant route of skin metabolism of these conjugates.

As will be noted from Figure 3, a preferred mono derivative of bioform 3 was formed after hydrolysis of *di*-PUFA ester of calcipotriol in porcine skin after its 8-hour application; after 8 hours of *mono*-PUFA application (form 2), bioform 3 was dominantly generated from the penetrating ester of form 2.

An enhanced antiproliferative activity of calcipotriol in combination of PUFA 18:3n-3 was also demonstreted. Figure 4 presents the antiproliferative activity of the mono calcipotriol-PUFA complexes (1:1 mol equivalent) in cultures of grown keratinocytes (cell number is expressed as absorbance of Alamara blue vital stain).

A complete hydrolysis of the complexes was simulated by preparing a mixture of free calcipotriol and free PUFA at the appropriate equimolar combinations. As shown, a significant anti-proliferative activity was obtained after complete hydrolysis of the monoester conjugate possessing PUFA 18:3n-3 compared to calcipotriol alone, while no antiproliferative contribution was made by PUFA 18:3n-6. It has been concluded therefore that apart of their enhanced skin permeability, conjugates consisting of n-3 PUFA produce a stronger proliferative inhibitory effect than that obtained by conjugates of n-6 PUFA.

From the above examples it can be seen that the permeation of calcipotriol through the skin can be markedly improved by the addition of fatty acid. The desirable combination of the complex for topical treatment of psoriasis in terms of its aqueous solubility and lipophilicity can be achieved by variation and proper selection of the acyl group. In this research, we have shown that derivation with n-3 PUFAs resulted in better penetration than derivation with n-6 PUFAs. It was shown that free n-3 PUFA by itself surprisingly possessed a significant antiproliferative activity in cultured keratinocytes. Up to date there is no previous work dealing with topical PUFAs treatment. In the present study, the release of n-3 PUFA have been shown to occur by mainly two steps of enzymatic processes – transesterification to new bioforms/isomers and ester hydrolysis. The released n-3 PUFA was found to be specifically and quantitatively localized into its target organ through topical treatment. In view of the ongoing on the use of vitamin D_3 derivatives in psoriasis, this research may lead to a new approach in drug development for treatment of psoriasis and perhaps of other skin disorders as well.

As will be noted, according to the present preferred embodiments of the present invention a conjugation was made between polyunsaturated fatty acids (PUFAs), such as linolenic acid or γ-linolenic acid and calcipotriol, a vitamin D_3 analog clinically used for topical treatment of psoriasis. These complexes were prepared by coupling the corresponding fatty acid with calcipotriol in the presence of dicyclohexyl-carbodiimide (DCC) and 4-(dimethylamino)-pyridine (DMAP) to obtain an ester bond. The conjugates were capable of enhancing penetration of the vitamin into the skin as well as inhibiting proliferation of keratinocytes in cultures. The antiproliferative activity was even increased after simulating the full hydrolysis of conjugates. *In vitro* skin penetration studies revealed that the conjugates penetrated

into the skin at higher levels relative to calcipotriol alone. It will also be noted that the conjugate containing n-3 fatty acid penetrated into the skin at higher levels as compared to the conjugate containing n-6 PUFA. HPLC analysis has shown that after penetration, a major portion of calcipotriol-PUFA conjugate was first converted mainly into another stable isomer form, presumably by trans-esterification, and only then it was hydrolyzed to form apparently high local concentrations of both calcipotriol and PUFA. This unique biotransformation that has occurred after penetration into the skin indicates that these conjugates are mutually prodrugs which are able to be bio-processed in the skin and fully converted to the parent therapeutic agents.

It will be evident to those skilled in the art that the invention is not limited to the details of the foregoing illustrative examples and that the present invention may be embodied in other specific forms without departing from the essential attributes thereof, and it is therefore desired that the present embodiments and examples be considered in all respects as illustrative and not restrictive, reference being made to the appended claims, rather than to the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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WHAT IS CLAIMED IS:

1. A topical composition comprising a conjugate of a vitamin D derivative and a fatty acid.

2. A topical composition according to claim 1 wherein said vitamin D derivative is a vitamin D_3 analog.

3. A topical composition according to claim 2 wherein said vitamin D_3 analog is calcipotriol.

4. A topical composition according to claim 1 wherein said fatty acid is a polyunsaturated fatty acid.

5. A topical composition according to claim 4 wherein said polyunsaturated fatty acid is an n-3 polyunsaturated fatty acid.

6. A topical composition according to claim 4 wherein said polyunsaturated fatty acid is linolenic acid.

7. A topical composition according to claim 4 wherein said polyunsaturated fatty acid is γ-linolenic acid.

8. A topical composition according to claim 1 for the treatment of a hyperproliferative skin disease.

9. A topical composition according to claim 8 wherein said hyperproliferative skin disease is psoriasis.

10. The use of a conjugate of a vitamin D derivative and a fatty acid for the manufacture of a topical pharmaceutical composition for the treatment of a hyperproliferative skin disease.

11. The use according to claim 10 wherein said vitamin D derivative is a vitamin D_3 analog.

12. The use according to claim 10 wherein said vitamin D_3 analog is calcipotriol.

13. The use according to claim 10 wherein said fatty acid is a polyunsaturated fatty acid.

FIGURE 1

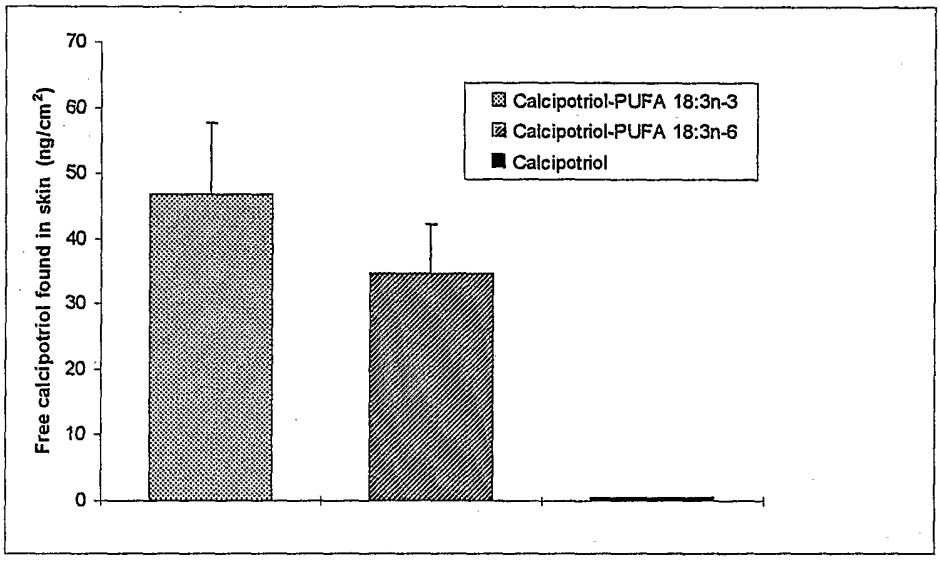


FIGURE 2

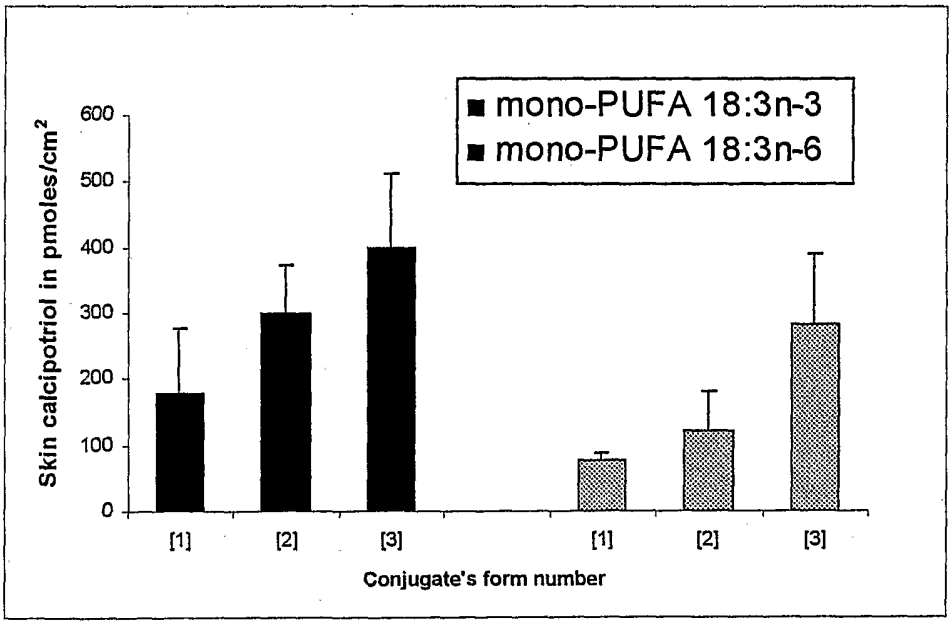


FIGURE 3

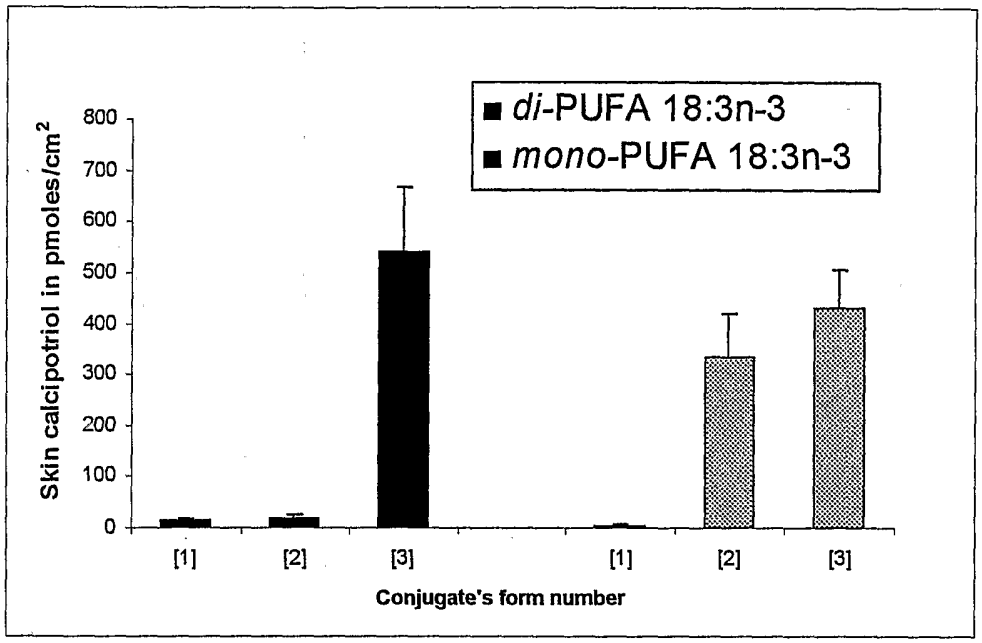


FIGURE 4

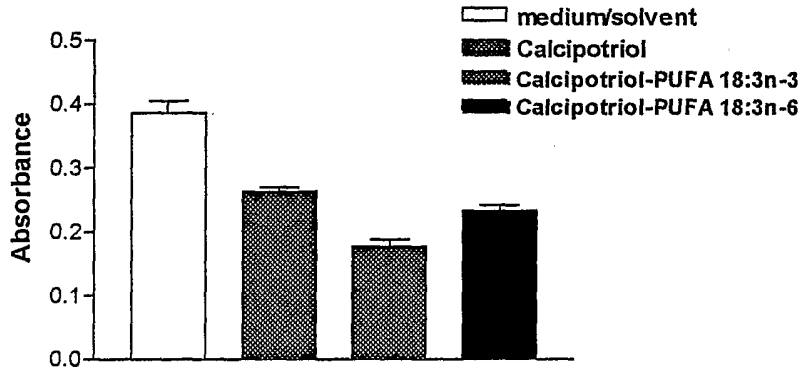


FIGURE 5

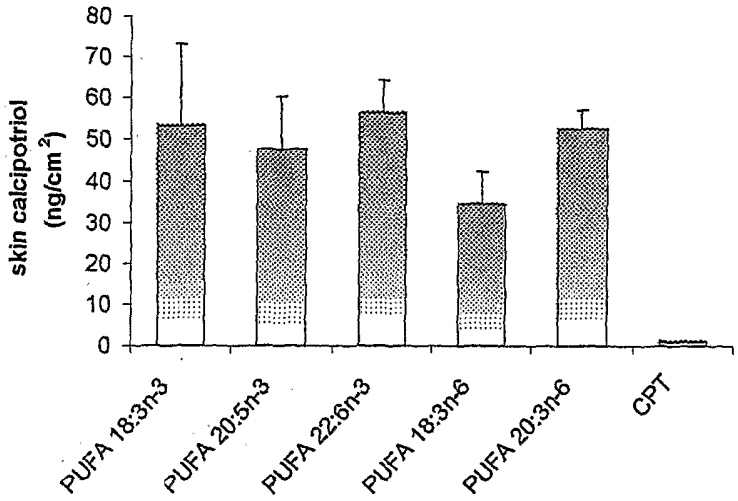


FIGURE 6a

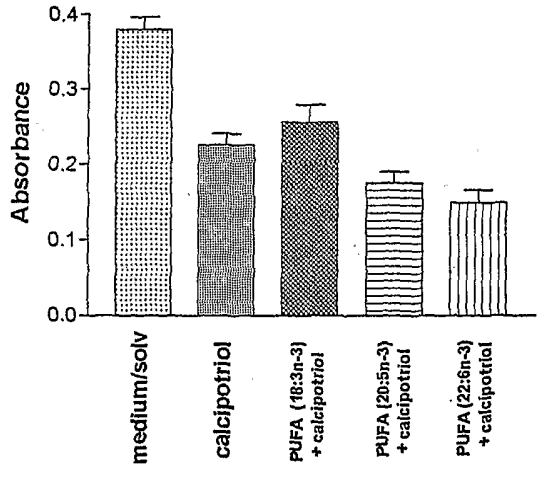
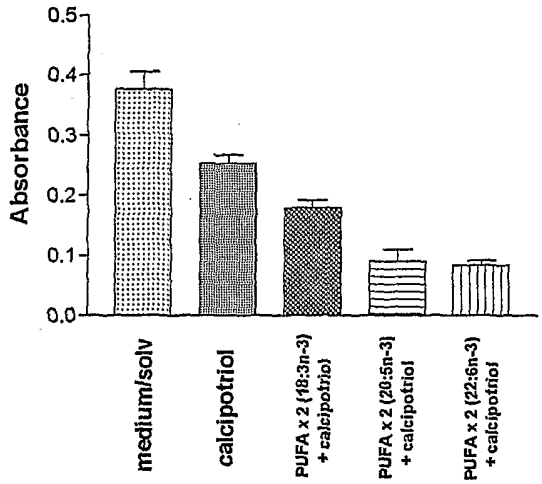


FIGURE 6b



INTERNATIONAL SEARCH REPORT

[redacted]ational Application No
PCT/IL2005/000405

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/01

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 491 007 A (EASTMAN KODAK COMPANY) 23 August 1938 (1938-08-23) page 3, lines 24-41; claim 1	1-13
P,X	BEN-SHABAT ET AL: "Vitamin D3 based conjugates for topical treatment of psoriasis" PHARMACOL.RES., vol. 22, no. 1, 2005, - 2005 pages 50-57, XP002333601 see "Conclusions"	1-13

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2005	08/07/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Cattell, James

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INTERNATIONAL SEARCH REPORT

Information on patent family members

[redacted]national Application No
PCT/IL2005/000405

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 491007 A	23-08-1938	NONE	