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Verproside inhibits TNF-α-induced MUC5AC expression through suppression of the TNF-α/NF-κB pathway in human airway epithelial cells

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ABSTRACT

Airway mucus secretion is an essential innate immune response for host protection. However, overproduction and hypersecretion of mucus, mainly composed of MUC5AC, are significant risk factors in asthma and chronic obstructive pulmonary disease (COPD) patients. Previously, we reported that verproside, a catalpol derivative iridoid glycoside isolated from *Pseudolysimachion rotundum* var. *subintegrum*, is a potent anti-asthmatic candidate drug *in vivo*. However, the molecular mechanisms underlying the pharmacological actions of verproside remain unknown.

Here, we found that verproside significantly reduces the expression levels of tumor necrosis factor alpha (TNF-α)-induced *MUC5AC* mRNA and protein by inhibiting both nuclear factor kappa B (NF-κB) transcriptional activity and the phosphorylation of its upstream effectors such as IκB kinase (IKK)β, IκBα, and TGF-β-activated kinase 1 (TAK1) in NCI-H292 cells. Moreover, verproside attenuated TNF-α-induced *MUC5AC* transcription more effectively when combined with an IKK (BAY11-7082) or a TAK1 (5z-7-oxozeaenol) inhibitor than when administered alone. Importantly, we demonstrated that verproside negatively modulates the formation of the TNF-α-receptor (TNFR) 1 signaling complex [TNF-RSC; TNFR1-recruited TNFR1-associated death domain protein (TRADD), TNFR-associated factor 2 (TRAF2), receptor-interacting protein kinase 1 (RIP1), and TAK1], the most upstream signaling factor of NF-κB signaling. *In silico* molecular docking studies show that verproside binds between TRADD and TRAF2 subunits.

Altogether, these results suggest that verproside could be a good therapeutic candidate for treatment of inflammatory airway diseases such as asthma and COPD by blocking the TNF-α/NF-κB signaling pathway.

1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) cause chronic inflammation of the respiratory tract [1]. Although the combination treatments of various bronchodilators and corticosteroids have had good outcomes in the management of asthma/COPD, corticosteroids have side effects such as osteoporosis and pneumonia [2]. Thus, recent studies have increasingly focused on finding safe, natural therapeutic alternatives for asthma/COPD [3].

Abbreviations: ChIP, chromatin immunoprecipitation; COPD, chronic obstructive pulmonary disease; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IKK, IκB kinase; NFκB, nuclear factor kappa B; RIP1, receptor interacting kinase 1; TAK1, transforming growth factor beta-activated kinase 1; TNF-α, tumor necrosis factor alpha; TNF-RSC, tumor necrosis factor receptor 1 signaling complex; TNFR, tumor necrosis factor receptor; TRADD, tumor necrosis factor receptor-associated death domain protein; TRAF2, tumor necrosis factor receptor associated factor 2.

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Verproside, a catalpol derivative iridoid glycoside isolated from the genus *Pseudolysimachion*, shows potent anti-inflammatory, antioxidant, and antinociceptive activities [4]. Additionally, we reported that verproside, one of the major components in *Pseudolysimachion rotundum* var. *subintegrum* extract, is a potent anti-asthmatic/COPD drug candidate *in vivo* [5]. However, the underlying pharmacological mechanism of verproside in inflammatory diseases such as asthma and COPD is still unknown.

Mucus secretion in airway epithelial cells is an essential innate immune response for host protection against pathogens and irritants at mucosal surfaces [6]. Both mucus overproduction and hypersecretion (sputum production) are prominent pathophysiological features in several patients with asthma and COPD [7]. Mucin glycoproteins are one of the major components of mucus. In particular, MUC5AC is a major mucin protein secreted from the airway surface epithelium and its expression is markedly upregulated in lung tissue from ovalbumin-induced asthmatic mice [8], as well as in the bronchiolar epithelium of COPD patients [9]. Thus, it is important to understand the mechanisms of inhibition of *MUC5AC* expression to treat lung diseases such as asthma and COPD [10,11].

Tumor necrosis factor alpha (TNF-α) is a potent pro-inflammatory cytokine that stimulates *MUC5AC* expression [12]. The TNF-α/Nuclear factor kappa B (NF-κB) cascade is also known to play a central role in regulating *MUC5AC* expression [13]. Thus, the regulation of *MUC5AC* expression by the TNF-α-activated transcription factor NF-κB has been proposed to be a therapeutic target in asthma and COPD [14]. Activated TNF-α-receptor (TNFR) recruits TNFR1-associated death domain protein (TRADD), TNFR–associated factor 2 (TRAF2), receptor-interacting protein kinase 1 (RIP1, also known as RIPK1), and transforming growth factor-β-activated kinase 1 (TAK1, also known as MAP3K7), forming the TNFR1 signaling complex (TNF-RSC) to initiate the TNF-α/NF-κB signaling cascade [8,15]. TRADD is a first adaptor protein that is recruited to TNFR1 [16]. Continuously, the E3 ubiquitin ligase TRAF2 catalyzes K63-linked polyubiquitination of RIP1, leading to the recruitment of TAK1 and IκB kinase (IKK; composed of two kinases, IKKα and IKKβ, and a regulatory subunit, NEMO/IKKγ) [17–19]. These protein–protein interactions are essential for IKK activation [20–22], a central regulator of NF-κB activation [23].

TNF-α-overexpression in a mouse model was associated with pathological features of COPD such as inflammatory cell infiltration, pulmonary fibrosis, and emphysema [12,24]. Moreover, TNF-α inhibitors are considered potential new medications for asthma and COPD management [25]. IKKβ overexpression resulted in increased secretion of inflammatory mediators and neutrophilic inflammation similar to that observed in COPD [26], while the inhibition of IKKβ activity resulted in reduced TNF-α-induced MUC5AC secretion *in vivo* and *in vitro* [14,27]. Furthermore, TAK1 (IKK activator) has been proposed as a novel target for the inhibition of cigarette smoke-induced inflammatory responses involved in the development and progression of COPD [28].

In this study, we showed that verproside represses TNF-α-induced *MUC5AC* expression by inhibiting NF-κB activation via the IKK/IκB signaling cascade. Moreover, we, to the best of our knowledge, demonstrated for the first time that verproside disturbs the interaction between TNFR1 and its interaction partners such as TRADD, TRAF2, RIP1, and TAK1, which are essential for NF-κB activation. Importantly, *in silico* molecular docking studies demonstrated that verproside binds to the TRADD–TRAF2 complex.

Collectively, these results suggest that verproside is a promising drug candidate for improving lung function in the treatment of asthma and COPD by reducing the expression of NF-κB-response genes such as *MUC5AC* via the suppression of TNF-RSC formation (the most upstream signaling component of the NF-κB signaling pathway).

2. Materials and methods

2.1. Chemicals and reagents

Verproside was obtained from the Korea Research Institute of Bioscience and Biotechnology (KRIBB; Daejeon, Korea) with a purity of more than 99.5% as determined by high-performance liquid chromatography [5]. TNF-α was purchased from Peprotech (Rocky Hill, NJ, USA). For the inhibition experiments, the IKK inhibitor (BAY 11-7082), TAK1 inhibitor (5z-7-oxozeaenol), and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (St. Louis, MO, USA). BAY 11-7082 and 5z-7-oxozeaenol were dissolved in DMSO. Anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH), anti-histone deacetylase 1, and anti-TRAF2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-RIP1 was purchased from BD PharMingen (San Diego, CA, USA). Anti-TRADD, anti-TAK1, anti-NF-κBp65, anti-TNFR1, anti-RIP1, anti-phospho-IKKα/β, anti-phospho-IκBα, and anti-phospho-TAK1 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell preparation and culture

NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from the American Type Culture Collection (CRL-1848; ATCC, Manassas, VA, USA). NCI-H292 cells were grown in growth medium (GM; RPMI 1640 medium Hyclone, GE Healthcare, United Kingdom) supplemented with 10% fetal bovine serum (FBS; Hyclone, GE Healthcare, United Kingdom) and 100 units/mL penicillin plus 100 μg/mL streptomycin (Hyclone, GE Healthcare, United Kingdom) at 37 °C under a humidified 5% CO_2 atmosphere. For the treatment with verproside or the respective inhibitors, NCI-H292 cells (1×10^4 cells/cm^2 well) were seeded in GM and incubated for 16 h. Subsequently, the medium was changed to RPMI supplemented with 0.1% FBS and 100 units/mL penicillin plus 100 μg/mL streptomycin and the cells were incubated for another 16 h.

2.3. Cell viability assay

The NCI-H292 cells were plated in 96-well plates in GM at a density of 5×10^3 cells/well and grown for 16 h. The GM was subsequently changed to serum-reduced medium (0.1% FBS). After 16-h incubation, cells were incubated with verproside in the presence or absence of TNF-α for 24 h. Cell viability was measured in triplicate using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, ML, USA) according to the manufacturer's protocol. The absorbance was measured using a VERSA max microplate reader (Molecular Devices, Sunnyvale, CA, USA) and the measured absorbance was converted to the percentage (%) of the control value.

2.4. Evaluation of the mRNA expression level

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration and purity of total RNA were calculated using the absorbance at 260 and 280 nm using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA). The first cDNA strand was synthesized with 2 μg of total RNA and 1 μM Oligo-dT_{18} primer using Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). SYBR green-based quantitative real-time PCR (qRT-PCR) amplification was performed using an S1000 thermal cycler real-time PCR system and iQ SYBR Green supermix (both from Bio-Rad, Hercules, CA, USA) in the presence of 1:25 diluted first-strand cDNA and 20 pmol of primers according to the manufacturer's protocols.

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