

## **C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> Suppressed Th17 Differentiation and the Pathogenesis of Imiquimod-Induced Psoriasis**

Guangqiang Li <sup>1, a</sup>, Baocheng Wang <sup>2, b, \*</sup>

<sup>1</sup>College of Pharmacy, Jinan University, Guangzhou 510632, P. R. China.

<sup>2</sup>Biomedical Translational Research Institute, Jinan University, Guangzhou 510632, P. R. China.

<sup>a</sup>guangq\_li@126.com, <sup>b</sup>Corresponding author: wbczhz@163.com

### **Abstract**

**Psoriasis is a chronic papulosquamous skin disease that is thought to be a T-cell-mediated autoimmune disorder of keratinocyte proliferation characterised by epidermal hyperplasia (acanthosis) and leukocyte infiltration of the skin. IL-17-producing T helper cells (Th17 cells) is found to have potential functions in the pathogenesis of psoriasis. Inhibition of IL-17 production can reduce psoriasis development. Herein, we found a chemical composition of traditional Chinese medicine (C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub>), which isolated from the deep-sea-derived fungus, could suppress Th17 cells producing inflammatory cytokines of IL-17. In vivo experiments, our results suggested this monomer compound might have an essential function in suppressing pathogenesis of psoriasis.**

### **Keywords**

**C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub>, Th17, anti-inflammation, imiquimod, psoriasis.**

### **1. Introduction**

Psoriasis, a papulosquamous skin disease, is originally thought of as a disorder primarily of epidermal keratinocytes, but is now recognised as one of the commonest immune-mediated disorders. Five types of psoriasis have been reported in the current study: plaque psoriasis; guttate or eruptive psoriasis, which is characterised by scaly teardrop-shaped spots; inverse psoriasis, also called intertriginous or flexural psoriasis that is usually found in folds of skin; pustular psoriasis, which can either take the form of palmoplantar pustulosis or generalised pustular psoriasis (a rare and serious form of psoriasis); and erythrodermic psoriasis, which is a rare but very serious complication of psoriasis [1].

Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), dendritic cells, and T-cells all contributed substantially to its pathogenesis [2]. About 70–80% of patients have mild psoriasis that can be controlled using topical therapies alone [3]. Climate, sun exposure, and ethnicity are thought to affect psoriasis prevalence; however, results from a recent study showed weak correlation between latitude and psoriasis prevalence [4]. HIV infection is also a trigger of psoriasis, because the prevalence of psoriasis in HIV-infected patients is the same or slightly higher than in the general population, and HIV-infected patients with pre-existing psoriasis often have a flare of lesions that are difficult to treat [5]. Moreover, genetic contributions to psoriasis is a nonnegligible factor. Population studies showed that the incidence of psoriasis vulgaris is greater in first and second degree relatives of patients than in the general population [6]. Psoriasis causes many challenges including high prevalence, chronicity, disfigurement, disability, and associated comorbidity.

In recent years, clinical and basic science observations have shown that innate as well as adaptive immunity is crucial in the initiation and maintenance of psoriatic plaques. Natural killer cells and natural killer T cells (NKT) are part of the cutaneous inflammation in psoriasis [7]. Three types of dendritic cells appear likely to be involved in the development of psoriasis: langerhans cells in the epidermis; dermal factor XIII a-positive dendritic cells; and a subset of dendritic cells, known as plasmacytoid dendritic cells, which are found in involved psoriatic skin but not in normal skin [8].

The leucocyte infiltrate in psoriasis consists predominantly of CD4-positive and CD8-positive T-cells, and may precede epidermal hyperplasia [9]. Psoriasis is classified as a Th1 disease, which is consistent with the relative under-representation of Th2 diseases, such as atopic dermatitis, in patients with psoriasis [10]. Th17 cells, a distinct subset of CD4+ T cells, secrete the cytokines IL-17A and IL-17F and express lineage-specific transcription factor ROR $\gamma$ t. Under certain conditions, their effector molecules, such as IL-17, IL-21 and IL-22 are associated with the pathogenesis of several autoimmune and inflammatory diseases [1]. Th17 cells, through the production of both IL-22 and IL-17, induce keratinocyte proliferation and other hallmark features of psoriasis [11].

Herein, we found a monomer compound C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> could suppress the production of proinflammatory cytokine IL-17 and the pathogenesis of imiquimod-induced psoriasis.

## 2. Materials and Methods

### 2.1 Mice

IL-17-GFP mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were males, aged 6–8 weeks old and weighed 17–22 g. All animals were maintained under specific pathogen-free (SPF) conditions in the animal facility of Jinan University (Guangzhou, China). The experimental procedures were approved by the Jinan University's Institutional Lab Animal Care and Use Committee. Maximum care was taken to limit the number of animals used in this study.

### 2.2 Reagents

The following reagents were used: Iscove's Modified Dulbecco's Medium (IMEM, Gibco), fetal bovine serum (FBS, Gibco), penicillin/streptomycin (P/S, 10,000  $\mu$ g/ml, Sigma), phosphate buffered saline (PBS, Hyclone), anti-CD3 antibody (Sungene, clone:145-2C11), anti-IL-4 antibody (Sungene, clone:11B11), anti-CD28 antibody (Sungene, clone:37.51), anti-IFN- $\gamma$  antibody (Sungene, clone:XMG1.2), TGF- $\beta$  (Biologend, cat#580702), IL-6 (PeproTech, cat#216-16), APC anti-mouse CD4 (Sungene, M10041-11A).

### 2.3 Th17 Cell Expansion in vitro

Naive CD4+ T cells were isolated from the spleen of male IL-17-GFP mice. Then the cells were activated with immobilized anti-CD3 (10 mg/ml) and soluble anti-CD28 (1 mg/ml) and were induced to differentiate into Th17 cells with TGF- $\beta$  (2 ng/ml), IL-6 (10 ng/ml), anti-IL-4 (10 ng/ml), and anti-IFN- $\gamma$  (10 ng/ml) for 96 h.

### 2.4 RNA Extraction and Quantitative RT-PCR Analysis

Total RNA was extracted from Th17 cells cultured in vitro. High-fidelity cDNA was generated from each RNA sample with the Bio-Rad S1000 Thermal Cycler system. Quantitative RT-PCR reaction samples were prepared as a mixture with Quantitect SYBR Green PCR kit (Takara) following the manufacturers' instructions. Reactions were performed using Bio-Rad CFX Connect Real-Time System. The primer sequences used are as shown in **Tab1**.

Table 1 The primer sequences

| Gene           | Sequence (5'-3')                 |
|----------------|----------------------------------|
| ROR $\gamma$ t | Forward: GACCCACACCTCACAAATTGA   |
|                | Reverse: AGTAGGCCACATTACACTGCT   |
| IL-17          | Forward: TTAACTCCCTTGGCGCAAAA    |
|                | Reverse: CTTTCCCTCCGCATTGACAC    |
| IL-23R         | Forward: TTCAGATGGGCATGAATGTTTCT |
|                | Reverse: CCAAATCCGAGCTGTTGTTCTAT |
| Hprt           | Forward: TCAGTCAACGGGGGACATAAA   |
|                | Reverse: GGGGCTGTACTGCTTAACCAAG  |

### 2.5 Animal Model of Psoriasis and the Therapeutic Effect Evaluation of C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub>

Male BALB/c mice were induced psoriasis by imiquimod. The details are described as follows. 12 mice were assigned to imiquimod and 6 to control treatment. In the imiquimod group, 62.5 mg imiquimod cream (5% Aldara®; 3M Pharmaceuticals) was applied to the shaved back and ear of the

mice with the following schedule, daily for 8 consecutive days. On day 2, 6 mice of imiquimod group were treated with  $C_{26}H_{26}N_4O_{10}$  (dissolved in PEG400) through hypodermic injection (10 mg/kg/day). In the control group, petroleum jelly (Vaseline®) was applied to the same skin sites according to the same schedule. On day 8, all mice were sacrificed and ear tissue were obtained for pathological analysis.

### 2.6 Immunohistochemistry

Ear tissue obtained on day 8 were used for histological analysis. Formalin-fixed, paraffin-embedded tissue blocks were sectioned at 4  $\mu$ m thickness and subjected to standard haematoxylin and eosin (H&E) staining.

### 2.7 Flow Cytometry

After incubation of  $C_{26}H_{26}N_4O_{10}$  with proliferating Th17 cells for 96 h, production of IL-17 and CD4+T cells were determined by APC-CD4 staining and analyzed by flowcytometry.

### 2.8 Statistical Analysis

The SPSS 20.0 statistical software was used to process and analyze the data and images. For ear thickness analysis, Man Whitney U test was used to identify the difference between groups. Other data were analyzed using student's t-test. All tests were two-sided. Datas were presented as the Mean  $\pm$  SEM.  $P < 0.05$  was considered to be statistically significant.

## 3. Results

### 3.1 The structure and molecular formula of the monomer compound

The dimeric nitrophenyl trans-epoxyamides were obtained from the deep-sea-derived fungus *Penicillium chrysogenum* SCSIO41001. Our previous studies have analyzed its chemical structure and the molecular formula was determined to be  $C_{26}H_{26}N_4O_{10}$  [12]. The structure was shown as follows:

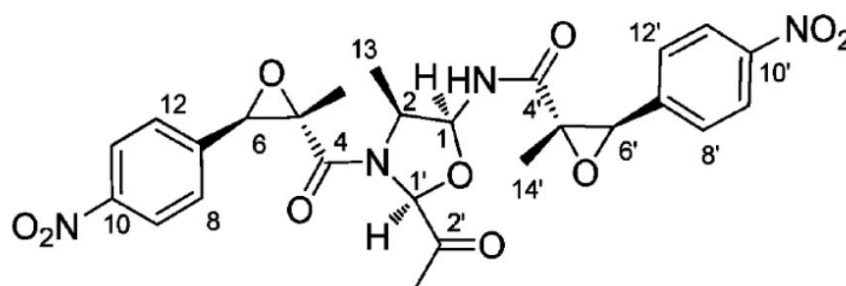


Fig.1. Structure of  $C_{26}H_{26}N_4O_{10}$

### 3.2 $C_{26}H_{26}N_4O_{10}$ suppressed Th17 cells producing inflammatory cytokines of IL-17

To investigate the anti-inflammatory role of  $C_{26}H_{26}N_4O_{10}$ , lymphocytes isolated from mice spleen were treated with  $C_{26}H_{26}N_4O_{10}$  under Th17-cell-polarizing conditions. (Details were described in Materials and Methods 2.3). There was no effect on CD4+ T cells differentiation when treated with  $C_{26}H_{26}N_4O_{10}$  (Fig.1A). And the statistical results of three independent experiments were shown no statistical significance at different drug concentrations (1  $\mu$ M, 5  $\mu$ M, 25  $\mu$ M) (Fig.1C). However, there was a significant change of IL-17 producing as shown in Fig.1B, treatment naive T cells with  $C_{26}H_{26}N_4O_{10}$  under Th17-cell-polarizing conditions resulted in significant inhibition of IL-17 producing compared to vehicle-treated (DMSO) cells (Fig.1B & D). These data clearly demonstrated that we have developed a compound that targets Th17 and was efficacious in inhibition of IL-17 producing.

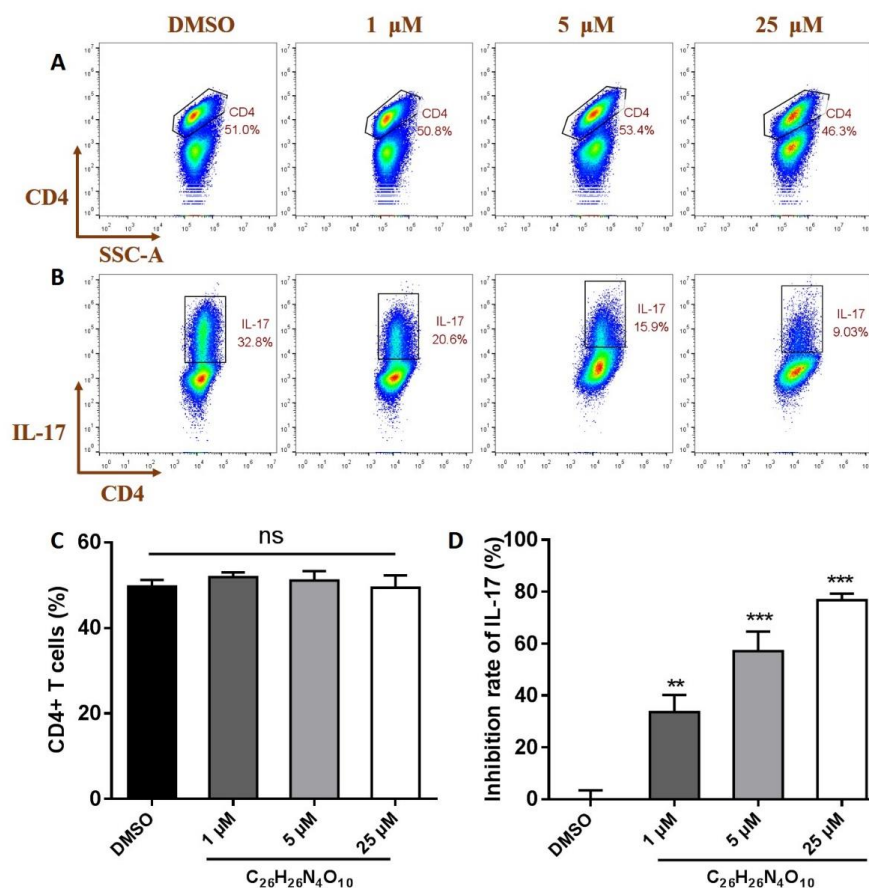


Fig.2. Naive T cells were activated under Th17-cell-polarizing conditions in the presence of different doses (1  $\mu M$ , 5  $\mu M$ , 25  $\mu M$ ) of  $C_{26}H_{26}N_4O_{10}$  or DMSO (vehicle control). After 4 days, polarized Th17 cells were stained by APC-CD4 and then the cytokine IL-17 were determined by flowcytometry. (A) Proportion of CD4 at different doses (1  $\mu M$ , 5  $\mu M$ , 25  $\mu M$ ) of  $C_{26}H_{26}N_4O_{10}$ . (B) Secretion of IL-17 at different doses of  $C_{26}H_{26}N_4O_{10}$ . (C) Statistical analysis of the effect of  $C_{26}H_{26}N_4O_{10}$  on CD4 differentiation. (D) The inhibitory rate on the production of IL-17 at different concentration of  $C_{26}H_{26}N_4O_{10}$ . The results are shown as mean  $\pm$  SEM; \*\*p < 0.01, \*\*\*p < 0.001, N=3.

### 3.3 $C_{26}H_{26}N_4O_{10}$ inhibited the expression of ROR $\gamma$ t, IL-17A and IL-23R in Th17 cells.

To confirm these results that  $C_{26}H_{26}N_4O_{10}$  could repress the Th17 differentiation. Naive T cells were activated under Th17-cell-polarizing conditions in the presence of  $C_{26}H_{26}N_4O_{10}$  (5  $\mu M$ ) or DMSO (vehicle control). After 4 days, polarized Th17 cells were collected for RNA extraction and quantitative PCR to further analysis the mRNA expression of Th17 differentiation-related gene. These results demonstrated that  $C_{26}H_{26}N_4O_{10}$  could inhibit the transcriptional activity of ROR $\gamma$ t (Fig.3A), resulting in the suppression of IL-17 (Fig.3B) and IL-23R (Fig.3C) gene expression.

### 3.4 $C_{26}H_{26}N_4O_{10}$ Suppressed the Pathogenesis of Imiquimod-Induced Psoriasis

To further study its immune regulation in vivo. Male BALB/c mice (6 week aged) were induced psoriasis by imiquimod and then treated with  $C_{26}H_{26}N_4O_{10}$ . On day 4, imiquimod treated mice started to demonstrate increased ear thickness. However, the increasing rate of ear thickness in  $C_{26}H_{26}N_4O_{10}$  treated group was lower compared to the IMQ group. Until day 8, mice ear thickness in  $C_{26}H_{26}N_4O_{10}$  treated group was significantly attenuated compared to the IMQ group (Fig.4A). On day 8, all mice were sacrificed and ear tissue were obtained for morphological analysis. Imiquimod treated mice shows marked epidermal acanthosis, hyperkeratosis. However,  $C_{26}H_{26}N_4O_{10}$  treated mice showed significantly reduced of psoriatic pathogenesis, compared to IMQ mice (Fig.4B). All results suggested that  $C_{26}H_{26}N_4O_{10}$  had a potential role in suppressing the pathogenesis of imiquimod-induced psoriasis.

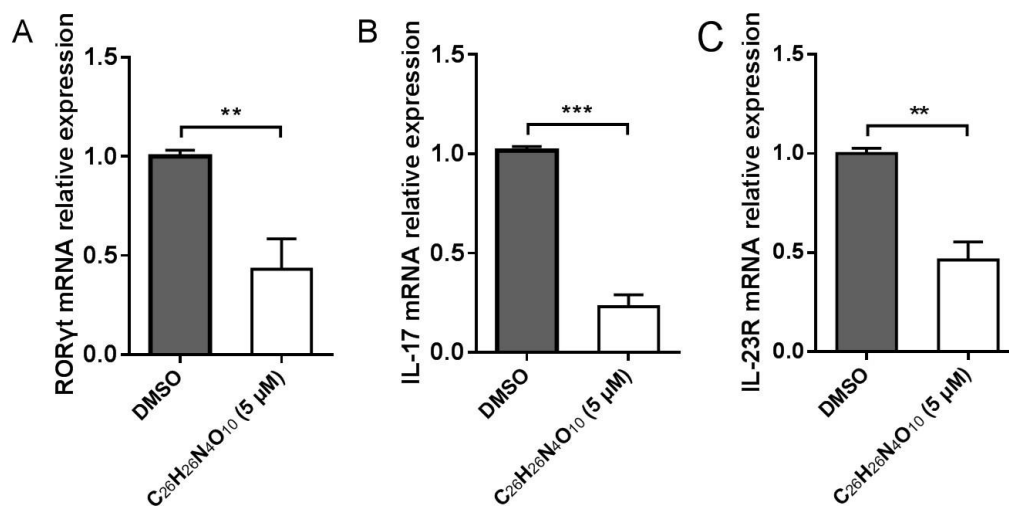


Fig.3. Th17 cells were pretreated with C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> (5  $\mu$ M) or DMSO for 96 h. ROR $\gamma$ t (Fig.2A), IL-17A (Fig.2B) and IL-23R (Fig.2C) mRNA expression were quantitated and normalized to Hprt. Data were representative of three independent experiments. The results were shown as mean  $\pm$  SEM; \*\*p < 0.01, \*\*\*p < 0.001.

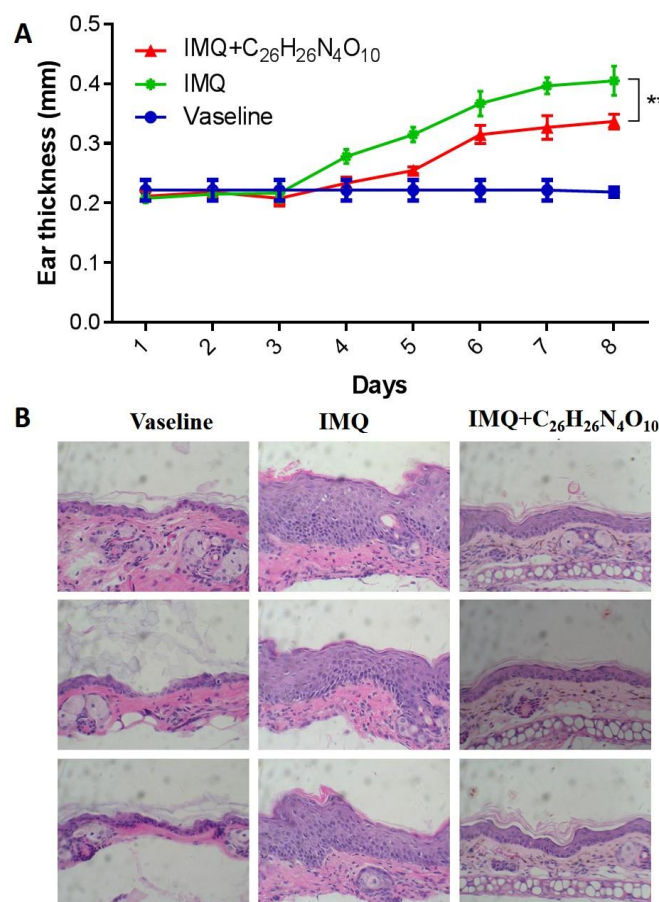


Fig.4. Evaluation of the therapeutic effect of C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> on psoriasis. (A) The ear thickness of the three groups of mice was measured daily. Man Whitney U test was used to identify the difference between groups. N=6 mice per group, \*\*p < 0.01. (B) On day 8, three groups of mice ear sections were analyzed by H&E staining.

#### 4. Conclusion

Psoriasis is a long-term chronic inflammatory skin disease. The accumulated experimental and clinical studies show that CD4<sup>+</sup>T cells, especially Th1 and Th17 subsets, and their cytokines play critical roles in the pathophysiology of psoriasis [13, 14]. Interleukin 17 is involved in the inflammatory cascade in several immune-mediated and inflammatory diseases such as psoriasis, psoriatic arthritis, and ankylosing spondylitis. Blocking interleukin 17 in severe psoriasis leads to superior efficacy over etanercept, a TNF inhibitor that is still widely prescribed based on its balanced benefit-to-risk profile, and on substantial experience in clinical practice acquired over more than a decade[15].

In our studies, C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> was used to co-incubate with proliferating Th17 cells. The results showed that the C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> suppressed Th17 cells producing inflammatory cytokines of IL-17. Furthermore, treatment of Th17 with C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> reduced the expression of ROR $\gamma$ t, IL-17 and IL-23R. These data strongly suggested that C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> was a potent and efficacious ROR $\gamma$ t modulator and repressed its transcriptional activity. In vivo experiment, we found the psoriasis could be alleviated through hypodermic injection of C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub>. Therefore, the present study may offer a potential utility of C<sub>26</sub>H<sub>26</sub>N<sub>4</sub>O<sub>10</sub> for the treatment of Th17-mediated immune disease, such as psoriasis.

#### Conflict of interests

All authors have no conflict of interest to declare.

#### References

- [1] W.H. Boehncke, M.P. Schon, Psoriasis, *Lancet*, 386 (2015) 983-994.
- [2] C.E. Griffiths, J.N. Barker, Pathogenesis and clinical features of psoriasis, *Lancet*, 370 (2007) 263-271.
- [3] M.P. Schon, W.H. Boehncke, Psoriasis, *The New England journal of medicine*, 352 (2005) 1899-1912.
- [4] N. Balato, L. Di Costanzo, C. Patrino, A. Patri, F. Ayala, Effect of weather and environmental factors on the clinical course of psoriasis, *Occupational and environmental medicine*, 70 (2013) 600.
- [5] L. Fry, B.S. Baker, Triggering psoriasis: the role of infections and medications, *Clinics in dermatology*, 25 (2007) 606-615.
- [6] E.M. Farber, M.L. Nall, W. Watson, Natural history of psoriasis in 61 twin pairs, *Archives of dermatology*, 109 (1974) 207-211.
- [7] A.A. Gaspari, Innate and adaptive immunity and the pathophysiology of psoriasis, *Journal of the American Academy of Dermatology*, 54 (2006) S67-80.
- [8] C.E. Griffiths, R.J. Dearman, M. Cumberbatch, I. Kimber, Cytokines and Langerhans cell mobilisation in mouse and man, *Cytokine*, 32 (2005) 67-70.
- [9] H. Valdimarsson, B.S. Baker, I. Jonsdottir, A. Powles, L. Fry, Psoriasis: a T-cell-mediated autoimmune disease induced by streptococcal superantigens?, *Immunology today*, 16 (1995) 145-149.
- [10] E. Landgren, L. Braback, G. Hedlin, A. Hjern, F. Rasmussen, Psoriasis in Swedish conscripts: time trend and association with T-helper 2-mediated disorders, *The British journal of dermatology*, 154 (2006) 332-336.
- [11] Y. Zheng, D.M. Danilenko, P. Valdez, I. Kasman, J. Eastham-Anderson, J. Wu, W. Ouyang, Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis, *Nature*, 445 (2007) 648-651.
- [12] S. Chen, J. Wang, X. Lin, B. Zhao, X. Wei, G. Li, K. Kaliaperumal, S. Liao, B. Yang, X. Zhou, J. Liu, S. Xu, Y. Liu, Chrysamides A-C, Three Dimeric Nitrophenyl trans-Epoxyamides Produced

- by the Deep-Sea-Derived Fungus *Penicillium chrysogenum* SCSIO41001, *Organic letters*, 18 (2016) 3650-3653.
- [13] A. Nikaiein, C. Phillips, S.C. Gilbert, D. Savino, A. Silverman, M.J. Stone, A. Menter, Characterization of skin-infiltrating lymphocytes in patients with psoriasis, *The Journal of investigative dermatology*, 96 (1991) 3-9.
- [14] A. Di Cesare, P. Di Meglio, F.O. Nestle, The IL-23/Th17 axis in the immunopathogenesis of psoriasis, *The Journal of investigative dermatology*, 129 (2009) 1339-1350.
- [15] H. Bachelez, Interleukin-17 inhibition: a route to psoriasis clearance?, *Lancet*, 386 (2015) 510-512.