Original Paper

Relaxing Effect of TSU-68, an Antiangiogenic Agent, on Mouse Airway Smooth Muscle

Honghao Tan^a Jun Lei^a Lu Xue^a Congli Cai^b Qing-hua Liu^a Jinhua Shen^a

^aInstitute For Medical Biology and Hubei Provincial Key Laboratory for Protection and Application of Special Plants in Wuling Area of China, College of Life Sciences, South-Central University for Nationalities, ^bWuhan Youzhiyou Biopharmaceutical Co., Ltd., Biolake, Wuhan, China

Key Words

TSU-68 • Small-molecule chemical compound • Pulmonary disease • VDCCs • NSCCs

Abstract

Background/Aims: Recently, some small-molecule compounds that were designed for cancer therapy have acquired new roles in the treatment of pulmonary diseases. However, drug screening aimed at abnormal muscle contraction is still limited. TSU-68 is a potent, orally administered, small-molecule agent that can reduce the vascular endothelial growth factor (VEGF)-induced Ca^{2+} increase in endothelial cells. We questioned whether TSU-68 could also affect calcium influx and relax airway smooth muscle (ASM) cells. The current study aimed to investigate these effects and to explore the underlying mechanisms. **Methods:** The effects of TSU-68 on ASM cells were studied in mice using a series of biophysiological techniques, including force measurement and patch-clamp experiments. **Results:** TSU-68 inhibited high K^+ or acetylcholine chloride (ACh)-induced pre-contracted mouse tracheal rings in a concentration-dependent manner. Further research demonstrated that the TSU-68-induced ASM relaxation was mediated by calcium, which was decreased by blocking voltage-dependent Ca^{2+} channels (VDCCs) and non-selective cation channels (NSCCs). **Conclusion:** Our data indicated that TSU-68 relaxes tense ASM by reducing the intracellular Ca^{2+} concentration through blocking VDCCs and NSCCs, which suggested that this small molecule might be useful in the treatment of abnormal smooth muscle.

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Introduction

Pulmonary diseases are a series of debilitating, life-threatening respiratory illnesses that have become severe worldwide public health problems and a financial burden [1-4]. Airway inflammation, excessive cell matrix proliferation, and especially the abnormal contraction of airway smooth muscle cells are the main symptoms of pulmonary diseases [5-

H. Tan, J. Lei and L. Xue contributed equally to this work.

Shen Jinhua, PhD

Institute For Medical Biology and Hubei Provincial Key Laboratory for Protection and Application of Special Plants in Wuling Area of China, College of Life Sciences, South-Central University for Nationalities, Wuhan, 430074 (China)
E-Mail shenjinhua2013@163.com/2011084@mail.scuec.edu.cn

7]. The development of more effective medications for pulmonary diseases and improving quality of life without side effects are urgently needed.

Recent studies have revealed that quite a few angiogenic and inflammatory signal pathways are shared in both pulmonary diseases and cancer. Fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), vascular endothelial growth factors (VEGF), and inflammatory factors (IF) and their receptors (FGFR, PDGFR, VEGFR, and IFR, respectively) as well as calcium and calcium permeable channels, which are pivotal targets of anticancer treatments [8-17], are also critical factors for inflammation, extracellular matrix proliferation, and abnormal smooth muscle contraction in pulmonary diseases [18-24]. Considering the roles of these processes in pulmonary diseases, we could readily screen for pulmonary disease medications using established molecular cancer drugs. Recently, several bioavailable small-molecule agents have been evaluated for their efficacy in a series of respiratory diseases and their complications, including chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), and asthma, etc [25]. For example, recent pharmaceutical and clinical research has demonstrated that BIBF1120, a potent triple blocker of PDGFR, VEGFR, and VEGFR, has significant antitumor efficacy in the treatment of non-small cell lung cancer (NSCLC) [26, 27] and reduces the loss of lung function in IPF [12, 28-30]. Gefinitib, which is also an effective small-molecule agent in NSCLC therapy, plays a new role in asthma treatment via inhibiting airway remodeling and inflammation [31]. As a CXC chemokine receptor (CXCR) antagonist, SCH-527123 can inhibit excessive CXCR activation of neutrophils and prevent pulmonary inflammation [32]. SCH-527123 has undergone clinical trials in a number of airway inflammatory diseases, such as COPD, allergen-induced asthma, and neutrophilia-induced asthma [32-34]. Metoprolol, a first-line drug for angina and hypertension, might be applied in cardiac complications during treatment of pulmonary diseases [35]. The breakthrough of using small-molecule compounds as treatments for respiratory diseases has demonstrated that further investigations of other therapies, including angiogenetic inhibitors, might lead to new treatments for these illnesses.

Nonetheless, drug screening aimed at mitigating excessive abnormal contraction in airway smooth muscle (ASM), which is a key symptom of pulmonary disease, has been limited. The small-molecule compound TSU-68, a known tyrosine kinase inhibitor that can reduce VEGF-evoked calcium and inhibit the kinase activity of VEGFR, has been developed as an angiogenesis inhibitor in oncotherapy, especially for solid tumor treatment [36-38]. In the current study, we investigated the roles of TSU-68 in pulmonary disease therapy with a focus on ASM relaxation. The results showed that contracted mouse ASM could be relaxed by TSU-68 in a concentration-dependent manner. Further research indicated that TSU-68 exerted its relaxant effects by decreasing intracellular calcium via voltage-dependent Ca^{2+} channels (VDCC) and non-selective cation channels (NSCC), probably through transient potential canonical (TRPC) channels.

Materials and Methods

Reagents and chemicals

TSU-68 was purchased from Selleck Chemical Reagent Co. (Shanghai, China). Acetylcholine chloride (ACh), gadolinium, pyrazole 3 (Pyr3), niflumic acid (NA), nifedipine, and tetraethylammonium chloride (TEA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Animals

Sexually mature male BALB/c mice were purchased from the Hubei Provincial Center for Disease Control and Prevention (Wuhan, China). The mice were housed in a specific pathogen-free (SPF)-grade laboratory under a 12 h light-dark cycle. All animal experiments were approved by the Animal Care and Ethics Committee of the South-Central University for Nationalities (Wuhan, China) and were performed under the supervision of the Institutional Animal Care and Use Committee of the South-Central University for Nationalities (Wuhan, China).

ASM contraction measurement

Mouse ASM contraction was measured in tracheal rings. The mice were sacrificed by cervical dislocation and the tracheas were isolated and quickly transferred to ice-cold PSS (in mM: NaCl 135, KCl 5, MgCl₂ 1, CaCl₂ 2, HEPES 10, glucose 10, pH 7.4). The connective tissues were removed, and the tracheal rings (5–7 mm) were cut from the bottom of the tracheas. Each ring was mounted with a preload of 0.5 g in a 10 mL organ bath containing PSS bubbled with 95% O₂-5% CO₂ at 37°C. Tracheal rings were equilibrated for 60 min, pre-contracted with either 80 mM of high K⁺ or 100 μM of ACh, washed, and rested 3 times. The experiments were performed following an additional 30 min of rest. Based on tension experiment design, particular channel inhibitors including nifedipine (10 μM), Pyr3 (30 μM), and gadolinium (30 μM) were applied in ASM contraction measurements, respectively.

Isolation of ASM cells

Mouse ASM cells were isolated as described previously [39–41]. Briefly, tracheas were isolated as described above in ASM dissociation buffer (in mM: NaCl 136, KCl 5.36, KH₂PO₄ 0.44, NaHCO₃ 4.16, Na₂HPO₄·12H₂O 0.34, HEPES 20, glucose 10, pH 7.1). The isolated tracheas were then digested in ASM dissociation solution containing 3 mg/mL papain, 0.15 mg/mL dithioerythritol, and 1 mg/mL bovine serum albumin (BSA) at 35°C for 22 min. The tissues were transferred to ASM dissociation buffer containing 1 mg/mL collagenase H, and 1 mg/mL BSA and were incubated at 35°C for 8 min. The tissues were washed and gently triturated with 1 mg/mL BSA to yield single ASM cells for use in subsequent experiments.

Measurement of VDCC currents

The VDCC currents were measured using an EPC-10 patch-clamp amplifier (HEKA, Lambrecht, Germany). Ba²⁺ was used as a charge carrier. The pipette was filled with intracellular solution containing 130 mM CsCl, 10 mM EGTA, 4 mM MgCl₂, 4 mM Mg-ATP, 10 mM HEPES, and 10 mM TEA, and was adjusted with CsOH to pH 7.2. The bath solution contained (in mM) 107 mM NaCl, 27.5 mM BaCl₂, 10 mM HEPES, 11 mM glucose, and 10 mM TEA, and was adjusted with NaOH to pH 7.4. ASM cells were patched and held at -70 mV. Currents were measured following depolarization for 500 ms from -70 to +40 mV in 10 mV increments every 1 s. Based on patch experiments design, nifedipine (10 μM) was applied in VDCC current measurement.

Measurement of NSCC currents

For the measurement of NSCC currents, the pipette solution contained (in mM) CsCl 126, MgCl₂ 1.2, HEPES 10, EGTA 3, and CaCl₂ 1 and was adjusted with Tris to pH 7.2. The composition of the bath solution contained (in mM) NaCl 126, CaCl 1.5, HEPES 10, and glucose 11 at pH 7.2. The free Ca²⁺ concentration was approximately 70 nM as calculated using WEBMAXC STANDARD (<http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm>). The bath solution was K⁺-free PSS. To block VDCC, Cl⁻, and K⁺ currents, 10 μM nifedipine, 100 μM NA, and 10 mM TEA, respectively, were added to the bath solution. NSCC currents were recorded with a ramp using a perforated whole-cell configuration with a holding potential of -60 mV. The ramp was performed over 500 ms from -80 to +60 mV. Based on patch experiments design, nifedipine (10 μM) was applied in VDCC current measurement. Based on experiments design, nifedipine (10 μM), NA (100 μM), TEA (10 mM) were applied in NSCC current measurement, respectively.

Statistical analysis

The results are expressed as the means ± SEM. For all analyses, the evaluations were performed with Student's *t*-test using Origin 9.0 software (OriginLab, Northampton, USA). *p* < 0.05 was considered to be statistically significant.

Results

TSU-68 relaxed high K⁺-induced pre-contraction in a dose-dependent manner

A high K⁺ concentration induces smooth muscle contraction through the membrane depolarization-VDCC opening-calcium influx pathway [42, 43]. For this reason, the relaxant characteristic of TSU-68 was tested under conditions of high K⁺. Before the experiment,

Fig. 1. Relaxant effects of TSU-68 on high K^+ -induced pre-contraction. (A) High K^+ induced a steady-state contraction in a mouse tracheal ring, which was inhibited by TSU-68 in a dose-dependent manner. (B) Dose-relaxation curve of TSU-68 based on the results of 7 different experiments (shown in A). (C) High K^+ -induced pre-contraction was completely reversed by nifedipine ($n = 6/6$ mice). (D) TSU-68 had no effect on resting tension ($n = 4/4$ mice).

Fig. 2. TSU-68 blocked high K^+ -induced Ca^{2+} influx. (A) Under Ca^{2+} -free conditions (0 mM Ca^{2+} and 0.5 mM EGTA), high K^+ did not evoke contraction in a tracheal ring. After restoration of 2 mM Ca^{2+} , a sustained contraction occurred, which was mostly inhibited by TSU-68 ($n = 6/6$ mice). (B) In the presence of 100 μM TSU-68, a Ca^{2+} restoration-provoked contraction was not noted ($n = 6/6$ mice).

a tension measurement to calculate a proper effective concentration (EC) of K^+ was performed (for all online suppl. material, see www.karger.com/doi/10.1159/000475653, Fig. S1). High K^+ was shown to induce the gradual contraction of ASM in a dose-dependent manner (see supplementary material, Fig. S1A). According to the dose-contraction curve (see supplementary material, Fig. S1B) and the results of our previous studies [39, 44], the effective concentration 100% (EC_{100}) was 80 mM, which could maximally activate Ca^{2+} permeable channels; thus, this concentration was applied. As shown in Fig. 1A, the pre-contraction induced by high K^+ (80 mM) was reversed by TSU-68 in a dose-dependent manner. According to the dose-contraction curve shown in Fig. 1B, the maximal relaxation was calculated as $98.46 \pm 1.63\%$. The half-maximal inhibition (IC_{50}) was $29.08 \pm 8.70 \mu M$ ($n = 7/7$ mice). Fig. 1C shows that 10 μM nifedipine, a selective blocker of VDCCs, completely reversed the high K^+ -induced contraction in ASM ($n = 6/6$ mice), which confirmed that the contraction was induced via the opening of VDCCs. Fig. 1D shows that 100 μM TSU-68 had no effect on resting mouse tracheas ($n = 4/4$ mice). These results indicated that TSU-68 inhibited high K^+ -induced pre-contraction in a dose-dependent manner. Furthermore, the

Fig. 3. TSU-68 blocked VDCC currents. (A) The VDCC currents of single mouse ASM cells were measured. (B) VDCC currents were recorded following depolarization and were blocked by TSU-68 or nifedipine. (C) The I - V relationship was constructed based on the results of 6 experiments.

relaxant effect of nifedipine suggested that VDCC participated in high K^+ -induced contraction and also might be involved in TSU-68-induced relaxation.

TSU-68 blocked high K^+ -evoked Ca^{2+} influx

A previous study indicated that contracted ASM was relaxed by blocking VDCC via an intracellular Ca^{2+} decrease [45]. Meanwhile, a VEGF-evoked Ca^{2+} release was blocked by pre-treatment with TSU-68 [16]. To further characterize the relaxant mechanism of TSU-68 shown in Fig. 1, the following experiments were designed to explore the role of calcium in TSU-68-induced relaxation. Under Ca^{2+} -free conditions, high K^+ -induced contraction did not occur (Fig. 2A), indicating that calcium influx was necessary for VDCC opening-induced contraction. Following Ca^{2+} restoration, the contraction that immediately appeared was almost completely inhibited by 100 μM TSU-68 (Fig. 2A) ($n = 6/6$ mice). Meanwhile, high K^+ failed to induce a pre-contraction under Ca^{2+} -free conditions in the presence of 100 μM TSU-68, and even after Ca^{2+} restoration, the ASM contraction was not obvious ($p = 0.54$) (Fig. 2B) ($n = 6/6$ mice). These data suggested that blocking high K^+ -induced Ca^{2+} influx was involved in the relaxant effects of TSU-68.

TSU-68 blocked VDCC currents

To further confirm the involvement of VDCC in TSU-68-induced relaxation on ASM, particular VDCC currents were measured using the whole-cell patch-clamp technique. As shown in Fig. 3A, the currents were recorded with voltage steps from -70 mV to +40 mV. As a positive control, the currents were blocked by the specific blocker nifedipine, which indicated that VDCC currents were recorded (Fig. 3B, top). The currents were then removed by 316 μM TSU-68 (Fig. 3B, bottom), while 100 μM TSU-68 partially blocked the currents (see supplementary material, Fig. S2). As a type of voltage-dependent channel, the voltage-dependent property of VDCC was examined by calculating the current-voltage (I - V) ($n = 6/6$ mice, Fig. 3C), and the results suggested that TSU-68 inhibited VDCC currents. Taken together, TSU-68 was able to relax high K^+ -induced ASM contraction by blocking VDCC and then decreasing intracellular Ca^{2+} .

TSU-68 relaxed ACh-induced pre-contraction in a dose-dependent manner

The regulation of calcium is a complicated process that involves a series of ion channels [16, 45]. We sought to determine whether any other ion channels besides VDCCs might participate in TSU-68-induced relaxation. ACh, a known muscarinic receptor agonist, induced ASM contraction through both VDCCs and NSCCs [46, 47]. In the following experiments, ACh was chosen to pre-contraction ASM. The ACh concentration was determined by measuring the dose-dependent tension, as shown in Fig. S3 (see supplementary material). ACh could stimulate ASM contraction gradually and in a dose-dependent manner (see supplementary material, Fig. S3A). The generated dose-response curve (see supplementary material, Fig.

Fig. 4. TSU-68 inhibited ACh-induced pre-contraction in mouse tracheal rings. (A) ACh induced a steady-state contraction in a mouse tracheal ring, which was inhibited by TSU-68 in a concentration-dependent manner. (B) Dose-relaxation curve of TSU-68 based on the results of 7 different experiments (shown in A).

Fig. 5. TSU-68 inhibited ACh-induced contraction, which was isolated by nifedipine. (A) Nifedipine partly reduced the ACh-induced contraction. The resistant contraction was inhibited by TSU-68 ($n = 7/7$ mice). (B) In the presence of nifedipine, ACh induced a typical contraction, which was dose-dependently inhibited by TSU-68. (C) The summary of results from 7 experiments.

S3B) and the results of our previous study [23, 40] indicated that 100 μM ACh ($EC_{75} = 97.93 \mu M$) was sufficient for our subsequent experiments. As shown in Figure 4, TSU-68 was able to completely relax 100 μM ACh-induced pre-contraction in a dose-dependent manner (Fig. 4A). The relaxation curve was then measured and was shown in Fig. 4B. The maximal relaxation was $97.86 \pm 1.86\%$, and the IC_{50} was $20.62 \pm 5.91 \mu M$ ($n = 7/7$ mice). As shown in Fig. 5, to identify the role of NSCCs, VDCCs were excluded with the specific blocker nifedipine before or after ACh addition. In the presence of ACh, induced pre-contraction was partially reversed by 10 μM nifedipine ($22\% \pm 3.98\%$, $p < 0.001$). Subsequently, 100 μM TSU-68 was able to almost completely relax resistant tension ($96.82\% \pm 4.85\%$) (Fig. 5A, $n = 7/7$ mice). In the presence of nifedipine, ACh-induced pre-contraction was also relaxed by TSU-68 in a dose-dependent manner (Fig. 5B). In Fig. 5C, the curve showed that in the presence of nifedipine, the maximal inhibition induced by 100 μM TSU-68 was $78.05\% \pm 1.5\%$, with an IC_{50} of $17.68 \mu M$ ($n = 7/7$ mice). These experiments indicated that in addition to VDCCs, NSCCs or intracellular calcium release could also be evoked by ACh and thus might participate in TSU-68-induced relaxation.

Fig. 6. TSU-68 blocked ACh-induced Ca^{2+} influx. (A) In the presence of ACh and under Ca^{2+} -free conditions (0 mM Ca^{2+} + 0.5 mM EGTA), ACh induced a rapid, transient contraction. Following the addition of 2 mM Ca^{2+} , a strong, sustained contraction occurred, which was fully relaxed by TSU-68. (B) In the presence of TSU-68, a Ca^{2+} restoration-induced contraction by ACh was not observed. (C) In the presence of nifedipine, TSU-68 relaxed the ACh-evoked contraction with 2 mM Ca^{2+} restoration.

Fig. 7. TSU-68 blocked NSCC currents. (A) The ramp clamp protocol was used to measure NSCC currents in single ASM cells. (B) I - t relationships were plotted at $V_m = -70$ mV. (C) The slope currents at times a, b, and c in B.

TSU-68 blocked ACh-evoked Ca^{2+} influx

In addition to NSCCs, calcium influx or intracellular calcium release could not be excluded from participating in TSU-68-induced relaxation. To further explore the role of calcium in TSU-68-evoked relaxation, Ca^{2+} influx was studied under ACh-induced pre-contraction. As shown in Fig. 6A, in the presence of nifedipine (10 μM) under Ca^{2+} -free conditions, ACh induced a sharp contraction, indicating that there was a transient release of intracellular calcium. Subsequently, the addition of 2 mM Ca^{2+} triggered a sustained contraction, which was completely reversed by 100 μM TSU-68 ($n = 6/6$ mice). In the presence of 100 μM TSU-68, an ACh-induced transient release of intracellular calcium was not observed. With the restoration of 2 mM Ca^{2+} , sustained contraction also did not occur (Fig. 6B). In presence of nifedipine, VDCCs were blocked and NSCCs were isolated, as shown in Fig. 6C. Under Ca^{2+} -free conditions, intracellular Ca^{2+} transiently released after the addition of 100 μM ACh. With the restoration of 2 mM Ca^{2+} , a sustained contraction was induced that was subsequently reversed by 100 μM TSU-68. These results indicated that Ca^{2+} influx played an important role

Fig. 8. ACh-induced contraction was inhibited by Pyr3, gadolinium, and TSU-68. (A) In the presence of nifedipine and 2 mM Ca^{2+} , Pyr3, gadolinium, and TSU-68 relaxed the ACh-induced contraction, sequentially. (B) The bar graph showed the average reduction of Pyr3, gadolinium, and TSU-68, respectively, from 6 experiments. NS, not significant; **, $p < 0.01$.

in ACh-induced contraction. Specifically, TSU-68 could inhibit ACh-induced intracellular Ca^{2+} release and extracellular Ca^{2+} influx.

TSU-68 blocked NSCC currents

To further confirm the role of NSCCs in TSU-68-induced relaxation, whole-cell patch-clamp was used to measure ACh-induced NSCC currents in the absence or presence of TSU-68 (Fig. 7). As shown in Fig. 7A, the NSCC current showed a ramp from -80 mV to +60 mV. Nifedipine, NA and TEA were applied to block currents from VDCCs, Cl^- channels and K^+ channels, respectively. Thus, the residual current was the ACh-induced NSCC current. Then, the currents at -70 mV were measured and a current-time trace was plotted (Fig. 7B). The results showed that NSCC currents could be completely blocked by 316 μ M TSU-68 ($n = 6/6$ mice). Three representative ramp current traces at time points a, b, and c are shown in Fig. 6C. Taken together, these results indicate that TSU-68 can inhibit ACh-induced NSCC currents.

TRPCs were involved in ACh-induced contraction

TRPC channels, which are important components of NSCC, played critical roles in mediating extracellular Ca^{2+} influx [48-52]. To further identify the NSCCs involved in TSU-68-blocked Ca^{2+} influx at the molecular level, the TRPC3 inhibitor Pyr3 [44, 53] and gadolinium, which is a blocker of TRPC1, 3, 5, 6, and 7 [54], were applied sequentially. As shown in Fig. 8A and B, in the presence of nifedipine, ACh induced a transient contraction under Ca^{2+} -free conditions, which indicated that intracellular Ca^{2+} was transiently released after the addition of ACh. With the restoration of 2 mM Ca^{2+} , a sustained contraction was induced by ACh and was partially reduced by 30 μ M Pyr3 (the average relaxation percentage was $27.41 \pm 5.02\%$, 6/6), 30 μ M gadolinium (the average relaxation percentage was $32.02 \pm 7.97\%$, 6/6), and finally almost completely eliminated by 100 μ M TSU-68 (the average relaxation percentage was $40.45 \pm 5.38\%$, 6/6). These results suggested that TRPC channels contributed to ACh-induced contraction. Combined with the result shown in Fig. 6C, TRPC channels might be identified as important components in the process of TSU-68-blocked Ca^{2+} influx via NSCC.

Discussion

Angiogenesis is a complex process that is critical for tumor growth, invasion, and metastasis [55]. Currently, several small-molecule chemical compounds have been designed

for oncology therapeutics according to their antiangiogenic characteristics [33]. Due to similar pathophysiological processes (inflammation, extracellular matrix proliferation, and angiogenesis) involved in both cancer and chronic disease, an increasing number of anti-cancer agents are being tested for treating chronic respiratory disease and particularly pulmonary disease [25]. For example, the antitumor drugs BIBF1120, SCH-527123, and gefitinib play new roles in the clinical treatment of chronic respiratory disease [27, 31, 56].

The tyrosine kinase inhibitor TSU-68 is now known as an antiangiogenic small-molecule compound and has been widely used in antitumor therapy [36, 37]. For decades, it has been known that tyrosine kinases induce muscle contraction, which indicated that tyrosine kinase inhibitors may play an important role in muscle relaxation [57-62]. In the current study, we investigated the relaxant effects of TSU-68 in agonist-induced ASM contraction and the underlying mechanisms. We first examined whether TSU-68 could relax pre-contracted ASM. A high concentration of K^+ and ACh were employed to evoke muscle contraction, and we found that TSU-68 relaxed high K^+ - or ACh-pre-contracted mouse ASM in a dose-dependent manner (Figs. 1, 4 and 5). A recent study demonstrated that TSU-68 inhibited Ca^{2+} release [16]. Meanwhile, NSCCs and VDCCs are two types of ion channels that are critical for Ca^{2+} signaling and were shown to be involved in ASM contraction [46, 47, 63, 64]. The relaxant mechanisms of TSU-68, VDCCs, NSCCs, and Ca^{2+} influx were further examined. The results indicated that calcium influx might participate in TSU-68-induced relaxation by blocking VDCCs and NSCCs (Figs. 2 and 6). Specially, TRPC channels, as an important component of NSCC, were likely to be involved in this process (Figs. 6C and 8). It should be noted that except for VDCC and NSCC, Na^+/Ca^{2+} exchangers (NCX) also play a critical role in mediating intracellular Ca^{2+} oscillation [65]. Whether NCX was involved in the blockage of Ca^{2+} influx by TSU-68 must be explored in additional research studies.

To further demonstrate the roles of VDCCs and NSCCs in TSU-68-induced relaxation, VDCC or NSCC currents were measured. We found that TSU-68 was able to completely inhibit both VDCC and NSCC currents (Figs. 3 and 7). Tyrosine kinase-induced smooth muscle contraction is a complicated process that involves plasma membrane depolarization [61] and the phosphorylation of a series of ACh-related receptors [66, 67]. In our study, TSU-68, as a tyrosine kinase inhibitor, may have indirectly polarized the plasma membrane to block VDCCs or may have directly dephosphorylated the ACh-related protein kinase K (PKC) pathway to block NSCCs. Both processes decrease calcium influx, thereby inducing muscle relaxation.

Overall, the tyrosine kinase inhibitor TSU-68 showed relaxant effects on pre-contracted ASM. These findings indicated that TSU-68 may be a potential treatment for abnormal airway contractions. However, the current conclusion was obtained based on data from *in vitro* mouse experiments. To definitively describe the effects of TSU-68 in clinical applications, further investigation is necessary. In future studies, additional experiments, including tissue toxicity detection, nasal inhalation in a pulmonary disease model, and others, will be needed. In the future, the drug should also be applied to human tracheas in the clinic.

Conclusion

In summary, our research indicated that TSU-68 can relax pre-contracted mouse ASM by inhibiting calcium influx through blocking VDCCs and NSCCs. Our research suggests a new role for TSU-68 in treating abnormally contracted ASM that should be further explored.

Abbreviations

ACh (acetylcholine chloride); ASM (airway smooth muscle); COPD (chronic obstructive pulmonary disease); CXCR (CXC chemokine receptors); EC (effective concentration); FGF (fibroblast growth factor); FGFR (fibroblast growth factor receptor); IC_{50} (half-maximal

inhibition); IPF (idiopathic pulmonary fibrosis); NA (niflumic acid); NSCCs (non-selective cation channels); NSCLC (non-small cell lung cancer); PDGF (platelet-derived growth factor); PDGFR (platelet-derived growth factor receptor); PKC (protein kinase C); Pyr3 (pyrazole 3); SPF (specific pathogen-free); TEA (tetraethylammonium chloride); TRP (transient receptor potential); TRPC (transient receptor potential canonical); VDCCs (voltage-dependent Ca^{2+} channels); VEGF (vascular endothelial growth factor); VEGFR (vascular endothelial growth factor receptor).

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Disclosure Statement

All authors report they have no conflicts of interest pertaining to this study.

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