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Calcipotriol and betamethasone dipropionate exert additive inhibitory effects on the cytokine expression of inflammatory dendritic cell–Th17 cell axis in psoriasis

Paola Lovato[a,*], Hanne Norsgaard[a], Yoshiki Tokura[c], Mads A. Røpke[b]

[a] Department of Skin Inflammation Pharmacology, LEO Pharma A/S, Industriparken 55, Ballerup, Denmark
[b] Department of Clinical Pharmacology, LEO Pharma A/S, Industriparken 55, Ballerup, Denmark
[c] Department of Dermatology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

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ABSTRACT

Background: Psoriasis vulgaris is characterised by epidermal hyper-proliferation and infiltration of immune cells including dendritic cells (DCs) and T cells. The inflammation is driven by a complex interplay between immune and skin cells involving interleukin (IL)-17A, IL-23 and TNF-α as key drivers. The calcipotriol/betamethasone dipropionate two-compound fixed combination product is widely used for topical treatment of psoriasis. However, the mechanism behind its high efficacy has not been elucidated in detail.
Objective: Here, we investigated and compared the immune modulatory effects of betamethasone, calcipotriol and the combination in *ex vivo* cultures of psoriatic skin and *in vitro* cultures of primary human cells that recapitulate key cellular activities of psoriatic inflammation.
Method: The immune modulatory effect of the treatments on psoriatic skin and on *in vitro* differentiated Th1/Th17 cells, Tc1/Tc17 cells, monocyte-derived inflammatory dendritic cells and primary keratinocytes was assessed by a panel of inflammatory and phenotypic related transcription factors and cytokines. The expression was evaluated by both gene and protein analysis.
Results: Compared to vehicle control or mono-treatments, the effect of calcipotriol/betamethasone combination was significantly better in inhibiting the secretion of IL-17A and TNF-α in psoriatic skin. Additionally, the two components showed additive inhibitory effects on secretion of IL-23 and TNF-α by DCs, of IL-17A and TNF-α by both $CD4^+$ and $CD8^+$ T cells and reduced inflammatory responses in Th17-stimulated keratinocytes. Furthermore, calcipotriol was found to enhance IL-10 secretion in psoriatic skin and in human T cells, to induce secretion of type 2 cytokines by T cells and, lastly, to significantly modulate the differentiation of DCs and T cells.
Conclusions: In summary, we demonstrate a unique and supplementary immune modulatory effect of calcipotriol/betamethasone combination on TNF-α and IL-23/Th17 immune axis, supporting the superior clinical efficacy of the combination product compared to the respective mono-treatments in psoriasis patients.

1. Introduction

Psoriasis is an immune-mediated, chronic inflammatory skin disease clinically characterised by well-defined, raised, red and scaly plaques that affects approximately 1–3% of the population worldwide although the prevalence is below 0.5% in Asia [1]. Histologically, psoriasis is characterised by epidermal hyper-proliferation with disordered keratinocyte differentiation, inflammatory cell infiltration and increased vascularity in the dermis [2]. An immune pathogenesis model of psoriasis involving dendritic cells (DCs), T cells and keratinocytes (KCs) has now been established. Activated inflammatory DCs produce tumour necrosis factor-α (TNF-α), interleukin (IL)-12 and IL-23, and these cytokines maintain or stimulate Th1, Th17 and Th22 cells [3]. Subsequently, IL-17, interferon-γ (IFN-γ) and IL-22 secreted from these cells

Abbreviations: ELISA, enzyme-linked immunosorbent assay; VDR, vitamin D receptor; Tc cell, T cytotoxic cell; Th cell, T helper cell; Treg cell, T regulatory cell; T_{cm}, central memory T cells; T_{em}, effector memory T cells; RORC, RAR-related orphan receptor C; GATA3, GATA binding protein 3; FOXP3, forkhead box P3.

* Corresponding author at: Department of Skin Inflammation Pharmacology, LEO Pharma A/S, Industriparken 55, 2750 Ballerup, Denmark.
E-mail address: paola.lovato@leo-pharma.com (P. Lovato).

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stimulate keratinocytes to hyper-proliferate and to produce additional inflammatory mediators such as chemokines, cytokines and antimicrobial peptides. Inhibition of TNF-α has been known to be an effective therapy in psoriasis for more than a decade. More recently, IL-23 and IL-17A are recognised as key cytokines of psoriasis pathogenesis through the documented clinical efficacy of antagonists to these cytokines that control disease in approximately 80% of treated patients with moderate–severe psoriasis [3].

The combination of the vitamin D receptor (VDR) agonist calcipotriol and betamethasone dipropionate is a first-line topical treatment for mild to moderate plaque psoriasis [4,5]. There are considerable differences between glucocorticoids and VDR agonists in modulation of the immune system [6–8]. Glucocorticoids are potent anti-inflammatory agents with broad immunosuppressive effects by blocking several inflammatory pathways [9,10], and by inducing apoptosis [11]. In contrast, VDR agonists promote a more immune modulatory profile by inducing a tolerogenic state in DCs and T cells [12–14], and by enhancing the immunosuppressive capacity of regulatory T cells [15,16]. Whereas corticosteroids broadly inhibit T cell activities, VDR agonists drive T cells towards a Th2 profile while inhibiting Th1/Th17 cells [17]. Studies using peripheral blood-derived T cells from patients with rheumatoid arthritis demonstrate decreased production of the Th1/Th17 cytokines IFN-γ and IL-17A and increased production of the Th2 cytokine IL-4 upon treatment with calcipotriol, which contrasts the more universal inhibitory effects of dexamethasone [18]. Calcipotriol also normalises the pro-inflammatory cytokine milieu in psoriasis, including a decrease of *IL17A*, *IL17F* and *IL8* transcripts in psoriatic skin and inhibition of Th17-induced expression of IL-6, IL-8, human beta defensin (HBD)-2, -3, S100A7, and S100A15 in KCs [19–21], thereby interrupting the pro-inflammatory feedback loop that drives the pathogenesis.

In light of the differentiated effects of VDR agonists and glucocorticoids on inflammatory responses and their confirmed clinical effect on psoriasis, it is relevant to explore the molecular effects of combining a corticosteroid and a VDR agonist on the key cell types involved in psoriasis pathogenesis. In this study, we investigated the effects of calcipotriol, betamethasone dipropionate and their combination on cytokine expression in *ex vivo* cultures of psoriatic skin that maintains the key cellular activities and inflammatory features as found in psoriatic skin *in vivo* in patients. Furthermore, to delineate the effects of the agents on specific cell types of central importance in psoriasis, studies were performed using *in vitro* cultures of primary human KCs, DCs and T cells, differentiated into subsets of relevance to psoriasis.

2. Materials and methods

2.1. Test compounds

Stock solutions (10 mM) of calcipotriol (MC 00903; LEO Pharma) and betamethasone dipropionate (provided by LEO Pharma) were prepared in dimethyl sulfoxide (DMSO). The stock solutions were diluted in cell culture medium at the time of use. The final concentration of the compounds was 100 nM of calcipotriol and 1 μM of betamethasone dipropionate both for single treatments and for the combination. The final concentrations of calcipotriol and betamethasone dipropionate used in this study are based on exposure data in *ex vivo* pig and human skin after topical application of the calcipotriol/betamethasone fixed-combination product.

2.2. Human specimens

Keratome biopsies, 500 μm thick, were obtained from non-treated lesional skin of patients with active plaque psoriasis after approval from local ethical committees and the Danish Data Protection Agency (No 271113-4E, 15-1427/2 and H-2-2013-011) and upon written informed consent by the patients.

Buffy coats from healthy adult blood donors were obtained from the Blood Bank of the National University Hospital Copenhagen after anonymisation of donor identity carried by the National University Hospital. The use of these buffy coats for research was approved by the ethical committee, Region H, The Capital Region of Denmark.

2.3. Psoriasis skin explant cultures

Keratome biospies were kept cold in Dulbecco's modified Eagle medium (DMEM) containing 100 μg/ml gentamicin and 2.5 μg/ml Fungizone (Gibco, Life Technologies, Carlsbad, CA, USA) and placed in culture within maximum 30 h. Three skin punches of 5 mm in diameter were taken from different areas of the keratome biopsy, pooled and cultured in EpiLife medium supplemented with 0.2 ng/ml human epidermal growth factor, 0.2% bovine pituitary extract, 5 μg/ml bovine insulin, 5 μg/ml bovine transferrin and gentamicin (all from Life Technologies), 10 ng/ml rhIL-2 (Gibco, Life Technologies, Minneapolis, MN, USA), 400 U/ml penicillin, 400 μg/ml streptomycin (Gibco, Life Technologies). *In situ* activation of T cells and inflammatory activity was resumed by stimulation with 1 μg/ml anti-CD3 and anti-CD28 antibodies (R&D System), and 50 ng/ml rhIL-23 (R&D System) resulting in a significant and reproducible increase of cytokine expression. The skin cultures were maintained at 37 °C in 5% CO_2 and stimulated skin cultures were treated with compounds or vehicle (0.1% DMSO) in triplicate for 24 h (gene expression analysis) and 72 h (protein analysis). These time points were selected based on time course studies to find the most optimal experimental conditions.

Supernatants from *ex vivo* skin cultures were harvested for protein analysis of IL-22 by ELISA (Human IL-22 Quantikine ELISA kit; R&D Systems) and of IL-17A (human IL-17A V-PLEX), IL-8 (Human IL-8 (HA) V-PLEX), IL-10 and TNF-α (Custom V-PLEX MSD for Proinflammatory Panel) using the MSD platform (Meso Scale Diagnostics, Rockville, MD, USA) according to the manufacturer's instruction. Skin samples were processed for RNA extraction and quantitative real time PCR. Independent experiments were performed using skin samples from six different donors for gene expression analysis and from five different donors for the protein analysis.

2.4. In vitro *differentiation and culture of Th1/Th17 and Tc1/Tc17 cells*

$CD4^+$ and $CD8^+$ T cells were isolated from buffy coat fractions by means of negative selection with RosetteSep™ Human $CD4^+$ T Cell Enrichment Cocktail and RosetteSep™ Human $CD8^+$ T Cell Enrichment Cocktail (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. The purity of isolated T cells routinely exceeded 95%. Purified $CD4^+$ and $CD8^+$ T cells were cultured in X-VIVO 15 medium (LONZA, Basel, Switzerland) containing 2 mM L-glutamine (GlutaMAX, Gibco, Life Technologies), 10 ng/ml rhIL-2 and 100 μg/ml penicillin/streptomycin (Gibco, Life Technologies) at a concentration of 1.5×10^6 cells/ml and activated with anti-Biotin MACSiBead particles loaded with anti-CD3 and anti-CD28 antibodies at 1:1 cell/bead ratio following manufacturer's instruction (Miltenyi Biotec Norden AB, Lund, Sweden). To achieve *in vitro* Th1/Th17 and Tc1/Tc17 differentiation, the cultures were supplemented with 10 ng/ml rhTGFβ (PeproTech Nordic, Stockholm, Sweden), rhIL-1β (Gibco, Life Technologies), rhIL-6, 20 ng/ml rhIL-23 and 1 mg/ml anti-IL-4 and anti-IL-13 antibodies (R&D System) and cultured at 37 °C in 5% CO_2 for 7 days. The expression level of all the transcription factors and