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Note

Stable dry powder inhaler formulation of tranilast attenuated antigen-evoked airway inflammation in rats

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ABSTRACT

Tranilast (TL) has been clinically used for the treatment of airway inflammatory diseases, although the clinical use of TL is limited because of its poor solubility and systemic side effects. To overcome these drawbacks, a novel respirable powder of TL (CSD/TL-RP) for inhalation therapy was developed using nanocrystal solid dispersion of TL (CSD/TL). Stability study on CSD/TL-RP was carried out with a focus on inhalation performance. Even after 6 months of storage at room temperature, there were no significant morphological changes in micronized particles on the surface of carrier particles as compared with that before storage. Cascade impactor analyses on CSD/TL-RP demonstrated high inhalation performance with emitted dose and fine particle fraction (FPF) of ca. 98% and 60%, respectively. Long-term storage of CSD/TL-RP resulted in only a slight decrease in FPF value (ca. 54%). Inhaled CSD/TL-RP could attenuate antigen-induced inflammatory events in rats, as evidenced by marked reduction of granulocytes in bronchoalveolar lavage fluid and inflammatory biomarkers such as eosinophil peroxidase, myeloperoxidase, and lactate dehydrogenase. These findings were consistent with decreased expression levels of mRNAs for nuclear factor-kappa B and cyclooxygenase-2, typical inflammatory mediators. Given these findings, inhalable TL formulation might be an interesting alternative to oral therapy for the treatment of asthma and other airway inflammatory diseases with sufficient dispersing stability.

1. Introduction

Tranilast (TL), *N*-(3,4-dimethoxycinnamoyl) anthranilic acid, is an anti-allergic agent used clinically for the treatment of inflammatory diseases, including bronchial asthma, atopic rhinitis, and atopic dermatitis [1]. In some cases, oral treatment of TL is often restricted owing to the systemic side effects such as liver dysfunction, abdominal discomfort, skin rash, and allergic cystitis [2]. Recently, dry powder inhaler (DPI) has attracted considerable attention since it can achieve a high drug concentration in lung with low systemic exposure [3]. DPI system for TL could reduce the systemic exposure of TL, possibly leading to the avoidance of the undesired systemic side effects. Previously, our group developed a crystalline solid dispersion of TL (CSD/TL) with improved solubility and high photostability [4], and the CSD/TL was also applied to a respirable powder formulation of TL (CSD/TL-RP) with a jet-milling strategy [5]. The new inhalable formulation could attenuate antigen-evoked inflammatory events in an experimental animal model of respiratory inflammation, without an excessive increase in systemic exposure of TL. Although the CSD approach might be efficacious to improve the therapeutic potential of TL, the inhalation performance and detailed pharmacological mechanism have never been fully elucidated.

The purpose of the present study was to evaluate the dispersing stability of CSD/TL-RP and to investigate the pharmacological effect of inhaled CSD/TL-RP in more detail. The CSD/TL-RP after storage was characterized in terms of surface morphology and simulated respirable fraction. Anti-inflammatory mechanism of inhaled CSD/TL-RP was also assessed by biomarker-based studies and expression of mRNAs for allergic mediators in experimental asthma/chronic obstructive pulmonary disease (COPD)-like rats after intratracheal administration.

Abbreviations: BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; COX-2, cyclooxygenase-2; CSD, nanocrystal solid dispersion; DPI, dry powder inhaler; ED, emitted dose; EPO, eosinophil peroxidase; FPF, fine particle fraction; HPMC, hydroxypropyl methylcellulose; LDH, lactate dehydrogenase; MPO, myeloperoxidase; NF-κB, nuclear factor-kappa B; OVA, ovalbumin; PBS, phosphate-buffered saline; PM, physical mixture; RP, respirable formulation; RT-PCR, reverse transcription polymerase chain reaction; SEM, scanning electron microscope; TL, tranilast; UPLC, ultra-performance liquid chromatography; XRPD, X-ray powder diffraction.

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2. Materials and methods

2.1. Respirable powder formulation of tranilast

The wet-milled tranilast (TL) formulation was prepared with a NanoMill®-01 system (Elan Drug Technologies, Dublin, Ireland) [4]. In brief, 882.0 mg of crystalline TL was weighed and added to a 100-mL stainless chamber. Polystyrene beads (46.7 g) with a diameter of 0.5 mm were put into the chamber, and 44.1 mL of 5 mg/mL HPC-SL solution with 0.2 mg/mL SDS was added. The TL colloidal suspension was micronized at 3600 rpm for 90 min with the NanoMill®-01 system upon cooling of the chamber to 5 °C. After micronization with wet-milling process, the TL wet-milled suspension was lyophilized with a LyoStar II® freeze-dryer (SP Industries Inc., Warminster, PA, USA). Respirable powder of CSD/TL (CSD/TL-RP) was prepared from jet-milled CSD/TL and carrier particles (Respitose® SV-003) [5]. Briefly, freeze-dried CSD/TL and lactose were initially mixed with a pestle and mortar and then milled with an A-O JET MILL (Seishin Enterprise Co. Ltd., Tokyo, Japan) at pusher nozzle pressure and grinding nozzle pressure of 0.60 and 0.55 MPa, respectively. The ratio (w/w) of CSD/TL to excipient was 1:4. The jet-milled particles were mixed with a 10-fold amount of carrier particle manually in a plastic bag for 3 min, providing a CSD/TL-RP. As a control-RP formulation, physical mixture of crystalline TL and HPC-SL (PM/TL-RP) was also jet-milled and mixed with lactose carrier. The amount of TL in the obtained dry powder was determined by an internal standard method using a Waters Acquity UPLC system (Waters, Milford, MA) as reported previously [5].

2.2. Scanning electron microscopy (SEM)

Representative scanning electron microscopic images of TL samples were taken using a scanning electron microscope, VE-7800 (Keyence Corporation, Osaka, Japan), without Au or Pt coating. For the SEM observations, each sample was fixed on an aluminum sample holder using double-sided carbon tape.

2.3. Cascade impactor

The dispersibility of dry powder was assessed according to USP 29 < 601 > AEROSOLS using an AN-200 system (Shibata Scientific Technology, Tokyo, Japan), consisting of a vacuum pump, a mass flow meter, and an eight-stage Andersen cascade impactor. Dry powders were filled into a JP No. 2 hard capsule of hydroxypropyl methylcellulose (HPMC; Qualicaps, Nara, Japan), and the capsule was installed in a JetHaler® (Hitachi Unisia, Kanagawa, Japan) powder inhaler. The dry powder formulations (30 mg) in each capsule were dispersed via the device with an inspiration rate of 28.3 L/min for an inhalation time of 30 s × 5 times, and the collection stages of the impactor (stages 0–7) were washed with 50% methanol. TL in each solution was determined by UPLC/ESI-MS as reported previously [5]. The fine particle dose (FPD) was defined as the mass of drug particles measuring less than 5.8 μm (particles deposited at stage 2 and lower). The fine particle fraction (FPF) was calculated as the ratio of FPD to total loaded dose.

2.4. Animals and drug inhalation

Male Sprague–Dawley rats, weighing ca. 400 g (11 weeks of age; Japan SLC, Shizuoka, Japan), were sensitized by the intraperitoneal injection of 100 μg of ovalbumin (OVA) with 5 mg of alum on days 0, 7, and 14. Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and intratracheally administered OVA (100 μg/rat)-RP at 24 h after the last OVA sensitization. At 1 h before the OVA-RP challenge, 5 mg of CSD/TL-RP (100 μg TL/rat), PM/TL-RP (100 μg TL/rat), or control-RP (micronized excipient and carrier powder) was administered via intratracheal insufflation using PennCentury insufflation powder delivery device (DP-4, INA Research Inc., Nagano, Japan). Blood samples were collected before and 3, 6, and 11 h after OVA-RP challenge from tail vein. All procedures used in the present study were conducted according to the guidelines approved by the Institutional Animal Care and Ethical Committee of the University of Shizuoka.

2.5. Bronchoalveolar lavage fluid (BALF)

At 24 h after OVA-RP challenge, BALF was obtained by flushing the airways with 5 mL of phosphate-buffered saline (PBS) through a tracheal cannula. BALF was pooled and immediately centrifuged at 112*g* for 5 min. The supernatant was used for biomarker profiling.

2.6. Measurement of MPO, EPO, and LDH activities

Enzymatic detection of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in plasma and LDH in BALF was performed in accordance with a previous report [6]. For MPO measurement, assay mixtures consisted of 40 μL of H_2O_2 (final concentration 0.3 mM) in 80 mM sodium phosphate buffer (pH 5.4) and 50 μL of plasma samples. The reaction was initiated by the addition of 10 μL of TMBZ (final concentration 1.6 mM) in dimethyl sulfoxide at 37 °C and stopped after 2 min by the addition of 0.18 M H_2SO_4. Subsequently, optical density was determined at 450 nm. For the detection of EPO activity in plasma, the reaction mixture was prepared by adding 500 μL of OPD (50 mM) to 24.25 mL of Tris buffer (pH 8.0), 3 μL of 30% H_2O_2, and 25 μL of Triton X-100. Then, 100 μL of reaction mixture was added to 50 μL of biological fluid sample in a 96-well plate and incubated for 30 min at room temperature. The reaction was stopped by the addition of 2 M H_2SO_4, and absorbance at 490 nm was measured. A titration curve of horseradish peroxidase was used for the calculation of MPO and EPO activities, which were expressed in arbitrary units. LDH leakage to BALF was determined using the Wako LDH-Cytotoxic test (Wako Pure Chemical, Osaka, Japan) in accordance with the manufacturer's directions. LDH from chicken heart was used as a standard. All samples were assayed in duplicate, and optical densities in all assays were measured using a microplate reader, Safire (Tecan, Männedorf, Switzerland).

2.7. PCR experiments

Total RNA was isolated from homogenized lung tissue using the TRI® reagent (Sigma, St. Louis, MO), and RNA was reverse-transcribed using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Tokyo, Japan). The resulting cDNAs were used for PCR with specific primers based on rat cDNA: 5′- and 3′-primers for NF-κB were 5′-AAGAT CAATG GCTAC ACGGG-3′ (sense) and 5′-CCTCA ATGTC TTCTT TCTGC-3′ (antisense), and those for COX-2 were 5′-CTGAG GGGTT ACCAC TTCCA-3′ (sense) and 5′-TGAGC AAGTC CGTGT TCAAG-3′ (antisense). PCR for NF-κB and COX-2 was performed for 30 and 40 cycles, respectively. After an initial denaturation at 94 °C for 3 min, the indicated cycles of amplification [1-min denaturing at 94 °C, 1-min annealing at 58 °C (NF-κB) or at 63 °C (COX-2), and a 1-min extension at 72 °C] were performed in a Takara PCR Thermal Cycler MP (Takara Bio, Shiga, Japan). The size of each PCR product was expected to be 493 bp for NF-κB and 209 bp for COX-2. The amplified PCR products were separated by electrophoresis (2.0% agarose gel in TAE buffer), stained with EtBr solution (0.5 μg/mL), and analyzed with image analyzing software Image J.

2.8. Statistical analysis

For statistical comparisons, one-way analysis of variance (ANOVA) with pairwise comparison by Fisher's least significant difference procedure was used. A P value of less than 0.05 was considered significant for all analyses.

3. Results and discussion

Fine inhalation properties of DPI system could be indispensable for sufficient clinical outcomes, therapy cost, and medication compliance. Variable inhalation properties during storage could affect the therapeutic outcomes from inhalation therapy, so the physicochemical stabilities need to be evaluated. In the present study, CSD/TL-RP was stored at room temperature for 6 months, and physicochemical stability data on the CSD/TL-RP before and after storage were compared with focus on potency, surface morphology, and cascade impactor analysis. UPLC/ESI-MS analysis demonstrated no significant degradation of TL after long-term storage (data not shown), suggesting high chemical stability of CSD/TL-RP. According to SEM images, there was no significant morphological change in micronized particles on the surface of carrier particles after storage compared with that before storage (Fig. 1). To clarify the possible changes in inhalation performance, cascade impactor analysis was carried out on CSD/TL-RP (Fig. 2). The emitted dose of the CSD/TL-RP from HPMC capsule was almost identical to that after storage (97.9% and 99.2% in CSD/TL-RP before and after storage, respectively). The fine particle fraction (FPF) value of CSD/TL-RP was found to be 59.4%, and there appeared to be only a slight decrease after storage (53.9%). Thus, CSD/TL-RP still showed a high dispersibility even after 6 months of storage. Interestingly, the aerodynamic particle size distribution of the aged CSD/TL-RP seemed to be slightly shifted to a lower stage. According to the Andersen cascade impactor analysis, mass median aerodynamic diameters (MMAD) of the freshly prepared and aged CSD/TL-RP were calculated to be 3.71 and 4.61 μm, respectively. It is well established that relative humidity (RH) during storage critically influences the aerosolization performance [7]. An environment of high RH tends to reduce the respirable fraction of inhalable powder formulations, which is possibly due to an increase in inter-particulate forces between the drug and carrier [8]. Therefore, the slight changes in FPF value, MMAD, and aerodynamic particle size distribution of the aged CSD/TL-RP might be attributable to the presence of environmental moisture during storage. On the basis of stability data such as purity, morphology, and inhalation performance, the present study indicates high physicochemical stability for CSD/TL-RP; however, to maintain the initial aerosolization performance, there might be a need to protect the CSD/TL-RP from moisture with storage in a controlled humidity environment.

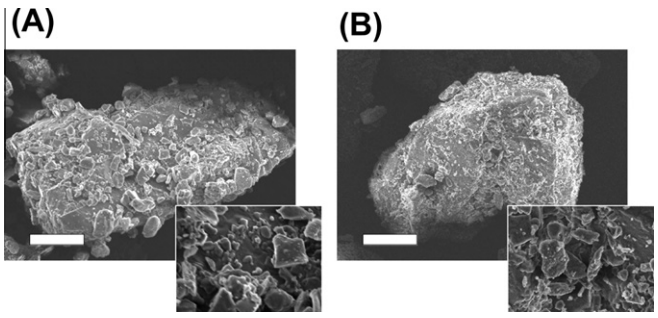

Fig. 1. Scanning electron microscopic images of CSD/TL-RP before storage (A) and after storage (B). Each scale bar represents 20 μm.

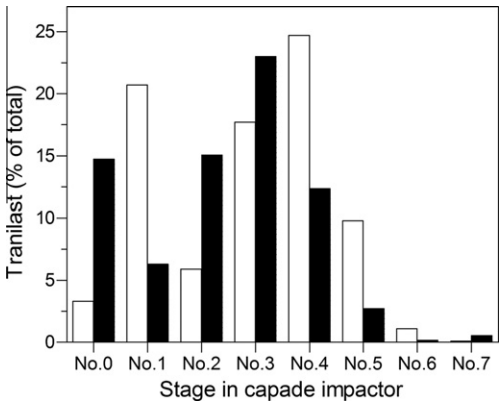

Fig. 2. Deposition pattern following the aerosolization of CSD/TL-RP. Open and filled bars represent before storage and after storage, respectively. Deposition pattern analysis of CSD/TL-RP was conducted using a cascade impactor connecting JetHaler® with an air flow rate of 28.3 L/min.

For pharmacological characterization, inflammatory biomarkers in plasma were monitored, including MPO and EPO mainly released from activated neutrophils/macrophages [9] and eosinophils [10], respectively. As shown in Fig. 3, both enzymatic activities in rat plasma were significantly increased after OVA-RP challenge, suggesting OVA-induced neutrophilia and eosinophilia.

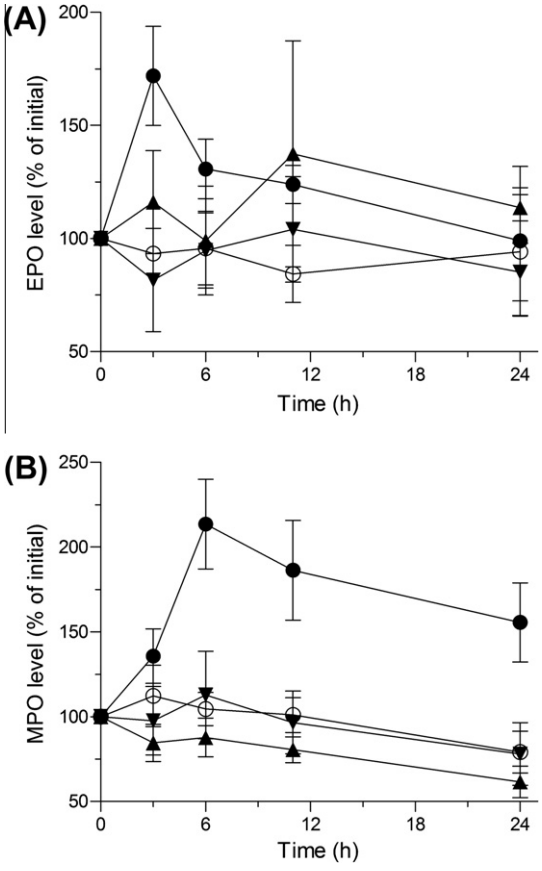

Fig. 3. Biomarker profiling in plasma in antigen-induced pulmonary inflammations. (A) Eosinophil peroxidase (EPO) activity in antigen-sensitized rat with or without TL-RP pretreatment. (B) Myeloperoxidase (MPO) activity in antigen-sensitized rat with or without TL-RP pretreatment. ○, control (non-sensitized); ●, OVA-sensitized rat; ▲, OVA-sensitized rat with pretreatment of PM/TL-RP; and ▼, OVA-sensitized rat with pretreatment of CSD/TL-RP. Data represent the mean ± SEM of 4–5 determinations.

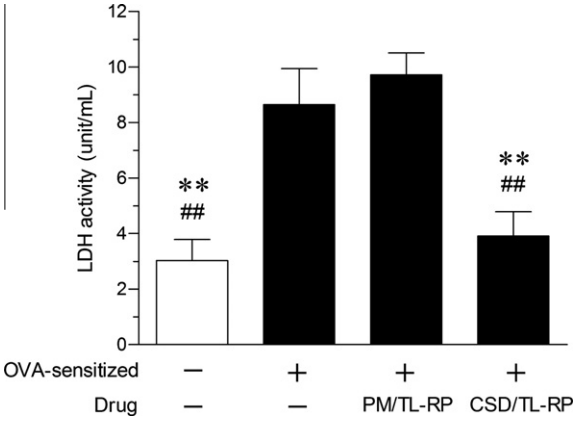

Fig. 4. Lactate dehydrogenase (LDH) activity in BALF of antigen-sensitized rat with or without TL-RP treatment. Data represent the mean ± SEM of 4–5 determinations. $^{\#\#}p < 0.01$ with respect to rats treated with OVA-RP; $^{**}p < 0.01$ with respect to rats treated with PM/TL-RP.

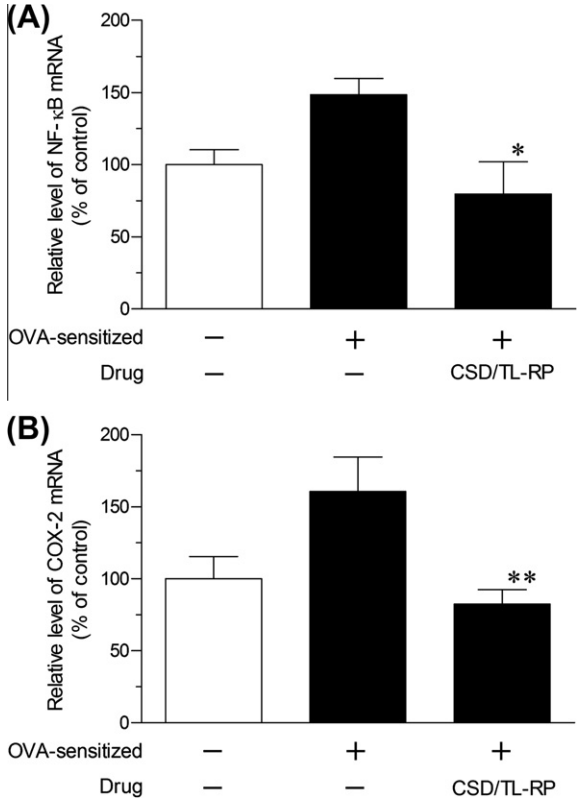

Fig. 5. Expression levels of mRNAs for inflammatory mediators. Relative expressions of mRNAs for (A) NF-κB and (B) COX-2 in the lung tissue were evaluated by RT-PCR. The mRNA expression ratio in the non-sensitized rat was established as 100%. Data represent the mean ± SEM of 4–5 determinations. $^{*}p < 0.05$; $^{**}p < 0.01$ with respect to rats treated with OVA-RP.

In contrast, the enzymatic levels of MPO and EPO in OVA-sensitized rats with inhalation of CSD/TL-RP (100 μg TL) were almost identical to those in the control group, whereas PM/TL-RP (100 μg TL), a physical mixture, was found to be less effective in attenuating the EPO level. Severe inflammation would lead to pulmonary injury, followed by the extracellular appearance of LDH, a cytoplasmic enzyme. The LDH activity level in BALF was determined as an indicator of lung damage, and the LDH activities in BALF were increased at 24 h after OVA-RP challenge (Fig. 4). Interestingly, the CSD/TL-RP could significantly suppress the increase in LDH activity; however, no significant suppression of LDH level in BALF was seen in OVA-sensitized rats with inhalation of PM/TL-RP. On the basis of these obtained data, the anti-inflammatory effect of CSD/TL-RP was shown to be much higher than that of PM/TL-RP, which was possibly due to the improved dissolution properties by micronization. To clarify the possible mechanism of inhaled CSD/TL-RP in more detail, expressions of mRNA for NF-κB and COX-2 were compared with or without CSD/TL-RP treatment by RT-PCR experiments (Fig. 5). NF-κB regulates the expression of IL-8 and other chemokines [11], and COX-2 is the inducible rate-limiting enzyme in the prostaglandin pathway [12]. OVA-RP challenge in OVA-sensitized rats resulted in ca. 50% increases in both mRNAs, suggesting the activation of inflammatory signaling. However, intratracheal administration of CSD/TL-RP led to significant decreases in the expressions of NF-κB and COX-2 mRNA compared with those of the OVA-RP-treated group. Thus, regulation of NF-κB and COX-2 expression might be involved in mechanisms by which inhaled CSD/TL-RP suppresses the inflammatory events in OVA-sensitized rats.

In conclusion, stability study demonstrated that CSD/TL-RP had a high inhalation property even after 6 months of storage at room temperature. Inhalation of CSD/TL-RP led to marked attenuation of antigen-evoked inflammatory events in the experimental asthma/COPD model, the mechanism of which was deduced to be the regulation of NF-κB and COX-2 expression. Given these findings, CSD/TL could be a promising formulation for the treatment of airway inflammatory diseases, particularly in combination with the use of a dry powder inhaler system.

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