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Pyronaridine Inhibited *MUC5AC* Mucin Gene Expression by Regulation of Nuclear Factor Kappa B Signaling Pathway in Human Pulmonary Mucoepidermoid Cells

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Abstract

In this study, the potential effects of pyronaridine, an antimalarial agent, on airway *MUC5AC* mucin gene expression were investigated. The human pulmonary epithelial NCI-H292 cells were pretreated with pyronaridine for 30 min and then stimulated with phorbol 12-myristate 13-acetate (PMA) for 24 h. The effect of pyronaridine on the PMA-induced nuclear factor kappa B (NF-κB) signaling pathway was also examined. Pyronaridine inhibited glycoprotein production and mRNA expression of *MUC5AC* mucins induced by PMA through the inhibition of degradation of inhibitory kappa Bα and NF-κB p65 nuclear translocation. These results suggest that pyronaridine suppresses gene expression of mucin through regulation of the NF-κB signaling pathway in human pulmonary epithelial cells.

Key Words: MUC5AC, Pulmonary mucin, Pyronaridine

INTRODUCTION

A thin layer of gel called mucus exists on the luminal surface of the respiratory tract. Mucus contains water and several molecules and ions that exert antimicrobial and antioxidant effects. Most macromolecules that impart viscoelasticity to mucus are mucins. Mucins maintain the normal function of the pulmonary system and protect it from inhaled noxious factors, including viruses, bacteria, various particles, and irritating gases. However, qualitative and/or quantitative changes in mucins, such as overproduction and/or over-secretion of respiratory mucus, interfere with the normal defensive action of the pulmonary system, thereby contributing to the pathogenesis of cystic fibrosis, bronchiectasis, chronic bronchitis, and asthma. Among the various subtypes of human mucins, MUC5AC is the major type of gel-forming pulmonary mucin (Mann *et al*., 2022; Kim *et al*., 2023; Ryu *et al*., 2023).

To efficiently control mucus in the pulmonary system, hypertonic saline solution, S-carboxymethyl cysteine, 2-mercaptoethane sulfonate sodium, bromhexine, erdosteine, thymosin β-4, glyceryl guaiacolate, ambroxol, N-acetyl L-cysteine, mannitol dry powder, azithromycin, dornase alfa, letocysteine, myrtol, and sobrerol have been used, despite their potential to induce diverse side effects, including rebound over-secretion of mucus and irritation of the luminal wall of the respiratory tract (Li *et al*., 2020).

Therefore, the development of novel agents that regulate the biosynthesis and/or degradation of mucin to control its production and/or secretion is warranted. Such development might be pivotal for controlling the over-secretion of pulmonary mucus. Although glucocorticosteroids are used to decrease the secretion and/or production of respiratory mucus, they have been demonstrated to exhibit various adverse pharmacological effects (Sprenger *et al*., 2011; Li *et al*., 2020; Ryu *et al*., 2023).

Given these challenges, we attempted to develop a novel candidate for altering the production and/or secretion of respiratory mucus. Accordingly, we aimed to determine the potential effect of pyronaridine on the expression of the airway MUC5AC mucin through a trial based on drug repurposing. Pyronaridine is a drug that has been used to treat malaria since the 1970s. It is administered to patients as a complex

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preparation of pyronaridine and artesunate, based on artemisinin combination therapy. The combination of pyronaridine and artesunate has shown potential as a therapeutic agent for controlling COVID-19 (Krishna *et al*., 2021). Pyronaridine has been used as an antiviral and anti-inflammatory agent (Ardanuy *et al*., 2023). Pyronaridine affects neuroblastomas and glioblastomas (Ghosalkar *et al*., 2022; Rank *et al*., 2022). Pyronaridine suppressed topoisomerase II expression in breast cancer cells (Villanueva *et al*., 2021) and induced apoptosis in lung cancer cells (Zhong *et al*., 2022).

However, there have been no reports on the effects of pyronaridine on mucin gene expression in airway epithelial cells. Thus, we suggest that investigating the potential activity of pyronaridine on mRNA expression and glycoprotein production of pulmonary MUC5AC mucin stimulated by phorbol ester in NCI-H292 cells is promising. The human pulmonary mucoepidermoid cell line NCI-H292 has been used to identify the intracellular signaling pathways involved in pulmonary mucin gene expression (Li *et al*., 1997; Takeyama *et al*., 1999; Shao *et al*., 2003). Phorbol ester provokes *MUC5AC* mucin gene expression, and intracellular nuclear factor kappa B (NF-κB) signaling is involved in the activity of phorbol ester in airway epithelial cells (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Fujisawa *et al*., 2009; Kim *et al*., 2012; Kurakula *et al*., 2015; Garvin *et al*., 2016; Li *et al*., 2020). Therefore, to elucidate the action mechanism of pyronaridine, we examined whether pyronaridine regulates the activation of the NF-κB signaling pathway stimulated by phorbol ester in NCI-H292 cells.

MATERIALS AND METHODS

Materials

Pyronaridine (purity: 98.0%) (Fig. 1) was donated by Shinpoong Pharmaceutical Company, Ltd. (Seoul, Korea). Phospho-specific anti-IκBα (serine 32/36, #9246), and phospho-specific anti-p65 (serine 536, #3036S) antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-β-actin (sc-8432), anti-NF-κB p65 (sc-8008), and anti-inhibitory kappa Bα (sc-371) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-nuclear matrix protein p84 (ab-487) was purchased from Abcam (Cambridge, MA, USA). Goat anti-mouse IgG (#401215) and goat anti-rabbit IgG (#401315) were purchased from Calbiochem (Carlsbad, CA, USA) and used as secondary antibodies. The other chemicals used in the current experiment were purchased from Sigma-Aldrich (St. Louis, MO, USA).

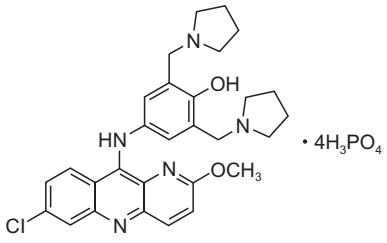

Fig. 1. Chemical structure of pyronaridine.

Culture of NCI-H292 cells

NCI-H292 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum in the presence of a pen-strep mixture (penicillin [100 units/mL] plus streptomycin [100 μg/mL]) and HEPES (25 mM) at 37°C in a humidified, 5% CO_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline (PBS) and cultured in RPMI 1640 medium supplemented with 0.2% fetal bovine serum for 24 h.

Cell viability assay

NCI-H292 cells were seeded at a density of 2×10^5/mL (0.1 mL/well) in a 96-well microtiter plate and allowed to attach for 24 h to maintain log phase growth at the time of drug treatment. After incubation with the indicated concentrations of pyronaridine for 72 h, cell proliferation was determined using a sulforhodamine B assay.

Treatment of cells with pyronaridine

After serum deprivation, the cells were pretreated with varying concentrations of pyronaridine for 30 min and then treated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) for 24 h in serum-free RPMI 1640. Pyronaridine was dissolved in dimethyl sulfoxide and added to the culture medium (final concentration of dimethyl sulfoxide was 0.5%). The final pH values of the solutions ranged from 7.0 and 7.4. Culture medium and 0.5% dimethyl sulfoxide did not affect mucin gene expression, activity, or expression of molecules involved in the NF-κB signaling pathway in NCI-H292 cells. After 24 h, the cells were lysed with a buffer solution containing 20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, and a protease inhibitor cocktail (Roche Diagnostics, IN, USA) and collected to measure the production of MUC5AC glycoproteins (in a 24-well culture plate). Total RNA was extracted to determine the expression of the *MUC5AC* gene (in a six-well culture plate) using RT-PCR. For western blot analysis, the cells were treated with pyronaridine for 24 h and then with PMA for 30 min.

Quantitative analysis of MUC5AC mucin

Airway MUC5AC mucin production was measured by enzyme-linked immunosorbent assay. Cell lysates were prepared with PBS at a 1:10 dilution, and 100 μL of each sample was incubated at 42°C in a 96-well plate until dry. The plates were washed three times with PBS and blocked with 2% bovine serum albumin (fraction V) for 1 h at room temperature. Plates were washed another three times with PBS and then incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, USA), which was diluted with PBS containing 0.05% Tween 20 and dispensed into each well. After 1 h, the wells were washed three times with PBS, and 100 μL of horseradish peroxidase-goat anti-mouse IgG conjugate (1:3,000) was dispensed into each well. After 1 h, the plates were washed three times with PBS. The color reaction was developed with 3,3',5,5'-tetramethylbenzidine peroxide solution and stopped with 1 N H_2SO_4. The absorbance was measured at 450 nm.

Isolation of total RNA and RT-PCR

Total RNA was isolated using an Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc., Sungnam, Korea) and reverse

transcribed using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. A total of 2 μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 50 μL (RT reaction). Two μL of the RT reaction product was PCR-amplified in a 25 μL mixture using ThermoPrime Plus DNA Polymerase (ABgene, Rochester, NY, USA). Primers for MUC5AC were (forward) 5′-TGA TCA TCC AGC AGG GCT-3′ and (reverse) 5′-CCG AGC TCA GAG GAC ATA TGG G-3′. Primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein and is a constitutively expressed housekeeping gene, were used as quantitative controls. Primers for Rig/S15 were (forward) 5′-TTC CGC AAG TTC ACC TAC C-3′ and (reverse) 5′-CGG GCC GGC CAT GCT TTA CG-3′. The PCR mixture was denatured at 94°C for 2 min followed by 40 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s. After PCR, 5 μL of PCR products were subjected to 1% agarose gel electrophoresis and visualized with ethidium bromide under a transilluminator.

Nuclear and cytosolic extracts preparation

NCI-H292 cells (confluent in a 150 mm culture dish) were pretreated for 24 h at 37°C with 0.5, 1, 2, or 5 μM of pyronaridine, and then stimulated with PMA (50 ng/mL) for 30 min in serum-free RPMI 1640. After treatment with pyronaridine, cells were harvested using trypsin-EDTA solution and centrifuged in a microcentrifuge (1,200 rpm, 3 min, 4°C). After discarding the supernatant, the cell pellet was washed with a suspension in PBS. The cytoplasmic and nuclear protein fractions were extracted using NE-PER® nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Both extracts were stored at −20°C. The protein content in the extracts was quantified using the Bradford method.

Western blotting for the detection of proteins

Cytosolic and nuclear extracts containing proteins (each 50 μg) were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane. The blots were blocked using 5% skim milk and probed with an appropriate primary antibody in blocking buffer overnight at 4°C. The membrane was washed with PBS and probed with a horseradish peroxidase-conjugated secondary antibody. The immunoreactive bands were detected using an enhanced chemiluminescence kit (Pierce ECL Western Blotting Substrate; Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The means of individual groups were converted to percent control and expressed as mean ± SEM. Differences between groups were assessed using one-way ANOVA and the Holm–Sidak test as a post-hoc test. Statistical significance was set at $p<0.05$.

RESULTS

Effect of pyronaridine on PMA-induced MUC5AC mRNA expression and glycoprotein production of MUC5AC mucin

Pyronaridine inhibited PMA-induced MUC5AC mucin glycoprotein production in a dose-dependent manner. The amounts of MUC5AC mucin in the cells of pyronaridine-treated cultures were 100 ± 11% (control), 296 ± 5% (10 ng/mL of PMA alone), 230 ± 2% (PMA plus pyronaridine 0.5 μM), 158 ± 1% (PMA plus pyronaridine 1 μM), 135 ± 4% (PMA plus pyronaridine 2 μM), and 85 ± 1% (PMA plus pyronaridine 5 μM), respectively (Fig. 2). PMA-induced MUC5AC mRNA expression was also inhibited by pretreatment with pyronaridine (Fig. 3). Cytotoxicity was checked by sulforhodamine B assay, and there was no cytotoxic effect of pyronaridine at 0.5, 1, 2, or 5 μM concentra-

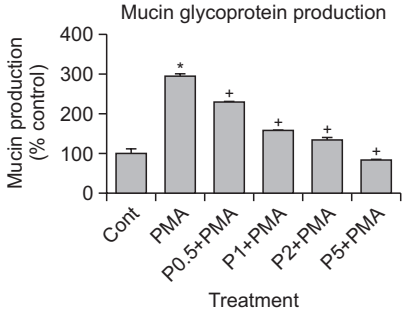

Fig. 2. Effect of pyronaridine on phorbol 12-myristate 13-acetate (PMA)-induced MUC5AC production in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of pyronaridine for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Cell lysates were collected for the measurement of MUC5AC mucin production by enzyme-linked immunosorbent assay (ELISA). Each bar represents a mean ± SEM of three culture wells compared with that of the control set at 100%. Three independent experiments were performed, and representative data are shown. *Significantly different from that of the control ($p<0.05$). ⁺Significantly different from that with PMA treatment alone ($p<0.05$). Cont: control, P: pyronaridine, concentration unit is μM.

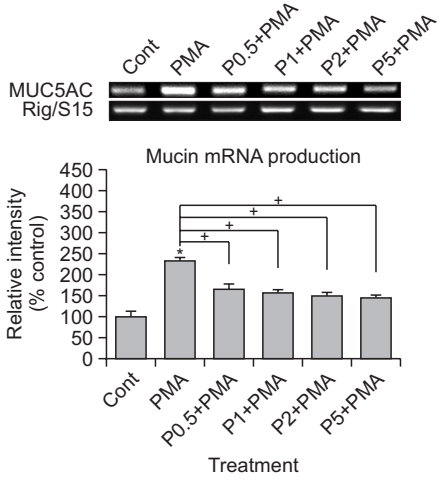

Fig. 3. Effect of pyronaridine on phorbol 12-myristate 13-acetate (PMA)-induced MUC5AC mRNA expression in NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of pyronaridine for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Cell lysates were collected for the measurement of *MUC5AC* gene expression using RT-PCR. Three independent experiments were performed, and representative data are shown. *Significantly different from that of the control ($p<0.05$). ⁺Significantly different from that with PMA treatment alone ($p<0.05$). Cont: control, P: pyronaridine, concentration unit is μM.

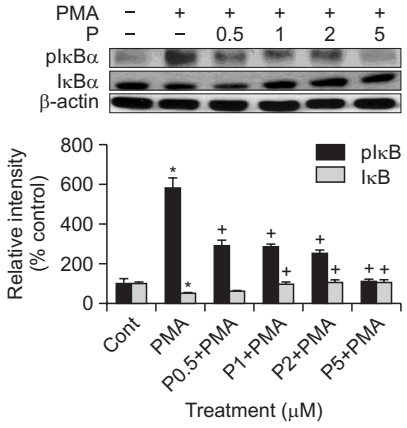

Fig. 4. Effect of pyronaridine on phorbol 12-myristate 13-acetate (PMA)-induced IκBα phosphorylation and IκBα degradation in NCI-H292 cells. NCI-H292 cells were incubated with varying concentrations of pyronaridine for 24 h, and then treated with 50 ng/mL PMA for 30 min. Cytoplasmic extracts were fractionated and then subjected to western blot analysis using a phospho-specific IκBα (Ser 32/36) antibody or an antibody against IκBα. Equal protein loading was evaluated by β-actin levels. Three independent experiments were performed, and representative data are shown (n=3). *Significantly different from that of the control (p<0.05). $^{+}$Significantly different from that with PMA treatment alone (p<0.05). Cont: control, P: pyronaridine, IκBα: inhibitory kappa Bα, concentration unit is μM.

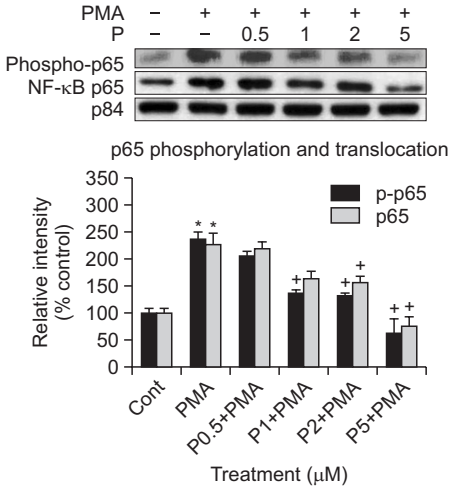

Fig. 5. Effect of pyronaridine on phorbol 12-myristate 13-acetate (PMA)-induced phosphorylation and translocation of NF-kB p65 in NCI-H292 cells. Nuclear protein extracts were prepared and subjected to western blotting using a phospho-specific p65 (Ser 536) antibody and an antibody against p65. p84 levels were analyzed as a loading control. Three independent experiments were performed, and representative data are shown (n=3). *Significantly different from that of the control (p<0.05). $^{+}$Significantly different from that with PMA treatment alone (p<0.05). Cont: control, P: pyronaridine, concentration unit is μM.

tion (data not shown).

Effect of pyronaridine on PMA-induced phosphorylation and degradation of IκBα

In order for NF-κB to be activated, PMA provokes the phosphorylation of IKK, and this phosphorylated IKK, in turn, phosphorylates IκBα. The phosphorylated IκBα dissociates from NF-κB and is degraded. Thus, we checked whether pyronaridine affects the phosphorylation and degradation of IκBα provoked by PMA. As shown in Fig. 4, PMA stimulated the phosphorylation of IκBα, whereas pyronaridine inhibited its phosphorylation. Additionally, PMA increased the degradation of IκBα, whereas pyronaridine inhibited its degradation (Fig. 4).

Effect of pyronaridine on PMA-induced phosphorylation and nuclear translocation of NF-κB p65

After the activation of NF-κB, it translocates from the cytosol to the nucleus, where it binds to specific sites on DNA. This assembly of DNA/NF-κB recruits RNA polymerase, and the resulting mRNA is translated into specific proteins, including MUC5AC mucins. The transcriptional activity of NF-κB p65 is dependent on its phosphorylation. As shown in Fig. 5, PMA stimulated the phosphorylation of p65, whereas pyronaridine suppressed its phosphorylation. Finally, pyronaridine blocked the nuclear translocation of NF-κB p65 stimulated by PMA.

DISCUSSION

As described in the Introduction, no specific compound efficiently controls the secretion and/or production of pulmonary mucus despite the use of several clinical medicines to exert such pharmacological activity. Controlling the inflammatory response may be the ultimate approach to efficiently regulate various inflammatory airway diseases. The effects of various natural compounds were also investigated. Notably, diverse natural anti-inflammatory compounds have a regulatory effect on the gene expression of *MUC5AC* mucin. Accordingly, we developed a novel candidate that alters the production and/or secretion of airway MUC5AC mucin (Kim *et al*., 2012; Ryu *et al*., 2013, 2014; Seo *et al*., 2014; Sikder *et al*., 2014; Lee *et al*., 2015; Kim *et al*., 2016; Li *et al*., 2020; Hossain *et al*., 2022a, 2022b; Kim *et al*., 2023; Ryu *et al*., 2023). However, the effective concentrations of these natural compounds are usually high, and the pharmacokinetic profile of each compound is generally unsatisfactory (Li *et al*., 2020).

In this study, we used drug repurposing to search for promising new drug candidates. Drug repurposing, also known as drug repositioning, is the process of identifying novel therapeutic uses for drugs approved and used clinically for human diseases. Instead of developing new drugs at the initial stage, drug repurposing involves identifying new uses for drugs that have already undergone extensive testing and have well-established safety profiles. Drug repurposing can be a cost-effective approach to drug development since it avoids many of the risks and costs associated with developing new drugs from the initial stage. Additionally, drug repurposing can decrease the time required to deliver new therapies to patients since the safety and pharmacokinetic profiles of the drugs have already been established (Kingsmore *et al*., 2020).

Our results showed that pyronaridine significantly suppressed the production of the MUC5AC mucin glycoprotein and the expression of MUC5AC mucin mRNA (Fig. 2, 3). To our knowledge, this is the first study to reveal the effects of pyronaridine on *MUC5AC* mucin gene expression in airway

epithelial cells. Pyronaridine was also found to suppress the phosphorylation and nuclear translocation of NF-κB p65 by affecting the phosphorylation and degradation of IκBα in NCI-H292 cells (Fig. 4, 5). Thus, the pharmacological effect of pyronaridine on the gene expression of *MUC5AC* may be manifested via the degradation of IκBa and nuclear translocation of NF-κB p65.

Additionally, pyronaridine may inhibit *MUC5AC* gene expression through intracellular signaling pathways other than NF-κB signaling. We examined whether pyronaridine inhibited *MUC5AC* mucin gene expression through an intracellular signaling pathway mediated by the epidermal growth factor receptor. Based on these results, pyronaridine did not affect the EGF-induced mitogen-activated protein kinase signaling pathway. Furthermore, pyronaridine did not affect EGF-induced epidermal growth factor receptor phosphorylation, p38 mitogen-activated protein kinase phosphorylation, extracellular signal-regulated kinase 1/2 phosphorylation, or the nuclear expression of specificity protein-1 (unpublished data).

Several studies have reported that the gene expression of *MUC5AC* mucin might be stimulated by inflammatory mediators that activate transcription factors, including NF-κB (Fujisawa *et al*., 2009; Kurakula *et al*., 2015; Garvin *et al*., 2016). PMA has been reported to stimulate *MUC5AC* mucin gene expression, and NF-κB signaling is involved in airway epithelial cell activity (Ishinaga *et al*., 2005; Laos *et al*., 2006; Wu *et al*., 2007; Kim *et al*., 2012). Based on these reports and the present experimental results, the pharmacological effect of pyronaridine on PMA-induced *MUC5AC* gene expression may be mediated, at least partly, by the degradation of IκBα and nuclear translocation of NF-κB p65.

The adverse effects of pyronaridine, including vomiting, headache, bradycardia, abdominal pain, mild transient elevation of liver enzymes, and hypoglycemia (Krishna *et al*., 2021), might be problematic. However, pharmacological agents that specifically control the production and/or secretion of airway mucus are currently unavailable. Therefore, it is crucial to identify specific compounds that regulate the abnormal secretion and/or production of mucus in the respiratory tract in pulmonary diseases via preclinical/clinical studies. Notably, this study was conducted during the early stages of drug development. Potential adverse effects should be resolved at each developmental step and novel drug stage. Using medicinal chemistry to modify and optimize the chemical structure of pyronaridine is a promising strategy for ensuring that it exerts adequate control on the secretion and/or production of pulmonary mucus.

In conclusion, a drug repurposing strategy should be adopted to identify new candidate compounds that exhibit an inhibitory effect on the gene expression of *MUC5AC*. The results of the present study suggest the possibility of using pyronaridine as an efficacious muco-regulatory agent for diverse respiratory diseases.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

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