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Icotinib inhibits EGFR signaling and alleviates psoriasis-like symptoms in animal models

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ABSTRACT

To investigate the effects of icotinib hydrochloride and a derivative cream on epidermal growth factor receptor (EGFR) signaling and within animal psoriasis models, respectively. The effect of icotinib on EGFR signaling was examined in HaCaT cells, while its effect on angiogenesis was tested in chick embryo chorioallantoic membranes (CAM). The effectiveness of icotinib in treating psoriasis was tested in three psoriasis models, including diethylstilbestrol-treated mouse vaginal epithelial cells, mouse tail granular cell layer formation, and propranolol-induced psoriasis-like features in guinea pig ear skin. Icotinib treatment blocked EGFR signaling and reduced HaCaT cell viability as well as suppressed CAM angiogenesis. Topical application of icotinib ameliorated psoriasis-like histological characteristics in mouse and guinea pig psoriasis models. Icotinib also significantly inhibited mouse vaginal epithelium mitosis, promoted mouse tail squamous epidermal granular layer formation, and reduced the thickness of the horny layer in propranolol treated auricular dorsal surface of guinea pig. We conclude that icotinib can effectively inhibit psoriasis in animal models. Future clinical studies should be conducted to explore the therapeutic effects of icotinb in humans.

1. Introduction

Epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptor tyrosine kinases. EGFR signals through a complex network of pathways and its activities play an essential role in physiological processes, including mammalian cell growth, survival, proliferation, and differentiation [1]. Based on EGFR's fundamental role in mammalian cells, it has been implicated in multiple diseases, including cancer and inflammatory diseases. EGFR has been found to be involved in the pathogenesis and progression of different carcinomas [2] as well as to play a crucial role in angiogenesis [3]. As a result, EGFR has emerged as a central therapeutic target for numerous diseases, and EGFR inhibitors are widely used to treat a variety of cancers and other diseases.

Psoriasis is defined as a non-contagious chronic inflammatory dermatosis. The overall prevalence of psoriasis is 2–3% worldwide with rates higher in American and Canadian populations (4.6–4.7%) versus African and Asian populations (0.4–0.7%) [4,5]. Psoriasis is characterized by epidermal hyper-proliferation, abnormal keratinization, and inflammation [6,7]. There are four different subtypes, which include guttate, pustular, erythrodermic, and inverse psoriasis [8,9]. The most common type of psoriasis is chronic plaque psoriasis or psoriasis vulgaris [10]. The pathogenesis of psoriasis is not fully understood, and heterogeneity within the disease is probably due to the interaction of multiple gene abnormalities with environmental factors [4,9,11]. However, dysregulated EGFR signaling is thought to cause epidermal hyper-proliferation and differentiation abnormalities, which in turn results in the development of psoriasis-like lesions [12]. Furthermore, EGFRs are overexpressed in psoriatic keratinocytes [13], indicating that suppressing EGFR activities with an inhibitor could be beneficial to this condition.

Icotinib is a highly selective EGFR tyrosine kinase inhibitor (EGFR-TKI) and has shown promising clinical efficacy and safety in patients with non-small-cell lung cancer (NSCLC) [14–17]. In addition, it has also been found to alleviate psoriasis symptoms effectively within our clinic (unpublished observations). These results suggested that icotinib

Abbreviations: CAM, chorioallantoic membranes; CK17/18, cytokeratin 17/18; EGFR, epidermal growth factor receptor; FCS, fetal calf serum; HE, hematoxylin and eosin; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NS, normal saline; NSCLC, non-small-cell lung cancer; RhEGF, recombinant human epidermal growth factor; Stat3, signal transducer and activator of transcription 3

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may have potential therapeutic effect in treating psoriasis. This study aims to investigate the effects of topically applied icotinib on psoriasis in animal models.

2. Material and methods

2.1. Animals

Adult female ICR mice (20–25 g) were purchased from Shanghai Sino-British SIPPR/BK Lab Animal Co., Ltd., (Shanghai, China). Adult male guinea pigs (190–200 g) were purchased from Shanghai Jiagan Biological Technology Co., Ltd., (Shanghai, China). All animals were housed under controlled conditions at room temperature of approximately 25 °C and exposed to a 12 h light/dark cycle with *ad libitum* access to food and water. All animal care and experimental procedures were performed in accordance with institutional (Shanghai Institute of Pharmaceutical Industry) animal ethical committee guidelines, which conform to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health.

2.2. Reagents

For *in vitro* studies, icotinib hydrochloride (Betta Pharmaceuticals Co., Ltd) was prepared as a DMSO stock solution and diluted in the relevant assay media. For *in vivo* studies, icotinib was applied at concentrations of 0.5%, 1%, 2%, and 4% as a cream (Betta Pharmaceuticals Co., Ltd). Calcipotriol ointment was purchased from LEO Pharma. Sorafenib was provided by the Shanghai Institute of Pharmaceutical Industry.

2.3. In vitro proliferation assay

HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, USA). Cells were seeded at a density of 2×10^4 cells/well in a volume of 100 μL/well in 96-well plates. Media that contained icotinib (0.001, 0.01, 0.1, 1, 10, 100 μg/mL) or DMSO was added 24 h after plating. Seventy-two hours after the addition of icotinib, cell proliferation was assessed using a Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay [18]. The IC_{50} was determined as the concentration of icotinib needed to reduce cell viability to 50% of DMSO control.

2.4. Immunoblot analysis

HaCaT cells were cultured in DMEM media (Gibco) supplemented with 10% (v/v) FCS. Cells were pretreated with icotinib at the indicated concentrations (0, 0.03, 0.1, 0.3, 1, 3 μM) for 24 h followed by treatment with 100 ng/mL recombinant human epidermal growth factor (rhEGF, R&D, USA) for 15 min. Cells were then collected and homogenized in radio-immunoprecipitation assay buffer (RIPA buffer, Thermo Fisher Scientific, USA) containing protease inhibitor cocktail (Thermo Fisher Scientific, USA), proteins were extracted according to the manufacturer's instructions. Equal amounts of protein extract (50 μg of total protein) were subjected to SDS-PAGE gel electrophoresis, followed by electrophoretic transfer of proteins to polyvinylidene fluoride membrane. The membrane was blocked in 3% BSA for 2 h, and then incubated with appropriate primary antibodies (1:1000 diluted in 3% BSA) overnight at 4 °C. The membrane was then washed and incubated with secondary antibodies (1:2000 diluted in 3% BSA) for 1 h at room temperature. Protein bands were visualized using an enhanced chemilunminescence detection system (Thermo Fisher Scientific, USA). Primary antibodies used include: β-actin, p-EGFR, EGFR, Signal transducer and activator of transcription 3 (Stat3), p-Stat3 (Tyr705), Akt and p-Akt (Ser473). Secondary antibodies included anti-rabbit IgG HRP-linked antibody and anti-mouse IgG HRP-linked antibody. All antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.5. Angiogenesis assay

The effect of icotinibon angiogenesis was evaluated using a chorioallantoic membrane (CAM) assay [19]. Fertilized eggs were incubated for 6 days at 37 °C and relative humidity of 50%. A hole of approximately 1 cm in diameter was created in the eggshell with sterile tweezers to provide access to the CAM. All test drugs were dissolved in 5% glucose. Membranes were treated with 5% glucose (vehicle control), icotinib and sorafenib. Drug drops were air dried on filter paper, and then gently placed on the CAM. Eggs were then covered with a laboratory wrapping film to prevent dehydration and possible contamination. Images of the CAM before and 48 h after treatment with different drugs were acquired with a camera (37XB/37 × BTV, Shanghai optical instrument factory, China). The number of new blood vessels was assessed by counting the branching points of the blood vessels. The relative inhibition rate was calculated as follows: inhibition rate (%) = 100 – [Drug treated group (48 h) − Drug treated group (0 h)]/[Control group (48 h) − Control group (0 h)] × 100.

2.6. Mitotic ratio analysis in mouse vaginal epithelium

Female mice were injected intraperitoneally with 35 mg/kg diethylstilbestrol once daily for 3 days. On the 4th day, mice were screened by vaginal smear and those in estrum were used in the study. Normal saline (NS), empty cream, icotinib hydrochloride cream at different concentrations (0.5, 1.0, 2.0, 4.0%), and calcipotriol ointment were topically applied once daily for 3 days. Two hours after the last application, mice were injected intraperitoneally with colchicine (15 mg/kg) and sacrificed 6 h after the injection. Vaginas were collected and fixed in 4% paraformaldehyde. Vaginal tissues were sectioned and stained with haematoxylin and eosin to identify mitotic epithelial cells, which were then counted. The mitotic index was defined as the number of mitotic cells found in 100 basal cells as imaged under the light microscope.

2.7. Histological analysis of the granular layer in mouse tail epidermis

NS, empty cream, calcipotriol ointment, and icotinib hydrochloride cream at 0.5%, 1%, 2%, or 4% were topically applied to mouse tails once daily for 7 days. One hour after the last application, mice were sacrificed and the tail skin was collected, fixed in 10% formalin, sectioned and stained with haematoxylin and eosin. For each tail section, the number of granular layers (SG) was counted per 100 scales. The granular layer ratio was calculated as 100 × (SG/100).

2.8. Histological analysis of guinea pig ear skin treated by propranolol

Propranolol was dissolved in an emulsifying ointment (5%, 200 μL) and was topically applied to the dorsal surface of the guinea pig auricular skin twice a day for 4 weeks. After the last application, animals were treated with a topical application of 200 μL of empty cream, calcipotriol ointment, or icotinib hydrochloride cream at 0.5%, 1%, 2%, and 4% twice daily for one week. Animals were sacrificed 24 h after the last application and ear specimens were collected, fixed in 10% formalin solution, and embedded in paraffin. Sections were obtained and stained with hematoxylin and eosin. Sections were examined for epidermal thickness by light microscopy.

2.9. Statistical analysis

The data were presented as mean ± standard error of the mean (SEM) unless otherwise noted. Statistical analysis of data was performed using SPSS 17.0 software. One way ANOVA test followed by