Repurposing Auranofin, an Anti-Rheumatic Gold Compound, to Treat Acne Vulgaris by Targeting the NLRP3 Inflammasome

Gabsik Yang1,ψ, Seon Joo Lee1, Han Chang Kang1, Yong-Yeon Cho1, Hye Suk Lee1, Christos C. Zouboulis2, Sin-Hee Han3, Kyung-Ho Ma3, Jae-Ki Jang1 and Joo Young Lee1,*

1BK21plus Team, College of Pharmacy, The Catholic University of Korea, Bucheon 14662, Republic of Korea
2Departments of Dermatology, Venereology, Allergology, and Immunology, Dessau Medical Center, Brandenburg Medical School Theodore Fontane, Dessau, Germany
3Department of Herbal Crop Research, National Institute of Horticultural & Herbal Science, Rural Development Administration, Eumsung 27709, Republic of Korea

Abstract
Activation of the NLRP3 inflammasome is critical for host defense as well as the progression of inflammatory diseases through the production of the proinflammatory cytokine IL-1β, which is cleaved by active caspase-1. It has been reported that overactivation of the NLRP3 inflammasome contributes to the development and pathology of acne vulgaris. Therefore, inhibiting activation of the NLRP3 inflammasome may provide a new therapeutic strategy for acne vulgaris. In this study, we investigated whether auranofin, an anti-rheumatoid arthritis agent, inhibited NLRP3 inflammasome activation, thereby effectively treating acne vulgaris. Auranofin suppressed NLRP3 inflammasome activation induced by Propionibacterium acnes, reducing the production of IL-1β in primary mouse macrophages and human sebocytes. In a P. acnes-induced mouse model, injection of P. acnes into the ears of mice induced acne symptoms such as redness, swelling, and neutrophil infiltration. Topical application of auranofin (0.5 or 1%) to mouse ears significantly reduced the inflammatory symptoms of acne vulgaris induced by P. acnes injection. Topical application of auranofin led to the downregulation of the NLRP3 inflammasome activated by P. acnes in mouse ear skin. These results show that auranofin inhibits the NLRP3 inflammasome, the activation of which is associated with acne symptoms. The results further suggest that topical application of auranofin could be a new therapeutic strategy for treating acne vulgaris by targeting the NLRP3 inflammasome.

Key Words: Skin disease, Inflammasome, Drug repurposing, Inflammation, Cytokine

INTRODUCTION
Acne vulgaris is a common skin disease that is characterized by papules, nodulocystic lesions, and inflammation of the pilosebaceous follicles. Acne vulgaris is mainly caused by Propionibacterium acnes (P. acnes), which is a key factor in the pathogenesis of acne, as it induces inflammatory responses in pilosebaceous follicles (Qin et al., 2014). Benzoyl peroxide and isotretinoin are commonly used to treat acne vulgaris. However, the effects of benzoyl peroxide are limited with many side effects (Gollnick and Schramm, 1998). Although isotretinoin is a potent treatment for acne vulgaris (Dispenza et al., 2012) and reduces sebum in the sebaceous glands (Peck et al., 1979), it has certain adverse psychological (Goodfield et al., 2010) and gastrointestinal effects (Lowenstein and Lowenstein, 2011). Therefore, safer and more effective drugs that potentially target new mechanisms are needed.

Pattern recognition receptors (PRRs) are the first line of defense against invading microbes as they recognize pathogen-associated molecular patterns and play an important role in host defense and the progression of various inflammatory diseases through the activation of the NLRP3 inflammasome. The NLRP3 inflammasome is a multi-protein complex that consists of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC), and procaspase-1, and is activated by many stimuli, including bacterial products, danger signals, and inflammasome activators. It is involved in the production of pro-inflammatory cytokines, such as IL-1β and IL-18, which play a crucial role in the development and progression of inflammatory diseases (Dey et al., 2019). Accumulating evidence has indicated that the NLRP3 inflammasome is critical for host defense as well as the progression of inflammatory diseases through the production of the proinflammatory cytokine IL-1β, which is cleaved by active caspase-1 (Itoh et al., 2017; Traber et al., 2018). Therefore, inhibiting activation of the NLRP3 inflammasome may provide a new therapeutic strategy for acne vulgaris.
role in the production of proinflammatory cytokines such as interleukin-1β (IL-1β) (Guo et al., 2015). PRRs are expressed in a variety of immune cells, including macrophages, epithelial cells, dendritic cells, neutrophils, and adaptive immune cells. Some of these PRRs are expressed on the cell surface and can be directly activated via external pathological signals known as pathogen-associated molecular patterns (PAMPs) (Patel et al., 2017). In particular, it has been reported that the NLRP3 inflammasome, whose activation is essential for the active form of IL-1β production, is closely associated with both the initiation process of acne and the inflammatory responses in acne lesions (Kistowska et al., 2014; Li et al., 2014; Qin et al., 2014). In addition, P. acnes-induced IL-1β production is dependent on the NLRP3 inflammasome, which is composed of NLRP3, apoptosis-associated speck-like protein containing CARD (ASC), and caspase-1 (Kistowska et al., 2014; Li et al., 2014; Qin et al., 2014). Therefore, inhibiting the NLRP3 inflammasome would be an effective therapeutic strategy for acne vulgaris by significantly reducing inflammation in the lesion area.

Auranofin, a gold (Au)-containing acetylated carbohydrate complex, is used in the treatment of rheumatoid arthritis. It has been widely studied as a potential treatment for cutaneous staphylococcal infections (Thangamani et al., 2016), plaque psoriasis, contact dermatitis (Marks et al., 1995), and lupus (Dalziel et al., 1986). In this study, we investigated whether auranofin could regulate the activation of the NLRP3 inflammasome associated with P. acnes using a cell system with primary mouse macrophages and human sebocytes (SZ95) and a mouse skin acne model to pursue the possibility of repurposing auranofin for acne vulgaris treatment.

MATERIALS AND METHODS

Animals

C57BL/6 mice were obtained from Raon Bio (Yongin, Korea). NLRP3-knockout mice (B6N.129-Nlrp3<sup>tm3Hhf/J</sup>) and caspase-1 knockout mice (B6N.129S2-Casp1<sup>tm1Flv/J</sup>) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed in an environment with controlled temperature (23 ± 3°C) and relative humidity (40–60%). The mice in each experiment were of similar age and weight and were randomly allocated into treatment groups. All experimental protocols followed the Institutional Animal Care and Use Committee (IAUC) of the Catholic University of Korea guidelines (Permit No. #2016-005).

Cell culture

Bone marrow cells were isolated from C57BL/6 mice and differentiated into macrophages in DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Carlsbad, CA, USA) and 20% L929 conditioned medium for seven days (Lee et al., 2019a). The adherent cells were used as bone marrow-derived macrophages (BMDMs). Immortalized human S295 sebocytes (Zouboulis et al., 1999) were cultured in Sebomed<sup>®</sup> medium (Biochrom, Berlin, Germany) containing 10% fetal bovine serum and 5 ng/mL recombinant human epidermal growth factor (Invitrogen, Grand Island, NY, USA). Cells were maintained at 37°C in a 5% CO<sub>2</sub>/air environment.

Bacterial culture

P. acnes (KCTC 3314) was obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea) and cultured anaerobically for 4-5 days in reinforced Clostridium medium followed by suspension preparation. A spectrophotometer (OD at 600 nm) was used to determine the log phase of bacterial growth. Bacterial cultures were diluted to an MOI of 20 and used to stimulate cells.

Reagents

Purified LPS from Escherichia coli was obtained from List Biological Laboratory Inc. (Campbell, CA, USA) and dissolved in endotoxin-free water. Auranofin was purchased from Biomol (Plymouth Meeting, PA, USA). ATP was obtained from Invivogen (San Diego, CA, USA). Nigericin was obtained from Sigma-Aldrich. Benzoyl peroxide was purchased from Kwang Dong Pharmaceutical Co (Seoul, Korea).

Immunoblot analysis for caspase-1 and IL-1β

This was performed as described previously (Lee et al., 2019b). BMDMs were plated in six-well plates (2×10<sup>6</sup> cells/mL) and primed with lipopolysaccharides (LPS, 100 ng/mL) for 4 h. After LPS was removed by washing with PBS, cells were treated with auranofin for 1 h. Cells were further stimulated with ATP or nigericin in serum-free medium. Cell culture supernatants were concentrated by methanol-chloroform protein precipitation. A cell culture supernatant:methanol:chloroform (4:4:1 ratio) mixture was centrifuged for 10 min at 20,000 g. Methanol was added to the interphase, and the mixture was centrifuged with the liquid phase being removed. The protein pellet was resuspended in 1X sample loading buffer. Equal volumes of sample were resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked to prevent nonspecific binding and incubated with the corresponding primary antibody and secondary antibody conjugated to HRP. Pro-caspase-1 and caspase-1 (p10) were detected by anti-caspase-1 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Pro-IL-1β and IL-1β were detected by anti-IL-1β antibody from R&D Systems (Minneapolis, MN, USA). The relative bands were visualized with an ECL-based detection method.

Enzyme-linked immunosorbent assays

IL-1β, IL-17, and TNF-α levels in culture media or ear homogenate supernatants were determined using a DuoSet ELISA kit (R&D Systems) according to the manufacturer’s instructions.

Experimental animal protocol

P. acnes (2×10<sup>7</sup> colony-forming units/20 µL) or phosphate-buffered saline (PBS, 20 µL) was intradermally injected into the ears of 8 weeks old C57BL/6 mice. Eight h after injection, auranofin (0.5% or 1% in 20 µL of a 3:1 mixture of acetone:olive oil) or benzoyl peroxide (5%) was topically applied on ears three times for two days (n=5/group). Ear thickness was measured with calipers on day two as described previously (Kang et al., 2019). Ear tissues were homogenized at 4°C in RIPA buffer, centrifuged at 10,000 g for 10 min at 4°C, and processed for further assays.

https://doi.org/10.4062/biomolther.2020.004
Histological analysis

Ear tissue specimens were fixed in 4% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for the histological examination of inflammation as described previously (Yang et al., 2018). For detecting protein expression in tissue, immunohistochemistry was performed as described previously (Jin et al., 2019). Images were captured using a microscope (Olympus, Tokyo, Japan).

Caspase-1 activity assay

The enzymatic activity of caspase-1 in mouse ear homogenates (50 μg) was measured using a caspase-1 assay kit from Bio-vision (Milpitas, CA, USA) according to the manufacturer’s instructions. Fluorescence was recorded at 400 nm after excitation at 505 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The enzymatic activity of caspase-1 in mouse ear homogenates was analyzed.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance was performed followed by Duncan’s multiple range test to determine any significant differences between groups. SPSS 12.0 software (IBM Co., Chicago, IL, USA) was used. Statistical significance was considered when p<0.05.

RESULTS

Auranofin suppresses NLRP3 inflammasome activation in macrophages and sebocytes

To investigate whether auranofin inhibited activation of the NLRP3 inflammasome in macrophages, mouse bone marrow-derived macrophages were stimulated with NLRP3 inflammasome activators, ATP or nigericin, in the presence or absence of auranofin. Activation of the NLRP3 inflammasome involves two signals. The first signal is to induce pro-IL-1β expression by TLR agonist such as LPS. The second signal is triggered by the NLRP3 inflammasome agonists to form the inflammasome complex consisting of NLRP3, ASC, and pro-caspase-1 resulting in the activation of caspase-1 to cleave pro-IL-1β to mature form of IL-1β (Swanson et al., 2019). Therefore, degradation of pro-caspase-1 or pro-IL-1β to caspase-1 or IL-1β, respectively, was determined as a hallmark of inflammasome activation. Auranofin reduced the levels of caspase-1 or IL-1β that were increased upon ATP or nigericin stimulation in a dose-dependent manner, as determined by immunoblotting (Fig. 1A, 1B). Auranofin decreased the secretion of IL-1β induced by ATP or nigericin as determined by ELISA (Fig. 1C, 1D). The inhibition of caspase-1 degradation and IL-1β production induced by ATP started to be observed even at 0.1 μM and was almost complete at 1 μM. These data demonstrate that auranofin is an inhibitor of NLRP3 inflammasome activation.

Next, we investigated whether auranofin could block P. acnes-induced activation of the NLRP3 inflammasome. P. acnes increased the secretion of IL-1β in human SZ95 sebocytes, while auranofin decreased IL-1β secretion induced by P. acnes in a dose-dependent manner (Fig. 2A). P. acnes induced IL-1β secretion in wild-type macrophages, with auranofin blocking IL-1β secretion (Fig. 2B). In contrast, P. acnes was not able to induce IL-1β secretion in NLRP3- or caspase-1-deficient macrophages (Fig. 2C, 2D), indicating that P. acnes-induced IL-1β secretion is mediated through the activation of the NLRP3 inflammasome. The results show that auranofin inhibits P. acnes-induced activation of the NLRP3 inflammasome.
We examined whether IL-1β reduction by auranofin was related to cytotoxicity. Auranofin exhibited no cell toxicity to primary macrophages up to 1 µM treatment, at which concentration the suppression of NLRP3 was observed (Supplementary Fig. 1A). Even when macrophages were treated with auranofin together with P. acnes, no cytotoxicity was observed (Supplementary Fig. 1B). The results show that the inhibition of the NLRP3 inflammasome by auranofin was not due to cell death.

**Auranofin suppresses P. acnes-induced activation of NLRP3 inflammasome activation in mice**

We examined whether auranofin suppressed acne-associated inflammation in a P. acnes-induced mouse acne model. P. acnes was intradermally injected into the ears of mice, and auranofin was topically applied three times (Fig. 3A). Benzoyl peroxide was used as a positive control for acne vulgaris treatment. P. acnes induced severe ear inflammation with redness and swelling (Fig. 3B, 3C). In contrast, topical application of auranofin 0.5% or 1% reduced ear redness and swelling (Fig. 3B, 3C). In addition, auranofin decreased the degree of neutrophil infiltration caused by P. acnes (Fig. 3C). Ear thickness, which was increased by auranofin, was decreased by auranofin treatment (Fig. 3D). Auranofin showed better efficacy in relieving inflammation induced by P. acnes than 5% benzoyl peroxide.

Immunohistochemistry results showed that the levels of IL-1β and caspase-1 in ear tissues were enhanced by P. acnes injection, whereas auranofin treatment reduced IL-1β and caspase-1 levels in ears (Fig. 4A). P. acnes increased the levels of IL-1β in ear homogenates, while auranofin significantly reduced IL-1β levels induced by P. acnes (Fig. 4B). Furthermore, auranofin blocked the increase in caspase-1 activity by P. acnes in ear homogenates (Fig. 4C). These results show that topical application of auranofin is effective in suppressing P. acnes-induced activation of the NLRP3 inflammasome in ear tissue. In contrast, benzoyl peroxide was not effective at blocking NLRP3 inflammasome activation.

Overall, our results demonstrate that topical application of auranofin on mouse ears alleviates inflammatory responses.
Caspase-1

performed to detect IL-1β from the mice described in Fig. 3. (A) Immunohistochemistry was performed to detect IL-1β or caspase-1 (magnification 100x). (B) The amounts of IL-1β or caspase-1 in ear tissue homogenates were determined by ELISA. (C) Caspase-1 activity was assayed in ear tissue homogenates. (B, C) Values represent the means ± SEM (n=3). *Significantly different from vehicle alone, p<0.05. #Significantly different from P. acnes alone, p<0.05.

induced by P. acnes injection into ear skin. The results suggest that auranofin could be an efficient therapeutic treatment for acne vulgaris, possibly mediated by suppression of the NLRP3 inflammasome.

DISCUSSION

Our study demonstrates that topical application of auranofin is effective for preventing acne vulgaris induced by P. acnes by reducing NLRP3 inflammasome activation. Auranofin suppressed the activation of the P. acnes-induced NLRP3 inflammasome in macrophages and sebaceous gland cells in a dose-dependent manner but not in NLRP3- or caspase-1-deficient macrophages. Consistently, topical application of auranofin on mouse ears prevented P. acnes-induced inflammatory responses such as redness and swelling, accompanied by downregulation of NLRP3 inflammasome activation in skin tissues. The results show that inhibition of the NLRP3 inflammasome could be an effective strategy for treating acne vulgaris. In addition to the well-established role of inflammation to explain the later stage pathology of acne, it has currently become clear that the inflammatory process participates in the initiation of acne lesion as a primary event (Jeremy et al., 2003). Therefore, our study provides a new approach to treat acne symptoms by regulating inflammatory processes of acne via targeting the NLRP3 inflammasome.

Auranofin is used for the oral administration of rheumatoid arthritis. In addition, the potential application of auranofin for other diseases, including skin complications, has been extensively studied. Topical application of auranofin (1% or 2%) suppressed Staphylococcus aureus infection in the skin lesions of BALB/c mice, suggesting that auranofin could be effective for cutaneous staphylococcal infections (Thangamani et al., 2016). Auranofin was found to be effective at significantly lowering the severity score of psoriasis when auranofin ointment (0.1%, 0.2%, or 0.6%) was topically administered to 101 volunteers with chronic plaque psoriasis twice daily for 8 weeks (Helm et al., 1995). Furthermore, auranofin significantly reduced lesional severity scores that included erythema, scaling, and thickness in a study with 23 patients with discoid lupus erythematosus being treated with auranofin (3 mg b.i.d monthly for 6 months) (Dalziel et al., 1986).

In addition, repurposing auranofin for the treatment of neurodegenerative disorders such as Alzheimer and Parkinson’s disease has been investigated (Madeira et al., 2014). These results suggest that the repurposing of auranofin can be beneficial for treating several diseases. Our study proposes the possibility of repurposing auranofin to treat acne vulgaris with NLRP3 inflammasome inhibition as a working mechanism.

Auranofin is orally administered for rheumatoid arthritis. However, bioavailability with the oral administration route is not effective since the absorption rate is 20 to 30% and 50% of the absorbed auranofin is excreted in the urine (Blocka et al., 1986). Moreover, side effects on the gastrointestinal system, such as loose stools and diarrhea, are reported with oral administration of auranofin. Therefore, dermal application to skin complications such as acne has the advantage of avoiding side effects derived from oral application. Benzoyl peroxide is used to treat mild to moderate acne and topically applied in concentrations of 2.5-5.0% and up to 10%. Our results show that the effectiveness of auranofin 0.5% is compatible with that of benzoyl peroxide 5.0%. Isotretinoin is prescribed to treat severe acne that has not responded to other treatments such as benzoyl peroxide or antibiotics. Isotretinoin is given orally with 0.25 to 0.5 mg/kg two times a day for adults. In future study, it would be needed to compare pharmacological efficacy and side effects between auranofin and isotretinoin to expand auranofin’s application to acne vulgaris. Isotretinoin as a pro-drug for retinoic acid, exhibits a low affinity for retinoic acid receptors (RAR) and retinoid X receptors (RXR) while it is suggested that its intracellular metabolites may act as agonists of RAR and RXR (Layton, 2009). There...
is a report suggesting that the regulation of *P. acnes*-induced immune responses is one of the mechanisms for isotretinoin’s long-term efficacy (Dispenza et al., 2012). However, to our best knowledge, little is known about whether isotretinoin affects inflammasome activity. Therefore, it will be interesting to investigate whether isotretinoin regulates the NLRP3 inflammasome in a direct or indirect way in a future study.

Collectively, our results show the efficacy of auranofin in the inhibition of the NLRP3 inflammasome in cells and animals, leading to the improvement in inflammatory symptoms of acne vulgaris. Our study suggests that the repurposing of auranofin via dermal application to treat acne vulgaris could be beneficial.

**CONFLICT OF INTEREST**

Christos C. Zouboulis is the owner of an international patent on the immortalized human sebaceous gland SZ95 cell line (WO0046353).

**ACKNOWLEDGMENTS**

This study was supported by the Research Fund, 2019 of The Catholic University of Korea, and grants from the National Research Foundation of Korea (NRF-2019R1A2C2085739, NRF-2017R1A4A1015036, and NRF-2020R1A4A202894) funded by the Korean government (Ministry of Science, ICT and Future Planning) and Cooperative Research Program for Agriculture Science and Technology Development (Project no. PJ01199002) from Rural Development Administration, Republic of Korea.

**REFERENCES**


