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Verticine, ebeiedine and suchengbeisine isolated from the bulbs of *Fritillaria thunbergii* Miq. inhibited the gene expression and production of MUC5AC mucin from human airway epithelial cells

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ABSTRACT

Background: The bulb of *Fritillaria thunbergii* has been utilised as mucoregulators and expectorants for controlling the airway inflammatory diseases in folk medicine.
Hypothesis/Purpose: We investigated whether verticine, ebeiedine and suchengbeisine isolated from the bulbs of *Fritillaria thunbergii* inhibit the gene expression and production of MUC5AC mucin from human airway epithelial cells.
Study design: Confluent NCI-H292 cells were pretreated with verticine, ebeiedine or suchengbeisine for 30 min and then stimulated with EGF, PMA or TNF-α for 24 h. The MUC5AC mucin gene expression was measured by RT-PCR. Production of MUC5AC mucin protein was measured by ELISA.
Results: (1) Verticine, ebeiedine or suchengbeisine inhibited the expression of MUC5AC mucin gene induced by EGF, PMA or TNF-α; (2) The production of MUC5AC mucin protein induced by EGF, PMA or TNF-α were also inhibited by treatment of verticine, ebeiedine or suchengbeisine.
Conclusion: These results suggest that verticine, ebeiedine and suchengbeisine isolated from the bulbs of *Fritillaria thunbergii* inhibit the gene expression and production of MUC5AC mucin, by directly acting on airway epithelial cells, and the results are consistent with the traditional use of *Fritillaria thunbergii* as remedy for diverse inflammatory pulmonary diseases.

Introduction

Mucus in respiratory system is pivotal for defensive action against invading particles, noxious chemicals and pathogenic microorganisms. The protective function of airway mucus is due to the physicochemical properties e.g. viscoelasticity of mucins. Mucins are multimillion-dalton glycoproteins present in respiratory mucus that are produced by goblet cells in the surface epithelium as well as mucous cells in the submucosal gland. However, any abnormality in the quality or quantity of mucins not only cause altered airway physiology but may also impair host defences often leading to severe airway pathology as exemplified in asthma, chronic bronchitis, cystic fibrosis, and bronchiectasis (Voynow and Rubin 2009). Therefore, we suggest it is valuable to find the possible activity of controlling (inhibiting) the excessive mucin secretion (production) by various medicinal plants. We investigated the activities of some natural products derived from several medicinal plants on mucin production and/or secretion from airway epithelial cells (Heo et al. 2007; Heo et al. 2009; Lee et al. 2011; Kim et al. 2012). According to traditional oriental medicine, the bulbs of *Fritillaria thunbergii* has been utilised as mucoregulators and expectorants for controlling the airway inflammatory diseases (Jang 2003). Among various compounds reported to be isolated and purified from the bulbs of *Fritillaria thunbergii*, verticine was reported to have an inhibitory activity on angiotensin converting enzyme (ACE), antitussive and antiinflammatory effects (Oh et al. 2003; Wang et al. 2011). However, to the best of our knowledge, there

Abbreviations: EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; RT-PCR, reverse transcription - polymerase chain reaction; TNF-α, tumour necrosis factor-α.

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is no report about the potential effects of verticine, ebeiedine and suchengbeisine, the compounds isolated from the bulbs of *Fritillaria thunbergii*, on the gene expression and production of mucin from airway epithelial cells. Among the twenty one or more MUC genes coding human mucins reported up to now, MUC5AC was mainly expressed in goblet cells in the airway surface epithelium (Rogers and Barnes 2006; Voynow and Rubin 2009). Therefore, we examined the effects of verticine, ebeiedine and suchengbeisine on EGF-, PMA- or TNF-α induced MUC5AC mucin gene expression and production in NCI-H292 cells, a human pulmonary mucoepidermoid cell line, which are frequently used for the purpose of elucidating mechanisms involved in airway mucin production and gene expression (Li et al. 1997; Takeyama et al. 1999; Shao et al. 2003).

Materials and methods

Materials

All chemicals and reagents used in this experiment were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.) unless otherwise specified. The bulbs of *Fritillaria thunbergii* (11 kg) were collected from Yeongam Province, Korea in January 2012, and authenticated by Prof. Dr. Je-Hyun Lee in Department of Herbology, College of Oriental Medicine, Dongguk University (Kyung-ju, Korea). A voucher specimen (SKKU NPL 1201) was deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea. For a preparation of the aqueous and 70% ethanol (EtOH) extracts, the bulbs of *F. thunbergii* (500.0 g) were extracted with water and 70% EtOH under reflux (2×3 h) and then filtered. The filtrates were evaporated under reduced pressure to give water extract (65.0 g, 13%) and EtOH extract (58.0 g, 11%). For an isolation of active constituents, the bulbs of *F. thunbergii* (11.0 kg) were extracted with 80% methanol, and evaporated under reduced pressure to give residues (967 g). The methanol extract (750 kg) was dissolved in water (800 ml) and partitioned with $CHCl_3$ and *n*-BuOH after pretreatment with 1 N hydrochloric acid (HCl), yielding a $CHCl_3$-fraction (21.0 g) and *n*-BuOH fraction (230.0 g), respectively. The $CHCl_3$-fraction (21.0 g) was chromatographed over a silica gel column with $CHCl_3$–MeOH (20:0–1:1) as the eluent to give ten fractions (C1–C10). The C6 fraction (1.5 g) was subjected to a Sephadex LH-20 (CH_2Cl_2 : MeOH = 1:1) and purified by RP-C_{18} prep. HPLC (60% MeOH in 0.05% TFA) to give suchengbeisine (25 mg). The C7 fraction (1.2 g) was subjected to a Sephadex LH-20 (CH_2Cl_2 : MeOH = 1:1) and purified by RP-C_{18} prep. HPLC (60% MeOH in 0.05% TFA) to give ebeiedine (30 mg). The BuOH-fraction (230 g) was chromatographed over a silica gel column with $CHCl_3$–MeOH (20:0–1:1) as the eluent to give four fractions (B1–B4). The B3 fraction (30 g) was also subjected to a RP-C_{18} silica gel column with 50% MeOH as the eluent to afforded five fractions (B31–B35). The B33 fraction (14 g) was subfractionated with a silica gel column with $CHCl_3$–MeOH (20:1–1:1) and purified by RP-C_{18} prep. HPLC (40% MeOH in 0.05% TFA) to give verticine (3.0 g). These compounds were identified to be verticine ((3β,5α,6α)-Cevane-3,6,20-triol) (Kaneko et al. 1979), ebeiedine ((3β,5α,6β)-Cevane-3,6-diol) (Lee et al. 1988) and suchengbeisine ((22*R*,25*R*)-13α,21-epoxy-5,6,12,13-tetrahydro-3β-hydroxy-5α-veratraman-6-one) (Huang et al. 2013) (Fig. 1A) by comparison of their spectroscopic and physical data with previously reported values (Fig. 1B).

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% foetal bovine serum (FBS) in the presence of penicillin (100 units/ml), 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 recultured in RPMI 1640 with 0.2% foetal bovine serum for 24 h.

Treatment of cells with aqueous extract, EtOH extract, verticine, ebeiedine and suchengbeisine

After 24 h of serum deprivation, cells were pretreated with varying concentrations of aqueous extract, EtOH extract, ebeiedine, suchengbeisine or verticine for 30 min and then treated with EGF (epidermal growth factor) (25 ng/ml), PMA (phorbol 12-myristate 13-acetate) (10 ng/ml) or TNF-α (tumour necrosis factor-α) (0.2 nM) for 24 h in serum-free RPMI 1640. Aqueous extract, EtOH extract, verticine, ebeiedine and suchengbeisine were dissolved in dimethylsulphoxide and treated in culture medium (final concentrations of dimethylsulphoxide were 0.5%). The final pH values of these solutions were between 7.0 and 7.4. Culture medium and 0.5% dimethylsulphoxide did not affect mucin gene expression and production in NCI-H292 cells. After 24 h, 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.

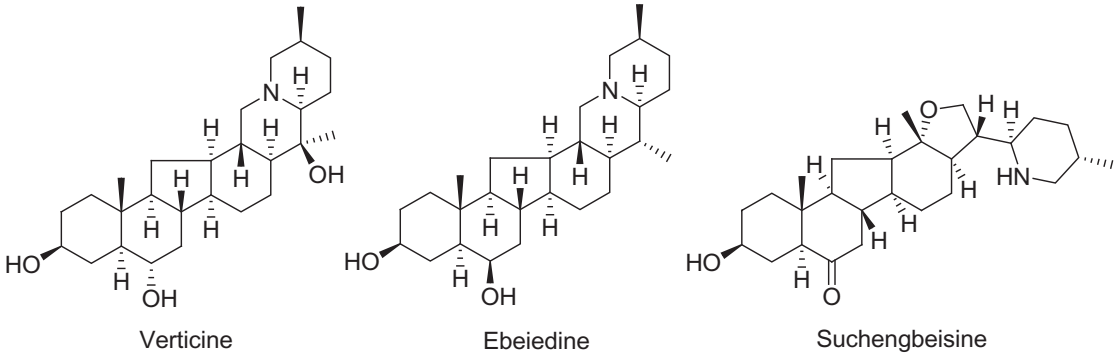

Fig. 1. Chemical structure of verticine, ebeiedine and suchengbeisine and their purity analysed by 1H, 13C NMR.
The isolated compounds were identified to be verticine ((3β,5α,6α)-Cevane-3,6,20-triol), ebeiedine ((3β,5α,6β)-Cevane-3,6-diol) and suchengbeisine ((22*R*,25*R*)-13α,21-epoxy-5,6,12,13-tetrahydro-3β-hydroxy-5α- veratraman-6-one) (Fig. 1A) by comparison of their spectroscopic and physical data with previously reported values (Fig. 1B).