Effects of Homogentisic Acid and Natural Products Derived from *Pinellia ternata* on Secretion, Production and Gene Expression of MUC5AC Mucin from Cultured Airway Epithelial Cells

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Abstract – In this study, we investigated whether adenosine, adenine, uridine and homogentisic acid derived from *Pinellia ternata* affect the secretion, production and gene expression of MUC5AC mucin from airway epithelial cells. Confluent NCI-H292 cells were pretreated with adenosine, adenine, uridine or homogentisic acid for 30 min and then stimulated with PMA (phorbol 12-myristate 13-acetate) for 24 h. The MUC5AC mucin gene expression, mucin protein production and secretion were measured by RT-PCR and ELISA, respectively. The results were as follows: (1) Adenine and homogentisic acid decreased PMA-induced MUC5AC mucin gene expression, although adenosine and uridine did not affect the mucin gene expression; (2) Adenosine, adenine, uridine and homogentisic acid inhibited PMA-induced MUC5AC mucin production; (3) Homogentisic acid inhibited the secretion of MUC5AC mucin from NCI-H292 cells. These results suggest that, among the four compounds examined, homogentisic acid showed the regulatory effect on the steps of gene expression, production and secretion of mucin, by directly acting on airway epithelial cells.

Keywords – Airway, MUC5AC, Mucin, Homogentisic acid

Introduction

Pulmonary mucus is pivotal in defensive action against invading pathogenic microorganisms, noxious chemicals and particles. This defensive action of pulmonary mucus is attributed to the physicochemical property of mucins i.e. viscoelasticity. Mucins are high molecular weight glycoproteins present in the pulmonary mucus and produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, hypersecretion of pulmonary mucus is one of the major symptoms associated with severe respiratory diseases including asthma, chronic bronchitis, cystic fibrosis and bronchiectasis.[1,2] Therefore, it is highly desirable to find the potential activity of regulating the excessive mucin secretion and/or production by the compounds derived from various medicinal plants. We have tried to investigate the possible activities of some natural products on mucin secretion and/or production from cultured airway epithelial cells. As a result of our trial, we previously reported that several natural compounds affected mucin secretion and/ or production from airway epithelial cells.[3-5] *Pinellia ternata* has been utilized empirically for controlling the pulmonary symptoms including cough and sputum in folk medicine and diverse biological effects of this medicinal plant and natural compounds derived from it has been reported. *Pinellia ternata* inhibited the allergic airway inflammation in animal model of asthma.[6,7] Some nucleotides derived from *Pinellia ternata* have been reported to affect the mucociliary clearance including mucin secretion.[8-11] However, to the best of our knowledge, there are no reports about the potential effects of adenosine, adenine, uridine and homogentisic acid derived from *Pinellia ternata* on the gene expression, production and secretion of mucin from airway epithelial cells. Among the twenty one or more MUC genes coding human mucins, MUC5AC was reported to be mainly expressed in goblet cells in the airway surface epithelium.[2,12] Therefore, in this study, we checked whether adenosine, adenine, uridine and homogentisic acid affect MUC5AC mucin secretion, production and gene expression from NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of studying the airway

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mucin production and gene expression.[13-15]

Experimental

General experimental procedures – All the chemicals and reagents used in this experiment were purchased from Sigma (St. Louis, MO, U.S.A.) unless otherwise specified.

Preparation of adenosine, adenine, uridine and homogentisic acid – Adenosine (purity: 98.0%), uridine (purity: 98.0%) and adenine (purity: 98.0%) were isolated, purified and identified by analytical chemist, Professor Dr. Eun Kyoung Seo, in the Laboratory of Pharmacognosy, Department of Pharmacy, Ewha Womans University (Daejeon, Korea). The tubers of *P. ternata* were collected in Jeju island, Korea and taxonomically identified by Professor Jae Hyun Lee (Herbologist) in Dongguk University, Kyoungju, Korea. A voucher specimen (No. EAB309) has been deposited at the College of Pharmacy, Ewha Womans University. Homogentisic acid (purity: 98.0%) was purchased from Sigma (St. Louis, MO, USA). Briefly, the tubers of *P. ternata* (5.4 kg) were extracted with MeOH (3 × 10 L over 24 h) at room temperature. The solvent was evaporated *in vacuo* to afford a MeOH extract (86 g), which was then suspended in MeOH-H_2O (9:1), and partitioned with *n*-hexane (3 × 1 L), giving *n*-hexane- and MeOH-H_2O-soluble fractions. The latter was suspended in distilled water, and further partitioned with EtOAc (4 × 1 L), and *n*-BuOH (3 × 1L), successively. The *n*-BuOH-soluble extract (16 g) was separated by silica gel CC, using gradient mixtures of CH_2Cl_2-MeOH (99:1 → 2:8) as mobile phases, affording 23 fractions (F1-F23). The fraction F12 and F14 were chromatographed using Sephadex LH-20 with 100% MeOH to give compound 1 (2 mg), 2 (150 mg) and 3 (200 mg), respectively. The structures of the isolated compounds were identified as uridine (1),[16] adenine (2),[17] and adenosine (3)[18] by physical and spectroscopic methods as well as by comparison of their data with those in the literature. UV and IR spectra were recorded on a U-3000 spectrophotometer (Hitachi, Japan) and a Nicolet iS10

FT-IR spectrometer (Thermo Fisher Scientific, MA). 1D and 2D NMR experiments were performed on a UNITY INOVA 400 MHz FT-NMR instrument (Varian, CA) with tetramethylsilane (TMS) as internal standard. HREIMS was performed with Waters ACQUITY UPLC system coupled to a Micromass Q-Tof Micro mass spectrometer and Agilent 6220 Accurate-Mass TOF LC/MS system. Silica gel (230 - 400 mesh, Merck, Germany), and Sephadex LH-20 (GE Healthcare, Sweden) were used for column chromatography (CC). Thin-layer chromatographic (TLC) analysis was performed on Kieselgel 60 F 254 plates (silica gel, 0.25 mm layer thickness, Merck, Germany), with visualization under UV light (254 and 365 nm) and 10% (v/v) H_2SO_4 spray followed by heating (120 ºC, 5 min).

NCI-H292 cell culture – NCI-H292 cells, a human pulmonary mucoepidermoid carcinoma cell line, were purchased from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) in the presence of penicillin (100 units/mL), streptomycin (100 μg/mL) and HEPES (25 mM) at 37 ºC in a humidified, 5% CO_2/95% air, water-jacketed incubator. For serum deprivation, confluent cells were washed twice with PBS and recultured in RPMI 1640 with 0.2% FBS for 24 h.

Treatment of cells with adenosine, adenine, uridine and homogentisic acid – After 24 h of serum deprivation, cells were pretreated with adenosine, adenine, uridine and homogentisic acid (1, 10 and 100 μM, the chemical structure of each compound can be seen in Fig. 1), for 30 min and then treated with phorbol 12-myristate 13-acetate (PMA) (10 ng/mL) for 24 h in serum-free RPMI 1640. Adenosine, adenine, uridine and homogentisic acid were dissolved in dimethylsulfoxide, diluted in PBS and treated in culture medium (final concentrations of dimethylsulfoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulfoxide in medium did not affect mucin secretion, production and gene expression from NCI-H292 cells. After 24 h, the spent media were collected to measure the
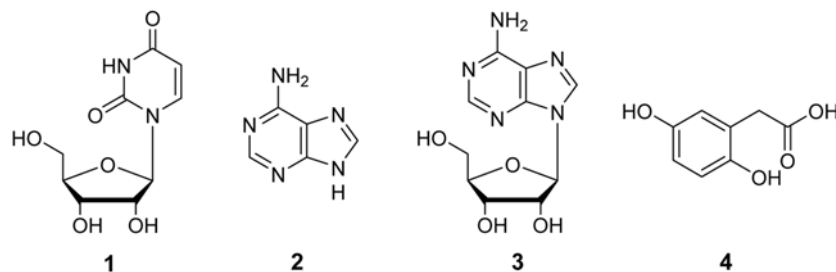

Fig. 1. Chemical structure of uridine (1), adenine (2), adenosine (3) and homogentisic acid (4).

secretion of MUC5AC protein and cells were lysed with buffer solution containing 20 mM Tris, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA and protease inhibitor cocktail (Roche Diagnostics, IN, U.S.A.) and collected to measure the production of MUC5AC protein (in 24-well culture plate). The total RNA was extracted for measuring the expression of MUC5AC gene (in 6-well culture plate) by using RT-PCR.

MUC5AC mucin analysis – MUC5AC mucin protein was measured by using ELISA. Cell lysates or spent media were prepared with PBS at 1:10 dilution, and 100 μL of each sample was incubated at 42 °C in a 96-well plate, until dry. Plates were washed three times with PBS and blocked with 2% BSA (fraction V) for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 100 μL of 45M1, a mouse monoclonal MUC5AC antibody (1:200) (NeoMarkers, CA, U.S.A.), 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, plates were washed three times with PBS. Color reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB) peroxide solution and stopped with 1 N H_2SO_4. Absorbance was read at 450 nm.

Total RNA isolation and RT-PCR – Total RNA was isolated by using Easy-BLUE Extraction Kit (INTRON Biotechnology, Inc. Kyung-gi-do, Korea) and reverse transcribed by using AccuPower RT Premix (BIONEER Corporation, Daejeon, Korea) according to the manufacturer's instructions. 2 μg of total RNA was primed with 1 μg of oligo (dT) in a final volume of 50 μL (RT reaction). 2 μL of RT reaction product was PCR amplified in a 25 μL by using Thermorprime Plus DNA Polymerase (ABgene, Rochester, NY, U.S.A.). 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'. As quantitative controls, primers for Rig/S15 rRNA, which encodes a small ribosomal subunit protein, a housekeeping gene that was constitutively expressed, were used. 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.

Statistics – Means of individual group were converted to percent control and expressed as mean ± S.E.M. The difference between groups was assessed using one-way ANOVA and Holm-Sidak test as a post-hoc test. $p < 0.05$ was considered as significantly different.

Result and Discussion

As aforementioned in introduction, there are no report about the potential effects of adenosine, adenine, uridine and homogentisic acid derived from *P. ternata* on mucin secretion, production and gene expression from airway epithelial cells. Among the twenty one or more MUC genes coding human mucins reported, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium.[2,12] Phorbol 12-myristate 13-acetate (PMA) was reported to stimulate the endogenous activator of protein kinase C (PKC), diacylglycerol (DAG)[19] and to be an inflammatory stimulant that can control a gene transcription[20], cell growth and differentiation.[21] PMA also can induce MUC5AC gene expression in NCI-H292 cells. PMA activates a type of isoform of PKC. This activates matrix metalloproteinases (MMPs), which cleave pro-EGFR ligands from the cell surface to become mature EGFR ligands. These ligands bind to the EGF receptor, provoking the phosphorylation of its intracellular tyrosine kinase. This leads to activation of MEK leading to ERK activation. Following is the activation of the transcription factor, Sp1, and binding of the factor to specific sites with the MUC5AC gene promoter. Eventually, the promoter is activated and produced the gene transcription and translation to MUC5AC mucin protein.[20] Based on these reports, we investigated the effects of adenosine, adenine, uridine and homogentisic acid on PMA-induced MUC5AC mucin secretion, production and gene expression from NCI-H292 cells, a human pulmonary mucoepidermoid cell line.

As shown in results, adenine and homogentisic acid decreased PMA-induced MUC5AC mucin gene expression, although adenosine and uridine did not affect the mucin gene expression (Fig. 2 (A), (B), (C) and (D)). Also, adenosine, adenine, uridine and homogentisic acid inhibited PMA-induced MUC5AC mucin production (Fig. 3 (A), (B), (C) and (D)). The amounts of mucin in the cells of adenosine-treated cultures were $100 \pm 7\%$, $387 \pm 20\%$, $200 \pm 31\%$, $133 \pm 3\%$ and $63 \pm 11\%$ for control, 10 ng/ml of PMA alone, PMA plus adenosine 10^{-6} M, PMA plus adenosine 10^{-5} M and PMA plus adenosine 10^{-4} M, respectively. The amounts of mucin in the cells of adenine-treated cultures were $100 \pm 5\%$, $472 \pm 36\%$, $326 \pm 14\%$, $57 \pm 2\%$ and $22 \pm 6\%$ for control, 10 ng/ml of PMA alone,
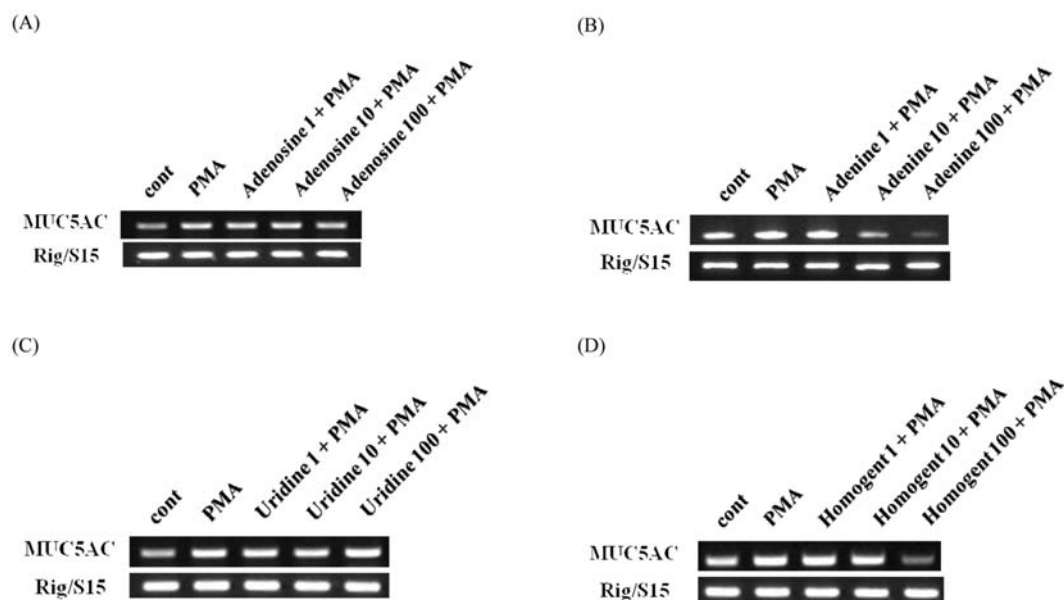
(A)

(B)

(C)

(D)

Fig. 2. Effect of adenosine, adenine, uridine or homogentisic acid on PMA-induced MUC5AC mRNA expression from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of adenosine, adenine, uridine or homogentisic acid for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. MUC5AC mRNA expression was measured by RT-PCR. Three independent experiments were performed and the representative data were shown.
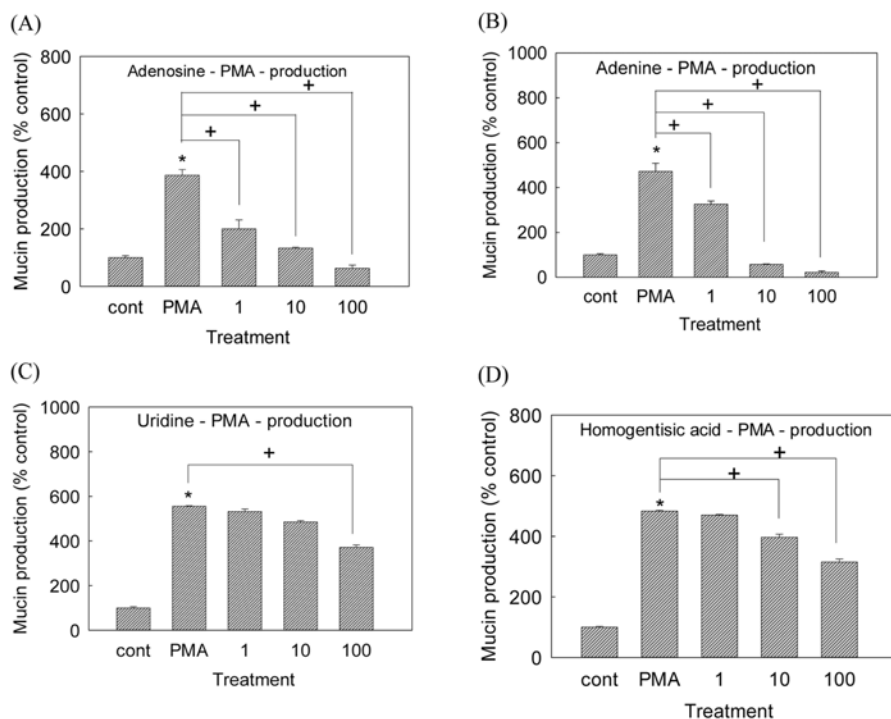
(A)

(B)

(C)

(D)

Fig. 3. Effect of adenosine, adenine, uridine or homogentisic acid on PMA-induced MUC5AC mucin production from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of adenosine, adenine, uridine or homogentisic acid for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Cell lysates were collected for measurement of MUC5AC mucin production by ELISA. Three independent experiments were performed and the representative data were shown. Each bar represents a mean ± S.E.M. of three culture wells in comparison with that of control set at 100%.

*significantly different from control ($p < 0.05$).

+significantly different from PMA alone ($p < 0.05$).
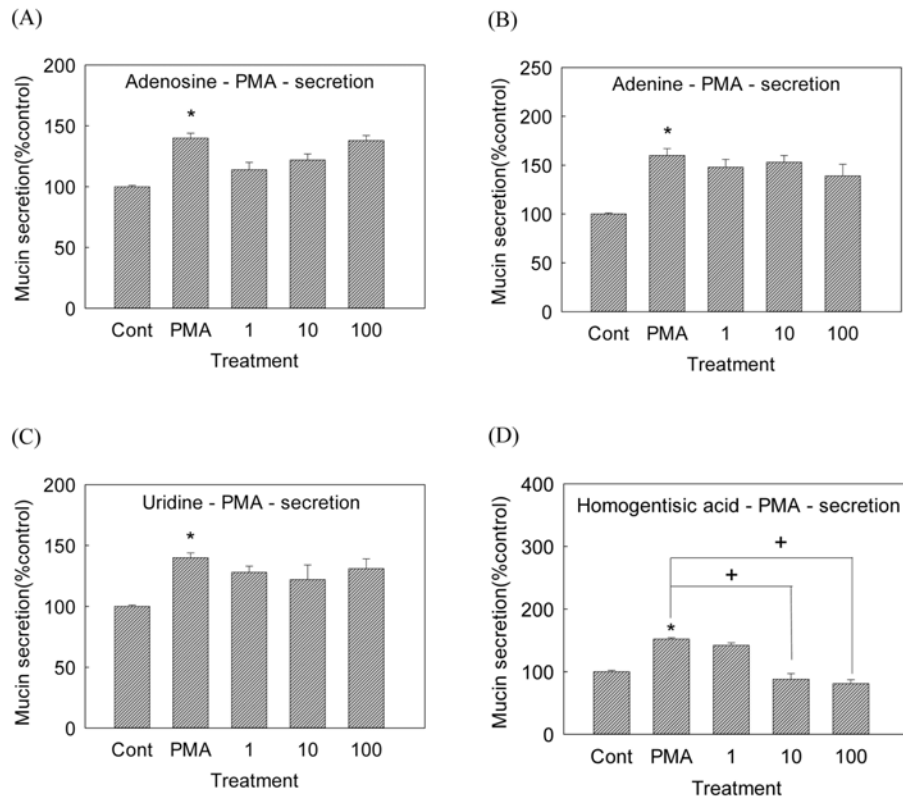
(cont: control, concentration unit is μM.)

Fig. 4. Effect of adenosine, adenine, uridine or homogentisic acid on PMA-induced MUC5AC mucin secretion from NCI-H292 cells. NCI-H292 cells were pretreated with varying concentrations of adenosine, uridine or homogentisic acid for 30 min and then stimulated with PMA (10 ng/mL) for 24 h. Spent media were collected for measurement of MUC5AC mucin secretion by ELISA. Three independent experiments were performed and the representative data were shown. Each bar represents a mean ± S.E.M. of three culture wells in comparison with that of control set at 100%.
*significantly different from control ($p < 0.05$).
+significantly different from PMA alone ($p < 0.05$).
(cont: control, concentration unit is μM.)

PMA plus adenine 10^{-6} M, PMA plus adenine 10^{-5} M and PMA plus adenine 10^{-4} M, respectively. The amounts of mucin in the cells of uridine-treated cultures were $100 \pm 5\%$, $556 \pm 2\%$, $532 \pm 11\%$, $485 \pm 6\%$ and $371 \pm 11\%$ for control, 10ng/ml of PMA alone, PMA plus uridine 10^{-6} M, PMA plus uridine 10^{-5} M and PMA plus uridine 10^{-4} M, respectively. The amounts of mucin in the cells of homogentisic acid-treated cultures were $100 \pm 3\%$, $483 \pm 3\%$, $470 \pm 2\%$, $397 \pm 10\%$ and $315 \pm 10\%$ for control, 10ng/ml of PMA alone, PMA plus homogentisic acid 10^{-6} M, PMA plus homogentisic acid 10^{-5} M and PMA plus homogentisic acid 10^{-4} M, respectively. However, among the four compounds, homogentisic acid only inhibited the secretion of MUC5AC mucin from NCI-H292 cells (Fig. 4 (A), (B), (C) and (D)). The amounts of mucin in the spent medium of homogentisic acid-treated cultures were $100 \pm 2\%$, $152 \pm 2\%$, $142 \pm 4\%$, $88 \pm 9\%$ and $81 \pm 6\%$ for control, 10ng/ml of PMA alone, PMA plus homogentisic acid 10^{-6} M, PMA plus homogentisic acid 10^{-5} M and

PMA plus homogentisic acid 10^{-4} M, respectively. Adenosine, adenine and uridine did not affect PMA-induced MUC5AC secretion. The amounts of mucin in the spent medium of adenosine-treated cultures were $100 \pm 1\%$, $140 \pm 4\%$, $114 \pm 6\%$, $122 \pm 5\%$ and $138 \pm 4\%$ for control, 10ng/ml of PMA alone, PMA plus adenosine 10^{-6} M, PMA plus adenosine 10^{-5} M and PMA plus adenosine 10^{-4} M, respectively. The amounts of mucin in the spent medium of adenine-treated cultures were $100 \pm 1\%$, $160 \pm 7\%$, $148 \pm 8\%$, $153 \pm 7\%$ and $139 \pm 12\%$ for control, 10ng/ml of PMA alone, PMA plus adenine 10^{-6} M, PMA plus adenine 10^{-5} M and PMA plus adenine 10^{-4} M, respectively. The amounts of mucin in the spent medium of uridine-treated cultures were $100 \pm 1\%$, $140 \pm 4\%$, $128 \pm 5\%$, $122 \pm 12\%$ and $131 \pm 8\%$ for control, 10 ng/ml of PMA alone, PMA plus uridine 10^{-6} M, PMA plus uridine 10^{-5} M and PMA plus uridine 10^{-4} M, respectively.

These results suggest that, among the four natural products, only homogentisic acid can regulate the secretion,

production and gene expression of mucin, by directly acting on airway epithelial cells. The underlying mechanisms of action of homogentisic acid on MUC5AC mucin secretion, production and gene expression are not clear at present, although we are investigating whether homogentisic acid act as potential regulators of the MAPK (mitogen-activated protein kinase) cascade after ligand binding to the EGF receptor and/or potential regulators of NF-kB signaling pathway, in mucin-producing NCI-H292 cells.[24]

Taken together, the inhibitory action of homogentisic acid on airway mucin secretion, production and gene expression might explain, at least in part, the traditional use of *P. ternata* as an anti-inflammatory agent for pulmonary inflammatory diseases, in traditional oriental medicine. We suggest it is valuable to find the natural products that have specific inhibitory effects on mucin secretion, production and gene expression - in view of both basic and clinical sciences - and the result from this study suggests a possibility of developing homogentisic acid as a candidate for the new efficacious mucoregulators for pulmonary diseases, although further studies are required.

Acknowledgements

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