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Periplogenin induces necroptotic cell death through oxidative stress in HaCaT cells and ameliorates skin lesions in the TPA- and IMQ-induced psoriasis-like mouse models

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ABSTRACT

Psoriasis is a multifactorial skin disease that inconveniences many patients. Considering the side effects and drug resistance of the current therapy, it is urgent to discover more effective and safer anti-psoriatic drugs. In the present study, we screened over 250 traditional Chinese medicine compounds for their ability to inhibit the cell viability of cultured human HaCaT keratinocytes, a psoriasis-relevant *in vitro* model, and found that periplogenin was highly effective. Mechanistic studies revealed that apoptosis and autophagy were not induced by periplogenin in HaCaT cells. However, periplogenin caused PI to permeate into cells, increased lactate LDH release and rapidly increased the number of necrotic cells. Additionally, the typical characteristics of necrosis were observed in the periplogenin-treated HaCaT cells. Notably, the necroptosis inhibitor Nec-1 and NSA were able to rescue the cells from necrotic cell death, supporting that necroptosis was involved in periplogenin-induced cell death. Furthermore, the ROS levels were elevated in the periplogenin-treated cells, NAC (an antioxidant) and Nec-1 could inhibit the ROS levels, and NAC could attenuate necroptotic cell death, indicating that the periplogenin-induced necroptotic cell death was mediated by oxidative stress. More importantly, in the murine models of TPA-induced epidermal hyperplasia and IMQ-induced skin inflammation, topical administration of periplogenin ameliorated skin lesions and inflammation. In sum, our results indicate, for the first time, that periplogenin is a naturally occurring compound with potent anti-psoriatic effects *in vitro* and *in vivo*, making it a promising candidate for future drug research.

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1. Introduction

Psoriasis is a common chronic inflammatory and hyperproliferative skin disorder characterized by patches of thick, red skin covered with silvery scales [1]. The disorder affects approximately 2–3% of the world's population, and there is no preventive or curative therapy [2,3]. It affects the psychological well-being of patients, leading to anger, embarrassment, low self-esteem and even depression [4]. These physical, social and psychological impacts ultimately affect psoriasis patients' quality of life [5].

It is generally acknowledged that psoriasis is a skin disease characterized chronic inflammation, hyperproliferation with incomplete differentiation and reduced apoptosis of epidermal keratinocytes [6,7]. Histologically, psoriasis is characterized by several unique features, such as epidermal hyperplasia with dysregulated keratinocyte differentiation, pronounced inflammatory cell infiltration and increased vascularization [8]. The etiopathogenesis of this skin disease remains unclear at present; however, hyperproliferation and abnormal differentiation of epidermal keratinocytes are essential in the pathophysiology of psoriasis [9]. Various pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukins (ILs) and interferon-γ (IFN-γ), are overexpressed in psoriasis [8]. Although the focus of studying therapeutic

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targets in psoriasis has recently shifted to the innate immune system, which plays important roles in the pathogenesis of psoriasis, compounds that inhibit keratinocyte proliferation are potentially useful in the management of psoriasis because the recovery from a psoriatic to a normal epidermis requires rebalanced homeostatic control of keratinocyte growth and differentiation [10]. Indeed, many commonly prescribed anti-psoriatic drugs, such as methotrexate, dithranol and vitamin D3 analogs, exert their therapeutic actions by counteracting keratinocyte hyperproliferation [1]. Therefore, it is important to identify anti-proliferative agents that act on keratinocytes to develop new and effective pharmaceutical agents for the treatment of psoriasis. It is now well known that HaCaT keratinocytes are proper candidates for studying the effects of drugs on hyperproliferative skin diseases such as psoriasis [11,12].

Currently, many treatment modalities are available for alleviating psoriatic lesions, ranging from topical therapy [13] for mild conditions to phototherapy [14] or systemic therapy [15]. However, Western medicines are difficult to use for a prolonged period because of their unsatisfactory effects or high toxicity or side effects and do not provide a complete cure [16]. Although there are traditional folk remedies for psoriasis in China [17], the active ingredients in these remedies and the mechanisms underlying their effects remain obscure [18], which makes their popularization and application difficult. Therefore, it is urgent to develop new drugs for the treatment of psoriasis.

In the present study, we investigated the cellular toxicity of traditional Chinese medicine compounds on cultured HaCaT keratinocytes as a psoriasis-relevant experimental model, and periplogenin was found to be a potent agent that could induce cell death. We performed additional experiments to elucidate the mechanism underlying the periplogenin-induced inhibition of HaCaT cell growth *in vitro*. Additionally, *in vivo* studies were performed in the psoriasis-like murine models [19] of 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced hyperplasia and imiquimod (IMQ)-induced skin inflammation.

2. Materials and methods

2.1. Materials

All natural compounds were purchased from the National Institutes for Food and Drug Control (NIFDC, Beijing, China), and their purity was greater than 95%. All of the compounds were dissolved in dimethyl sulfoxide (DMSO) as a 1 mg/ml stock solution and stored at −20 °C. The antibodies against cleaved caspase-3, Beclin-1 and LC3 were purchased from Cell Signaling Technology (Beverly, MA, USA). The mouse monoclonal antibody against GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was obtained from Kangcheng Biotech (Shanghai, China). Doxorubicin (ADM) was purchased from Hisun (Zhejiang, China). 4,6-Diamidino-2-phenylindole (DAPI) was purchased from Beyotime (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and TPA were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture and treatments

The HaCaT human keratinocyte cell line was obtained from the Chinese Academy of Sciences Shanghai Institute for Biological Sciences-Cell Resource Center. Hs-68, a human fibroblast cell line established from the foreskin of a normal Caucasian newborn male, was purchased from the American Type Culture Collection (Rockefeller, Maryland, USA). The cell lines had been characterized by short tandem repeat profiling, cell morphology and karyotyping assays. The HaCaT cells were maintained in RPMI 1640 medium (Corning, New York, USA) supplemented with 10% fetal bovine serum (FBS; TBD Science, Tianjin, China) and 200 U/ml gentamycin sulfate (Huangzhong Pharmaceutical Co., Ltd., XiangYang, China). The Hs-68 cells were routinely maintained in minimum essential medium (MEM; Corning, New York, USA) supplemented with 10% FBS (TBD Science, Tianjin, China) and 200 U/ml gentamycin sulfate. The cells were grown at 37 °C in a humidified atmosphere with 5% CO_2. In some experiments, the cells were exposed to various inhibitors for 1 h prior to the periplogenin treatment, including 40 μM N-benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (z-VAD-fmk) to inhibit the activation of caspase; 1 mM 3-methyladenine (3-MA) and 10 μM 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride (LY294002) to inhibit phosphoinositide 3-kinase (PI3K); 40 μM chloroquine diphosphate salt (CQ) to prevent autophagosome and lysosome fusion; 50 μM necrostatin-1 (Nec-1) to inhibit RIPK1; 1 μM necrosulfonamide (NSA) to inhibit MLKL; and 10 mM N-acetyl-cysteine (NAC) to inhibit oxidation. NSA was purchased from Abcam (Cambridge, UK), and all other inhibitors were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Compound screening

The compounds were screened using the MTT assay. The HaCaT cells were plated in triplicate in 96-well plates (1.5×10^4 cells/well). Twelve hours later, the cells were treated with 5 μg/ml of the compounds or DMSO in media supplemented with 3% serum. After 44 h of incubation, 20 μl of 5 mg/ml MTT in phosphate-buffered saline (PBS) was added to each well for an additional 4 h of incubation. The supernatant was removed, and the MTT formazan precipitate was dissolved in 100 μl of DMSO. The absorbance was measured on a microplate reader (Bio-Rad) at 570 nm. The inhibition ratio was determined by the following equation: inhibition ratio = (1 − OD value of experimental group/OD value of control group) × 100%.

2.4. Cell viability assay

The HaCaT cells were plated in triplicate in 96-well plates at 1.5×10^4 cells per well, whereas the Hs-68 cells were plated in triplicate in 96-well plates at 5.0×10^3 cells/well. Twelve hours later, the cells were treated with periplogenin for 44 h at the indicated concentrations. Cell viability was measured using the MTT assay. The cell viability was determined by the following equation: cell viability = OD value of experimental group/OD value of control group × 100%.

2.5. Cytotoxicity assay by real-time cell analysis (RTCA)

To validate our previous cytotoxicity and proliferation results, the Real-Time Cell Analysis (RTCA) xCELLigence System (ACEA Biosciences, San Diego, CA, USA) was used to dynamically monitor the cell proliferation rates. The system monitors cellular events at set intervals by measuring the electrical impedance across microelectrodes on the bottom of tissue culture E-plates. The impedance measurement provides information about the cell number, viability, morphology and adhesion. The RTCA Software was supplied by the manufacturer, and the measurements were analyzed based on the cell index (CI). The CI integrates the number and size of living cells. The method is established and has been described in the literature [20]. The HaCaT cells were seeded at a density of 1.5×10^4 cells/well on an E-Plate. After 12 h of incubation, the cells were treated with DMSO or periplogenin (0.5 μg/ml, 1 μg/ml, and 2 μg/ml). The dynamic CI values were monitored at 15-min intervals for 38 h after the treatments.

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