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Original Article

Tedizolid inhibits MUC5AC production induced by methicillin-resistant *Staphylococcus aureus* in human airway epithelial cells

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ABSTRACT

The innate immune system plays an important role in early immunity against respiratory tract infection. Although airway epithelial cells produce mucus to eliminate pathogens and irritants, hypersecretion of mucus is harmful for the host as it may cause airway obstruction and inhibit influx of antimicrobial agents. It has been reported that several antimicrobial agents have an immunomodulatory effect *in vitro* and *in vivo*, but little is known about whether tedizolid, a novel oxazolidinone, can modulate immune responses. In this study, we evaluated whether tedizolid can suppress MUC5AC production in human airway epithelial cells stimulated by methicillin-resistant *Staphylococcus aureus* (MRSA). Compared with the control, tedizolid significantly inhibited MUC5AC protein production and mRNA overexpression at concentrations of both 2 and 10 μg/mL (representative of trough and peak concentrations in human epithelial lining fluid). Among the mitogen-activated protein kinase inhibitors tested, only extracellular signal-regulated protein kinase 1/2 (ERK1/2) phosphorylation was inhibited by tedizolid as indicated by western blot analysis. These results indicate that tedizolid inhibits the overproduction of MUC5AC protein by inhibiting phosphorylation of ERK1/2. This study revealed that tedizolid suppresses excessive mucin production in human airway epithelial cells. The immunomodulatory effect of tedizolid may improve outcomes in patients with severe respiratory infectious diseases caused by MRSA.

1. Introduction

The innate immune system plays an important role in early immunity against respiratory tract infections. Airway epithelial cells produce mucus to protect epithelial cell surfaces, and to trap pathogens and irritants. Foreign particles are then eliminated from respiratory tract by ciliary movement. In this way, mucus is an indispensable part of airway host defense mechanisms.

Mucus consists mainly of mucin proteins and water. MUC5AC and MUC5B are two members of the mucin protein family that are strongly expressed in airway epithelial cells [1], and MUC5AC overexpression is observed in patients with chronic respiratory diseases such as diffuse panbronchiolitis and asthma [2,3]. Mucin overexpression is also observed in patients with acute respiratory infectious diseases such as ventilator-associated pneumonia (VAP) [4]. Excessive mucus induced by these diseases is harmful for the host because it results in airway obstruction, atelectasis, inhibition of oxygenation, and reduction of drug permeability. Hence, it is important to control MUC5AC production.

Several pathogens such as *Pseudomonas aeruginosa*, *Haemophilus influenzae*, *Acinetobacter baumannii*, *Fusobacterium nucleatum*, *Chlamydophila pneumoniae*, and methicillin-resistant *Staphylococcus aureus* (MRSA), stimulate airway epithelial cells and induce MUC5AC overexpression *in vitro* [5–10]. Several studies have reported that direct immunomodulatory effects of antibiotics on airway epithelial cells can reduce excessive MUC5AC production [6–10]. MRSA is the major causative microorganism associated with VAP, and isolation of MRSA from the respiratory tract of patients with cystic fibrosis is associated with the poorest survival

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[11,12]. MRSA is associated with both acute and chronic severe respiratory infections, and therefore it is important to evaluate whether anti-MRSA drugs have an immunomodulatory effect. It has been reported that linezolid, the first member of the oxazolidinones to be introduced, can suppress the MUC5AC overexpression induced by supernatant from MRSA cultures, but it is unclear whether other anti-MRSA drugs also have an immunomodulatory effect [10].

Tedizolid, a novel oxazolidinone, is approved for treatment of bacterial skin and soft tissue infections caused by MRSA and vancomycin-resistant enterococci. Tedizolid possesses several advantages over linezolid such as fewer adverse events, longer half-life, and greater *in vitro* susceptibilities [13,14]. Because the treatment efficacy of tedizolid is equivalent to linezolid in a murine pneumonia model, tedizolid is expected to become the main therapeutic modality for MRSA pneumonia and VAP [15]. Linezolid, the first-line drug for MRSA pneumonia, has been shown to have several immunomodulatory effects such as suppression of inflammatory cytokine production, and inhibition of MUC5AC overexpression [10,16–18]. However, the immunomodulatory effect of tedizolid has not been demonstrated *in vitro*. In this study, we evaluated the immunomodulatory effect of tedizolid on MUC5AC overexpression in human airway epithelial cells.

2. Materials and methods

2.1. Materials

Tedizolid was supplied by Bayer HealthCare AG. Tedizolid was diluted in dimethyl sulfoxide (DMSO). The following antibodies were used: mouse anti-MUC5AC monoclonal (clone 45M1; Monosan); goat anti-mouse horseradish peroxidase-conjugated secondary (Bio-Rad); and anti-ERK, anti-phospho-ERK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-JNK, anti-phospho-JNK, anti-IκBα, and anti-phospho-IκBα (Cell Signaling Technology). The following inhibitors were diluted in DMSO and used: extracellular signal-regulated protein kinase (ERK) inhibitor (U0126; Promega); and p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580), c-JUN N-terminal kinase (JNK) inhibitor II (SP600125), and caffeic acid phenethyl ester, a specific NF-κB inhibitor (CAPE; Calbiochem).

2.2. Bacterial strains

The MRSA strain NUMR101 was obtained from a blood sample isolated clinically at the Nagasaki University Hospital [19]. The strain was stored at −80 °C in a Microbank bead-based preservation system (Pro-Lab Diagnostics) until use. NUMR101 was characterized genetically by real-time polymerase chain reaction (PCR) using a previously described method [20]; the staphylococcal cassette chromosome *mec* (SCC*mec*) was type II, and the strain carried virulence genes such as *sec* and *tsst*, but did not carry *etb* and *pvl* genes.

2.3. Preparation of MRSA supernatant

To avoid direct antibiotic effects of tedizolid on MRSA, we used an MRSA culture supernatant as a stimulator. The MRSA supernatant was prepared according to our previously published method [6]. NUMR101 strain was cultured in Mueller-Hinton II medium (BD) at 37 °C with shaking at 250 rpm for 72 h. After incubation, the bacteria were centrifuged at 10,000× *g* for 10 min at 4 °C, and the supernatant was filtered using a 0.22-μm Millex-GP filter (Millipore). The MRSA supernatant was stored at −80 °C until use.

2.4. Cell culture

NCI-H292 human airway epithelial cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were grown at 37 °C with 5% CO_2. When the cells reached confluence, they were serum-starved for 24 h and then stimulated with the MRSA supernatant. During inhibition studies, the cells were treated with tedizolid simultaneously with MRSA supernatant stimulation. Based on previously reported trough and peak concentrations of tedizolid in the epithelial lining fluid (ELF) of healthy volunteers, tedizolid was used at concentrations of 2 and 10 μg/mL [21]. Because there is a possibility that LB broth induces MUC5AC overexpression, controls were incubated with a volume of LB broth equivalent to the MRSA supernatant. Cells were also pretreated with signal transduction inhibitors at a concentration of 10 μM for 30 min before stimulation. Cells in controls were incubated with medium plus the same amount of DMSO without the inhibitors.

2.5. Enzyme-linked immunosorbent assay (ELISA)

MUC5AC protein levels were measured using ELISA [5]. NCI-H292 cells were cultured in 24-well plates until confluent. After stimulation with MRSA supernatant for 24 h as described above, the culture medium (cell supernatant) was collected and incubated at 40 °C in a 96-well plate until dried. The plates were blocked with 2% FBS for 1 h at room temperature and then incubated with anti-MUC5AC antibody diluted in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 1 h. Horseradish peroxidase-conjugated anti-goat immunoglobulin G was then dispensed into each well. After 1 h, immunoreactivity was detected colorimetrically using 3,3′,5,5′-tetramethylbenzidine peroxidase (TMB) solution before the reaction was stopped by adding 1 N H_2SO_4, and the absorbance read at 450 nm.

2.6. RNA extraction and real-time quantitative reverse transcription-PCR (qRT-PCR)

NCI-H292 cells were stimulated for 6 h before the level of MUC5AC mRNA was assayed by qRT-PCR according to a previously published method [10]. Total RNA was extracted from NCI-H292 cells cultured in 6-well plates, using the ISOGEN II (Nippon Gene) and the PureLink RNA micro scale kit (Invitrogen) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into cDNA using oligo (dT) primers and SuperScript III reverse transcriptase (Invitrogen) and then treated with RNase H. To quantify expression of the MUC5AC gene, PCR primers and TaqMan probes were designed and used as reported previously (forward primer, 5′–CAGCCACGTCCCCTTCAATA–3′; reverse primer, 5′–ACCGCATTTGGGCATCC–3′: TaqMan probe, 5′–6–carboxyfluorecein [6-FAM]–CCACCTCCGAGCCCGTCACTGAG–6–carboxytetramethylrhodamine [TAMRA]–3′) [9]. The *MUC5AC* transcript was amplified for 40 cycles (each cycle consisted of 15 s at 95 °C and 30 s at 60 °C), using a LightCycler system (Roche Diagnostics). To normalize *MUC5AC* expression, human porphobilinogen deaminase (*PBGD*) expression was also measured using specific PCR primers and TaqMan probes ([22]; forward primer, 5′–AACCAGCTCCCTGCGAAGA–3′; reverse primer, 5′–6–FAM –ACTCCTGAACTCCAGATGCGGGAACT–TAMRA–3′).

2.7. Western blot analysis

NCI-H292 cells were harvested at 0, 60, and 120 min after MRSA stimulation and then washed and homogenized at 4 °C in lysis buffer (0.1% sodium dodecyl sulfate, 1% Igepal CA-630, 0.5% sodium deoxycholate). Cell lysates (40 μg) were resolved by electrophoresis

on a 12% polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking the membrane in 10% FBS and 0.1% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature, blots were hybridized overnight at 4 °C with primary antibodies. Hybridization with secondary antibodies was then performed and the immunocomplexes were visualized using ECL enhanced chemiluminescence western blotting detection reagent (GE Healthcare).

2.8. Statistical analysis

GraphPad Prism 5.0b statistic software (GraphPad) was used for all statistical comparisons. All data are expressed as the mean ± standard deviation (SD). One-way analysis of variance was used to determine statistically significant differences between groups. The Tukey test was used for pairwise comparisons. All tests of significance were two-tailed. The alpha level for denoting statistical significant was set at <0.05.

3. Results

3.1. MRSA supernatant induces MUC5AC protein production

Three dilutions of MRSA culture supernatant were tested (80-fold, 40-fold and 20-fold). The 40-fold and 20-fold dilutions significantly increased production of MUC5AC protein after 24 h of stimulation (Fig. 1; 287.4% ± 91.4% [$P < 0.001$] and 349.1% ± 53.2% [$P < 0.001$] percentage increase compared to the control, respectively). Based on this result, a 40-fold dilution of the MRSA supernatant was used for further studies.

3.2. Tedizolid inhibited MRSA supernatant-induced MUC5AC protein production and gene expression

We evaluated the effect of tedizolid on MRSA supernatant-induced MUC5AC protein production and gene expression. Compared with the untreated control group, tedizolid significantly reduced MUC5AC protein production at concentrations of 2 and 10 μg/mL (Fig. 2; 209.5% ± 124.2% versus 61.1% ± 48.5% of the control [$P < 0.01$] and 209.5% ± 124.2% versus 40.0% ± 18.5% of the control [$P < 0.01$], respectively). Ten μg/mL tedizolid also significantly reduced mRNA expression in comparison with the control group (Fig. 3; 102.9% ± 22.6% versus 284.8% ± 166.8% of the control [$P < 0.05$]).

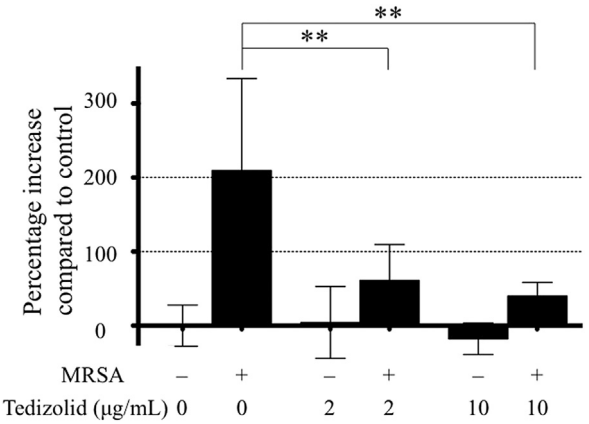

Fig. 2. Effect of tedizolid on MRSA supernatant-induced MUC5AC protein production. NCI-H292 cells were stimulated with 40-fold dilution of MRSA supernatant or LB broth, and treated with tedizolid (2 or 10 μg/mL). MUC5AC protein was measured by ELISA 24 h after treatment. Tedizolid significantly reduced MUC5AC protein production in a dose-dependent manner compared with the untreated control group. Data are expressed as percentage increase compared to the control and are presented as the mean ± SD (n = 5). ** indicates P < 0.01compared with the control group.

3.3. Effect of MAPK and NF-κB inhibitors on MRSA supernatant-induced MUC5AC protein production

Next, we examined the cell signaling pathways associated with MUC5AC protein production. To reveal MRSA-induced activation of cell signaling, cells were treated with MAPK inhibitors (ERK1/2, p38 MAPK, and JNK inhibitors) and a specific inhibitor for NF-κB, caffeic acid phenethyl ester (CAPE), before MUC5AC protein production was evaluated by ELISA. Compared with the untreated control group, the ERK1/2 and p38 MAPK inhibitors significantly reduced MUC5AC protein production when used at concentration of 10 μM (Fig. 4; 250.2% ± 66.2% versus −3.5% ± 30.3% increase compared to the control [$P < 0.001$], and 250.2% ± 66.2% versus 133.3% ± 21.1% increase compared to the control [$P < 0.001$], respectively). The ERK1/2 inhibitor reduced MUC5AC protein production in a dose dependent manner (Fig. 5). In contrast, the JNK and NF-κB

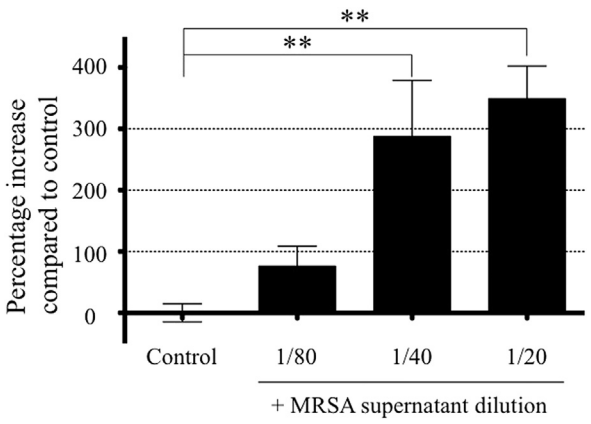

Fig. 1. Influence of MRSA supernatant on MUC5AC protein production. NCI-H292 cells were stimulated with 80-fold, 40-fold, and 20-fold diluted MRSA supernatant, or the same amount of LB broth as a control. The amount of MUC5AC protein was measured by ELISA 24 h after the addition of MRSA supernatant or LB broth. The 40-fold and 20-fold diluted MRSA supernatants significantly increased MUC5AC protein production. Data are expressed as percentage increase compared to the control and are presented as the mean ± SD (n = 6). ** indicates P < 0.01compared with the control group.

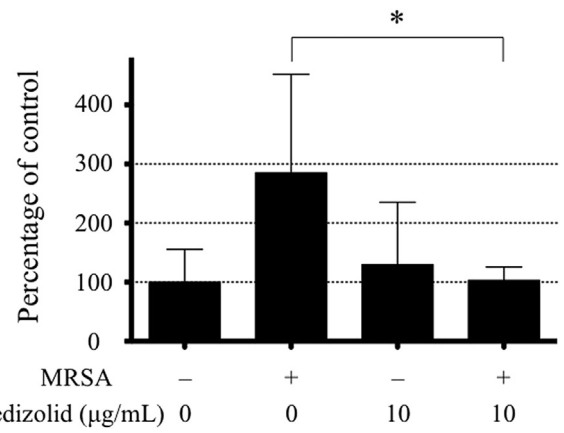

Fig. 3. Effect of tedizolid on MRSA supernatant-induced MUC5AC gene expression. NCI-H292 cells were stimulated with 40-fold dilution of MRSA supernatant or LB broth, and treated with 10 μg/mL tedizolid. MUC5AC mRNA levels were measured by qRT-PCR 6 h after treatment. Tedizolid significantly inhibited MUC5AC mRNA expression compared with the untreated group. Data are expressed as a percentage of the control and are presented as the mean ± SD (n = 6). * indicates P < 0.05 compared with the untreated group.

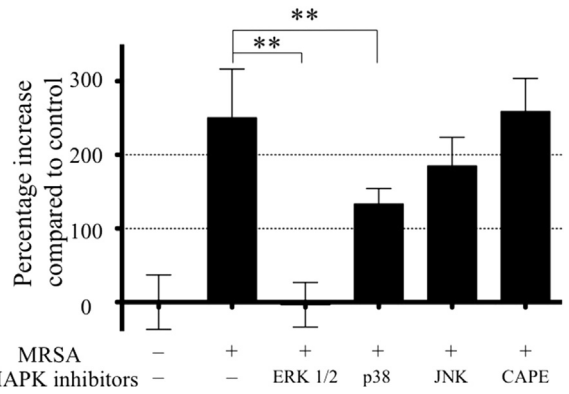

Fig. 4. Effect of MAPK inhibitors on MUC5AC protein production. NCI-H292 cells were treated with 40-fold dilution of MRSA supernatant and inhibitors (10 μM) of ERK1/2, p38 MAPK, JNK, and NF-κB (CAPE) for 24 h, and MUC5AC protein levels were evaluated by ELISA. The ERK1/2 and p38 MAPK inhibitors significantly reduced MRSA-induced MUC5AC protein production compared with the untreated control group. Data are expressed as the percentage increase compared to the control and are presented as the mean ± SD (n = 6). ** indicates $P < 0.01$ compared with the untreated group.

inhibitors did not reduce MUC5AC protein production when used at 10 μM.

3.4. Tedizolid inhibited the phosphorylation of ERK1/2 pathway

To investigate the effects of tedizolid on cell signaling pathways, we also examined the phosphorylation of ERK1/2, p38 MAPK, JNK, and IκBα by western blotting. Tedizolid suppressed the phosphorylation of ERK1/2 when compared to stimulation by the MRSA supernatant alone (Fig. 6A). The other cell signaling pathways (p38 MAPK, JNK, and IκBα) did not show changes in phosphorylation compared to stimulation by the MRSA supernatant alone (Fig. 6B).

4. Discussion

This study indicated that tedizolid suppresses MRSA-induced MUC5AC protein production by inhibition of phosphorylation of ERK1/2 pathway. Mucin overproduction is often observed in patients with chronic respiratory infectious diseases such as cystic fibrosis and diffuse panbronchiolitis (DPB), and contributes to poor outcomes [2]. Increased MUC5AC protein production is associated with acute exacerbation of airway bacterial load and disease severity in the patients with cystic fibrosis [23,24]. The long-term low-dose administration of macrolides has been shown to suppress MUC5AC overproduction in bronchoalveolar lavage fluid and to significantly improve the outcomes of DPB patients [2,25]. Mucin overexpression has also been observed in patients with acute respiratory infectious diseases, and mucin protein levels in the bronchoalveolar lavage fluid of VAP patients was significantly increased compared with non-VAP patients [4]. As with chronic respiratory infectious diseases, inhibition of excessive mucus might improve the outcomes of acute respiratory infectious diseases such as VAP.

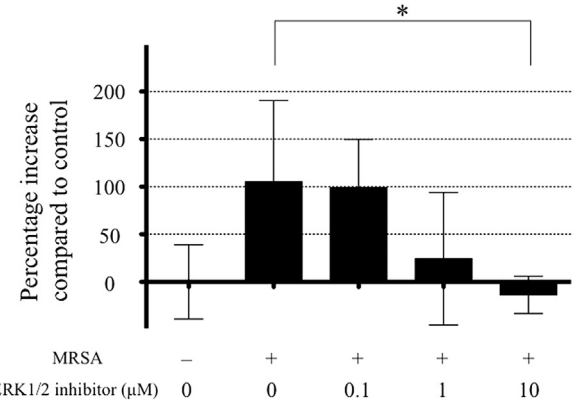

Fig. 5. Effect of ERK1/2 inhibitor on MRSA supernatant-induced MUC5AC protein production. NCI-H292 cells were treated with 40-fold dilution of MRSA supernatant and ERK1/2 inhibitors (0.1, 1, and 10 μM), and MUC5AC protein was measured by ELISA after 24 h. The ERK1/2 inhibitor significantly reduced MUC5AC protein production in a dose-dependent manner compared with the untreated control group. Data are expressed as percentage increase compared to the control, and are expressed as the mean ± SD (n = 6). * indicates $P < 0.05$ compared with the untreated group.

Tedizolid is a novel oxazolidinone that is approved for bacterial skin and soft tissue infections caused by MRSA and vancomycin-resistant enterococci. The bioavailability of oral and intravenous tedizolid are nearly equivalent, and the concentration of epithelial lining fluid and alveolar macrophages are approximately 40-fold and 20-fold greater, respectively, than free-drug exposures in the plasma of healthy adult volunteers [21,26]. The minimal inhibitory concentration (MIC) of tedizolid against gram-positive bacterial isolates *in vitro* has been shown to be lower than those of linezolid or vancomycin [14]. A phase III clinical trial for acute bacterial skin and skin structure infections showed that tedizolid was not inferior to linezolid in terms of clinical responses, and that the incidence of abnormally low platelet counts was significantly lower in the tedizolid-treated group than in the linezolid-treated group [13]. The potent *in vitro* activities and low incidence of adverse events are advantages of tedizolid over linezolid in clinical situations. By contrast, although there are no available clinical data on the use of tedizolid for MRSA pneumonia, tedizolid and linezolid showed similar efficacies in mice with MRSA pneumonia [15]. Moreover, in mice with hematogenous pulmonary infection, linezolid and tedizolid had equivalent antimicrobial efficacy, and tedizolid was also observed to have immunomodulatory effects that inhibited the production of inflammatory cytokines [27].

There are some reports on the immunomodulatory effects of linezolid *in vitro* and *in vivo*. In agreement with this study, Kaku et al. reported that linezolid suppresses excessive MUC5AC production in human airway epithelial cells by inhibiting phosphorylation of ERK1/2 pathway proteins [10]. *In vivo* studies have shown that linezolid significantly suppresses Panton-Valentine Leukocidin production and IL-6 in comparison with vancomycin in a murine MRSA sepsis model, and that sub-MICs of linezolid reduce IL-6 release in a dose-dependent manner in a murine MRSA pneumonia model [17,18]. The immunomodulatory effects of the oxazolidinones may contribute to improved outcomes in VAP caused by MRSA in comparison with vancomycin [28].

To investigate the mechanism by which tedizolid suppresses MUC5AC overproduction, we performed ELISA and western blot analysis. Reduction of MUC5AC protein level determined by ELISA in cells treated with ERK1/2 inhibitor indicates that the ERK1/2 pathway is involved in MRSA-induced MUC5AC production. Although p38 MAPK inhibitor reduced MUC5AC protein production, phosphorylation of p38 MAPK was not observed in western blot analysis. This inconsistency indicates that p38 MAPK pathway might have insignificant effect on MRSA-induced MUC5AC production. Western blot analysis revealed that tedizolid suppresses MUC5AC production by inhibiting phosphorylation of proteins in the ERK1/2 pathway. Although factors upstream of ERK1/2 were not examined in this study, western blot analysis suggests that tedizolid directly affects airway epithelial cells. This is the similar to other antibiotics including macrolides and linezolid [5–10].

There are some limitations to this study. We used MRSA culture supernatant as a stimulator to avoid direct antimicrobial effects of tedizolid on MRSA. The MRSA supernatant contains cell wall

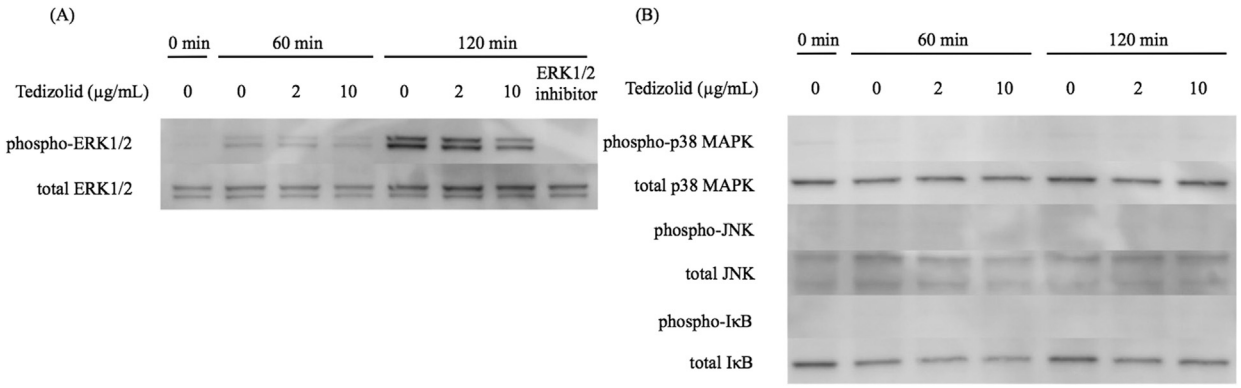

Fig. 6. Effect of tedizolid on the MAPK pathway. NCI-H292 cells were treated with tedizolid for 0, 60, and 120 min and evaluated by western blotting. (A) 40-fold dilution of MRSA supernatant increased phosphorylation of ERK1/2, and 10 μg/mL tedizolid inhibited phosphorylation of ERK1/2. (B) Tedizolid did not affect the phosphorylation of the p38 MAPK, JNK, and IκB pathways.

components and metabolic products, but we did not identify the components that stimulate MUC5AC production. Therefore, we cannot clarify which cell membrane receptor and signaling pathway upstream of ERK1/2 are involved in MUC5AC production. As we did not evaluate the immunomodulatory effects of other anti-MRSA agents, it is not clear whether the effects of tedizolid are superior to other anti-MRSA agents such as linezolid. Therefore, we should examine whether other anti-MRSA agents have the similar effect in the future. However, we speculate that tedizolid contributes to the outcomes of refractory respiratory infectious diseases in terms of both antimicrobial and immunomodulatory effects.

In conclusion, this study revealed that tedizolid can suppress MUC5AC overexpression by inhibiting phosphorylation of proteins in the ERK1/2 pathway. Tedizolid directly affects human epithelial cells, and suppression of excessive mucus may contribute to improved outcomes in MRSA-induced pneumonia.

Funding

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Conflict of interest

None.

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