Original Research

Allergen Release Profiles of Fast-Dissolving Freeze-Dried Orodispersible Sublingual Allergy Immunotherapy Tablets

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ARTICLE INFO

Article history:
Received 26 April 2022
Accepted 15 June 2022

Keywords:
Allergen delivery
Allergen release
SLIT-tablet
SLIT-tablet formulation
Sublingual allergy immunotherapy

ABSTRACT

Background: Sublingual allergy immunotherapy tablets (SLIT-tablets) provide a well-tolerated and clinically efficacious treatment for allergic disease such as allergic rhinitis and allergic asthma. In SLIT, uptake of allergen by immune-competent cells in the oral mucosa activates the immune system and leads to tolerance toward the sensitizing allergen. The ability to deliver the full allergen content into solution within the recommended sublingual holding time is therefore an essential quality of SLIT-tablets that must be supported by the tablet formulation for all relevant allergen sources. SLIT-tablets based on a fast-dissolving orodispersible freeze-dried formulation (2ydis) are currently available for 5 of the most prevalent allergens: tree (birch and related species from the birch-homologous group), grass, ragweed, Japanese cedar, and house dust mite.

Objectives: The purpose of this study was to examine the allergen release properties of three freeze-dried SLIT-tablets containing tree, ragweed, and Japanese cedar extracts, respectively. The correlation between SLIT-tablet allergen release and the level of allergen-specific T-cell activation was examined for the tree SLIT-tablet.

Methods: Allergen release kinetics and tablet disintegration times for the 3 freeze-dried SLIT-tablets were examined. For all 3 tablets, the magnitude of solubilized major allergen relative to time in solution was compared to external controls to achieve a measure of the total allergen release. Additional assessments of allergen release occurring after the initial timepoint (15 or 30 seconds in solution) were done independently of external controls by linear regression analyses. For the tree SLIT-tablet, the immunological potency of the released major allergen was assessed at each experimental timepoint by a Bet v-specific T-cell activation assay.

Results: All 3 SLIT-tablets disintegrated within 1 second after contact with assay buffer without any detectable residue. Complete release of major allergens (Bet v 1, Amb a 1, and Cry j 1, respectively) was seen at the earliest experimental timepoints (15 or 30 seconds). For the tree SLIT-tablet, full T-cell activation was achieved at 30 seconds (earliest experimental time point).

Conclusions: The freeze-dried SLIT-tablet formulation consistently provides rapid and complete release of allergen from a wide range of species in a standardized in vitro assay. Full release of the SLIT-tablet allergen content within the sublingual holding time is a prerequisite for maximal exposure of allergens to the sublingual mucosa immune system. The freeze-dried SLIT-tablet formulation examined here supports short sublingual holding times and furthermore offers a convenient administration form of allergy immunotherapy.

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Introduction

The prevalence of respiratory allergic disease, including allergic rhinitis and allergic asthma, has increased dramatically over the past 40 years and with 10% to 30% of the world's population af-
ected, allergic diseases are now considered a global health problem.1,2 The burden of allergy in both adults and children is substantial and affects the general quality of life, including reduced work and school performance.3 In addition, allergy is a known risk factor for development of allergic asthma.4 Most commonly, allergy is treated with symptomatic medications such as antihistamines and corticosteroids. Although these medications may provide relief for some but not all allergic individuals, their effects are symptomatic and short term.5 Allergy immunotherapy (AIT) is the only treatment option for respiratory allergic disease that unlike the symptomatic medications provides disease-modifying and long-lasting effects.6-8 Allergy is an immunologic disease and through repeated administration of extracts of the sensitizing allergen, the immune-modulatory effects of AIT lead to a series of events starting with rapid desensitization of effector cells (ie, mast cells and basophils) followed by production of protective allergen-specific antibodies (eg, immunoglobulin G4 and immunoglobulin A) and suppression of allergen-specific effector T-cell subsets by regulatory B-cells and T-cells.9,10

AIT is available in 2 main forms: subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT). SLIT is available as liquid formulations of allergen extracts (SLIT-drops) or as allergen extracts formulated as dry tablets (SLIT-tablets). With both SCIT and SLIT, repeated administrations, typically over a period of 3 years, are required to obtain lasting effect of the treatment.6,7 Unlike SCIT that requires repeated injections at the doctor’s office, SLIT is a needle-free self-administration treatment option where only the initial dose is administered under medical supervision.11 Although SCIT has been the mainstay of AIT for many years, a larger body of evidence of clinical safety and efficacy in the form of Phase I, II, and III trials is now available for SLIT-tablets than for SCIT. Large clinical trials have demonstrated the safety and efficacy of a range of SLIT-tablets based on a freeze-dried fast-dissolving orodispersible tablet formulation covering the 5 most prevalent respiratory allergies worldwide; that is, house dust mite (HDM)12-15 and pollens from grass,5,16-19 trees,18,19 ragweed,20,21 and Japanese cedar.22 Studies include allergic rhinitis with or without conjunctivitis in both adults and children,8,20,23,24 and allergic asthma.25

In SLIT, the allergen extract is placed under the tongue either in the form of allergen extracts that are prediluted in an aqueous buffer before administration (SLIT-drops) or in the form of dry tablets (SLIT-tablets) where the allergen content of the tablet is dissolved in saliva directly at the surface of the sublingual mucosa. For SLIT-tablets, the recommended sublingual holding time is about 1 minute,26-28 during which allergens become solubilized and subsequently internalized by immune-competent cells in the sublingual mucosa such as antigen-presenting oral Langerhans cells.29,30 For a dry SLIT-tablet, the ability to deliver the full content of allergen extract into soluble form in a limited amount of saliva within a short period of time is key, which must be reflected in the physical properties of the SLIT-tablet formulation.

The physical properties of 2 different SLIT-tablet formulations, a freeze-dried and a compressed formulation, respectively, regarding tablet disintegration time and major allergen release kinetics have previously been examined.31,32 Both formulations were examined under identical in vitro experimental conditions in the form of SLIT-tablets containing extracts of grass pollen (Phleum pratense) or HDM (mixtures of Dermatophagoides pteronyssinus and D. farinae). For both grass pollen and HDM, the freeze-dried formulation was shown to provide full release of major allergens in a matter of seconds in contrast to the compressed formulation where slower and incomplete release of the allergen content during the experiment were seen.31,32 These experiments indicated that the allergen-release properties of the fast-dissolving freeze-dried SLIT-tablet formulation were independent of the allergen extract species examined. However, allergens from different allergen sources are proteins with unique biochemical properties with different potential for interactions with the tablet matrix, which could potentially prevent or delay allergen release. It is therefore relevant to study the actual SLIT-tablet disintegration and allergen release properties for all allergen sources.

The purpose of this study was to examine the disintegration times, allergen dissolution properties, and release kinetics of 3 fast-dissolving freeze-dried SLIT-tablets containing allergen extracts from tree (birch, Betula verrucosa, and related species from the birch homologous group) pollen, ragweed (Ambrosia artemisiifolia) pollen, and Japanese cedar (Cryptomeria japonica) pollen, respectively. To demonstrate the biological potency of allergen released from a SLIT-tablet, data on T-cell activation in relation to dissolution time was done for the tree SLIT-tablet.

Materials and methods

Test samples

Freeze-dried SQ ragweed SLIT-tablets (12 SQ-Amb, lot No. 3702086) (ALK-Abelló A/S, Hørsholm, Denmark), SQ tree SLIT-tablets (12 SQ-Bet, lot No. 1721798) (ALK-Abelló A/S, Hørsholm, Denmark) and Japanese cedar SLIT-tablets (5000 JAU, lot Nos. 1619238, 1625155, and 1625156) (Torii Pharmaceutical Co. Ltd., Tokyo, Japan) were obtained from the manufacturer.

Assay buffer

A 100-mmol/L phosphate buffer (pH 6.8) supplemented with 0.125% casein was used for all disintegration and dissolution experiments. The composition of the assay buffer was similar to human saliva with regard to pH value, ionic strength, and total protein content.33

SLIT-tablet disintegration test

Tablet disintegration was done according to the Japanese pharmacopeia. Briefly, tablets (tree and ragweed SLIT-tablets [n = 6] and Japanese cedar SLIT-tablet [n = 9]) were deposited into a submersible mesh basket (1.8–2.2 mm mesh size) and agitated vertically in assay buffer at 37 °C until disintegrated. Disintegration was considered complete when all the tablet residues had passed through the mesh.

Total SLIT-tablet allergen content

The control samples for complete allergen content were prepared by dissolving 1 SLIT-tablet in 10 mL (ragweed SLIT-tablet [n = 4]) followed by vortexing for 1 minute or 100 mL (tree SLIT-tablet [n = 4] and Japanese cedar SLIT-tablets [n = 3]) assay buffer followed by stirring at 50 rpm for 1 minute, then leaving for 10 minutes. Major allergen concentrations were determined for Bet v 1 (tree SLIT-tablet) and Amb a 1 (ragweed SLIT-tablet) by ELISA (Indoor Biotechnologies, Charlottesville, Virginia). Concentration of Cry j 1 [Japanese cedar SLIT-tablet] was determined by ELISA developed by the Japanese Society of Allergology.34,35 All major allergen concentrations were calculated as micrograms major allergen per tablet. Full release of the SLIT-tablet major allergen content was confirmed by comparing the released amounts of major allergen to the manufacturer’s respective in-house references using in-house ELISAs and controls (Amb a 1 and Bet v 1) or ELISA developed by the Japanese Society of Allergology.34,35
SLIT-tablet dissolution and allergen release

The dissolution test was performed according to the paddle method of the Japanese pharmacopoeia. Allergen dissolution was measured in assay buffer using a minivesSEL (200 mL) Distek model 2500 instrument (Distek Inc, North Brunswick, New Jersey) (50 rpm paddle speed). Aliquots were removed at 15, 30, 60, 90, 120, 180, and 300 seconds (ragweed SLIT-tablet and tree SLIT-tablet) or 30, 45, 60, 90, 120, 180, and 300 seconds (Japanese cedar SLIT-tablet) and the amounts of soluble major allergens were analyzed by ELISA (n = 4 [tree and ragweed SLIT-tablets] or n = 3 [Japanese cedar SLIT-tablets] [Amb a 1 and Bet v 1 ELISAs [Indoor Biotechnologies] or Cry j 1 ELISA [Japanese Society of Allergology]34,35).

Allergen release kinetics

Allergen release data obtained between 15 seconds (tree and ragweed SLIT-tablets), or 30 seconds (Japanese cedar SLIT-tablet) and 300 seconds were analyzed by linear regression assuming linearity of any residual release occurring after these time points. The linear regression curves are mathematical approximations of the allergen release rates and provide a relative measure of the magnitudes of allergen release that may occur after 15 or 30 seconds, respectively. The ideal regression curve where no further allergen release occurred after the first time point would be a horizontal line with a slope = 0.

T-cell proliferation assay

DC preparation

Peripheral blood mononuclear cells were isolated from whole blood and adjusted to 5 × 10⁶ cells/mL in RPMI 1640 + Glutamax medium ( Gibco 72400-021, Live Technologies Europe B.V. the Netherlands) supplemented with 1% penicillin/streptomycin and 15% AB serum (Sigma-Aldrich, St Louis, Missouri). A total of 1 × 10⁹ cells/well (2 mL) was seeded in a 6-well culturing plate, incubated for 1.5 hours in 5% carbon dioxide at 37 °C, and washed 4 times with 1 mL prewarmed medium. For culturing, 2 mL culture medium supplemented with 50 ml/gucocyte-macrophage colony-stimulating factor (GM-CSF) and 25 ng/mL IL-4 (Peprotech, Rocky Hill, New Jersey)) was added to each well and incubated in 5% carbon dioxide at 37 °C for 7 to 10 days. At day 4 or 5, 500 μL of the medium was removed and replaced with 750 μL fresh culture medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and recombinant interleukin 4 (Peprotech, Rocky Hill, New Jersey). The DCs were detached from the plate with 1 mL/well 10 mM EDTA (Invitrogen, Waltham, Massachusetts) in phosphate buffered saline followed by incubation at 4 °C for 45 minutes. After centrifugation at 300 g for 10 minutes, the cells were collected in 1 mL RPMI and adjusted to a concentration of 2.5 × 10⁵ cells/mL.

T-cell stimulation

Tree SLIT-tablets (batch 1592972 7DU) were deposited into T-cell assay buffer (0.5% human serum albumin in phosphate buffered saline). After 30, 60, 90, and 120 seconds, aliquots were removed and sterilized by filtration (0.2 μM). An amount of 50 μL tablet solution was added to each well of a 96-well flat Clear Bottom White Polystyrene TC-treated Microplate (Corning, Glendale, Arizona). 25 μL of 4 × 10⁵ DC/mL was added to each well as antigen presenting cells, combined with 25 μL T-cells (3 × 10⁶ cells/well) from a previously established autologous Bet v-specific T-cell line47 and incubated at 37 °C in 5% carbon dioxide for 3 days. T-cell proliferation was measured using an adenosine triphosphate-based cell assay (CellTiter-Glo 2.0 (cat. no. G9241) Promega, Madison, Wisconsin) according to supplier protocol. In brief, an equal volume of CellTiter-Glo 2.0 reagent was added to each well of cell culture, and the plate was placed on an orbital plate shaker for 2 minutes at 200 RPM followed by incubation for 10 minutes at room temperature. Luminescence was recorded with a GloMax Explorer Multimode Microplate Reader (Promega). Phytomagemglutinin was used as an unspecific assay control. We have found that this method correlates with and can replace conventional 3H-thymine methods for measuring proliferation (unpublished data). Use of this method for lymphocyte cell proliferation assessment was recently reported by others.38

Statistical analyses

Estimation of slope and test for significance were done using ordinary linear regression (Table 1). Comparison of allergen release at individual timepoints were done using a Welch t test. All calculations were made using R software version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

SLIT-tablet disintegration time

When deposited into assay buffer, all 3 freeze-dried fast-dissolving SLIT-tablets disintegrated within 1 second (Table 1). No solid residue could be detected for any of the tablets after disintegration in assay buffer (data not shown).

Ragweed, tree, and Japanese cedar SLIT-tablet dissolution profiles

The freeze-dried SLIT-tablets were deposited into assay buffer under constant mixing and aliquots were removed at 15, 30, 60, 90, 120, and 300 seconds (tree and ragweed SLIT-tablets) or at 30, 45, 60, 90, 120, and 300 seconds (Japanese cedar SLIT-tablets). The amounts of the respective major allergens (Bet v 1, Amb a 1, Cry j 1) released into solution at each time point were determined by ELISA (Fig. 1). The total major allergen contents of each SLIT-tablet were determined in parallel experiments that included prolonged dissolution in assay buffer followed by measurements of released major allergen by ELISA. Complete release of allergen was confirmed by comparison to the manufacturer’s in-house references (data not shown). This 100% allergen release control is shown as dotted lines in Fig. 1. At each experimental time point, the amounts of major allergen released during the dissolution experiment were compared with the total major allergen content (Fig. 1). For all 3 SLIT-tablets, complete allergen release compared with the control was achieved at the first point of measure (15 seconds for tree and ragweed SLIT-tablets and 30 seconds for the Japanese cedar SLIT-tablet), although some experimental variations occurred, especially at the early time points (Fig. 1).

Allergen release kinetics

To establish the kinetic parameters of major allergen release from the 3 freeze-dried SLIT-tablets independently of the controls, the major allergen release data were examined with the amounts
Fig. 1. Sublingual allergy immunotherapy tablet (SLIT-tablet) dissolution. Recovered amounts of soluble major allergens are indicated for each timepoint as % of the control (mean [SD]). (A) Tree SLIT-tablet. (B) Ragweed (RW) SLIT-tablet. (C) Japanese cedar (JC) SLIT-tablet. Tree and RW SLIT-tablets: Ncontrol = 4. Ntimepoint = 4. JC SLIT-tablet: Ncontrol = 3. Ntimepoint = 3. NS = nonsignificant. *P > 0.05.
of released major allergens at the first experimental time points as references. The rapid release of allergen seen for all 3 SLIT-tablet species prevented assessment of the initial release rates due to lack of experimental data before 15 seconds (30 seconds for the Japanese cedar tablet). Instead, allergen release data obtained between 15 seconds (tree and ragweed SLIT-tablets), or 30 seconds (Japanese cedar SLIT-tablet) and 300 seconds were analyzed by linear regression assuming linearity of any residual release occurring after these time points, as suggested by the shapes of the curves in Fig. 1. For the ragweed SLIT-tablet and 3 different batches of the Japanese cedar SLIT-tablet, no statistically significant differences (P > 0.05) between the slopes of the respective regression curves and the ideal regression curve (slope = 0) were seen (Table 2), indicating that no measurable allergen release occurred after the first timepoints (15 and 30 seconds, respectively). For the tree SLIT-tablet, a slight upward trend in allergen release after the initial time point of 15 seconds was seen (Fig. 1A) and the slope of the linear regression curve was determined to be statistically significantly different from 0 (P = 0.011) (Table 2). However, although the calculated slope of the linear regression curve was statistically significantly different from 0, the magnitude of additional allergen release occurring from the 15-second time point was marginal. A calculated slope of the regression line of 2.07 × 10⁻² μg/sec as seen for the tree SLIT-tablet (Table 2) corresponds to a mean calculated release <2% of the SLIT-tablet major allergen content between the 15- and 60-second time points; the 60-second time point corresponding to the recommended sublingual holding time of 1 minute for the tree, ragweed, and Japanese cedar SLIT-tablets. 26–28

T-cell activation

The biological potency of the allergen released at each time point was examined for the tree SLIT-tablet in a T-cell activation assay. Aliquots of solubilized allergen were collected after 30, 60, 90, and 120 seconds in solution and mixed with peripheral blood mononuclear cells-derived DCs and T-cells from an autologous Bet v-specific cell line. After incubation at 37 °C for 3 days, the levels of T-cell proliferation resulting from DC uptake and presentation of solubilized tree SLIT-tablet allergen was quantified by chemiluminescence. Representative data from 1 of 5 donors is shown in Fig. 2. No statistically significant differences in the T-cell activation achieved after 30 seconds and 60, 90, or 120 seconds, respectively, were seen, indicating full immunological activity of the released amounts of Bet v allergens at all time points (Fig. 2).

Discussion

Rapid (15–30 seconds) and complete release of allergen from the 3 freeze-dried SLIT-tablets examined here was supported by 3 lines of experimental evidence. At almost all time points during the SLIT-tablet dissolution experiments, the released amounts of major allergens showed no statistically significant difference compared with the full allergen release controls, in line with results obtained with the freeze-dried HDM and grass SLIT-tablets that are based on the same formulation as the 3 SLIT-tablets examined here. 31,32 An additional approach where the rates of allergen release for each of the 3 SLIT-tablets were determined independently of the 100% major allergen release control also supported that full major allergen release had been achieved for all the SLIT-tablets at the first experimental time points (15 seconds for the tree and ragweed SLIT-tablets and 30 seconds for the Japanese cedar SLIT-tablet). In these analyses, the amounts of allergen released between earliest experimental time points (15 or 30 seconds) and 300 seconds were fitted to a linear regression curve, and the calculated slopes of these lines were for each tablet tested for statistically significant differences from a horizontal line with a slope = 0 (Table 1). For the ragweed and Japanese cedar (3 different batches) SLIT-tablets, the calculated slopes of the liner regression lines were not statistically significantly different from 0, meaning that no additional allergen release from 15 seconds (ragweed) or 30 seconds (Japanese cedar) could be demonstrated. For the tree SLIT-tablet, the slope of the linear regression line turned out statistically significantly different from 0. However, although statistically significantly different, the magnitude of the deviation from 0 of the calculated tree SLIT-tablet allergen release regression line slope was marginal with a maximal calculated residual allergen release <2% between 15 and 60 seconds (60 seconds corresponding to the 1-minute recommended sublingual holding time). 26–28 In combination, the results obtained by comparing SLIT-tablet major allergen release at each time point to the 100% major allergen release controls established in separate experiments and the results obtained by assessing the intra-assay major allergen release kinetics with the earliest time point because the reference shows that rapid and complete major allergen release is achieved with the freeze-dried SLIT-tablet formulation for all 3 species. The T-cell proliferation data obtained with the tree SLIT-tablets provide additional support for full release of Bet v allergen after 30 seconds in solution (30 seconds was the earliest time point in this experiment) and furthermore demonstrate that the amounts of allergen released from the SLIT-tablet at different time points correlate with biological activity.

The data shown here for the ragweed, tree, and Japanese cedar, and elsewhere for HDM 32 and grass pollen 31 freeze-dried SLIT-tablets demonstrated that under the experimental conditions employed, allergen release from the freeze-dried SLIT-tablets were highly reproducible: All 5 freeze-dried SLIT-tablets disintegrated within 1 second when applied to assay buffer and full release of major allergens was achieved within 15 seconds for HDM, grass, tree, and ragweed and within 30 seconds for Japanese cedar, where 30 seconds was the earliest experimental time point for Cry j 1 release quantification.

The concentration of solubilized allergen and the mucosal contact time are believed to be essential parameters for mucosal allergen uptake. 39 Sublingual holding times of 1 to 2 minutes are generally recommended by the SLIT-tablet manufacturers. 26–28,40–42 which emphasizes the need for rapid SLIT-tablet allergen release. When placed under the tongue, SLIT-tablets need to disintegrate and become completely solubilized in a small amount of saliva to deliver the full allergen extract content to the surface of the oral mucosa. Consequently, SLIT-tablet dosing not only depends on the amounts of allergen contained in the tablet but also on the properties of the tablet formulation. 43 Rapid and complete release of allergen extracts from the freeze-dried SLIT-tablets immediately after sublingual administration will ensure both the highest concentration and maximal contact time between the solubilized allergen extract and the sublingual mucosa within the recommended sublingual holding time. 32 Prolonging the sublingual holding time to extend the mucosal contact time may seem an attractive option for increasing mucosal allergen exposure, but due to continuous saliva production that occurs throughout the sublingual holding
period, the allergen concentration will be rapidly reduced, a process known as saliva washout,\textsuperscript{34} which is likely to have a negative influence on allergen uptake efficiency due to dilution.\textsuperscript{39}

Controlled and efficient delivery of pharmaceutical drugs to the target organ in the intended dose and with an acceptable benefit-to-risk ratio is a key element in drug development. In addition to actual drug delivery performances, particular drug formulations and administration forms may be preferred by individual patients or patient groups on the grounds of more subjective parameters such as convenience, fear of needles, and stigmatization. Choosing the right formulation and administration form for a given drug may lead to higher patient compliance and overall satisfaction with the treatment.

According to Food and Drug Administration guidance, orally dispersing/orodispersible tablets (ODTs) should disintegrate within 30 seconds or less in saliva without the need for chewing or intake with water.\textsuperscript{45} Advantages offered by the formulation of pharmaceutical drugs in the form of ODTs include quick absorption of the drug, rapid onset of action, convenience, and increased patient compliance.\textsuperscript{46,47} ODTs are generally well suited for pediatric use due to factors like noninvasive administration and ease of administration.\textsuperscript{48} Within the pediatric segment, it is important to define and use the most appropriate dose form according to age, and in a recent survey, a preference for ODTs among children and adolescents was seen.\textsuperscript{49} In an European Medicines Agency reflection paper based on clinical practice and the experience of hospital pediatricians and parents, ODTs with rapid dispersion were considered “the preferred dosage form” for children aged 2 to 5 years and “the dosage form of choice” for children aged 6 to 11 years.\textsuperscript{50} In AIT, where the more conventional regimens of repeated subcutaneous injections may be considered invasive and resource-demanding for both children and parents/caregivers, fast-dissolving SLIT-tablets provide a treatment option with daily self-administration after the first dose has been taken under medical supervision.\textsuperscript{10,11,18,20}

Conclusions

The tree, ragweed, and Japanese cedar freeze-dried SLIT-tablets were shown to provide rapid and complete allergen release in vitro in a highly reproducible manner that, together with previously published reports, demonstrate that the allergen-release properties of the freeze-dried SLIT-tablet formulation are reproducible across a wide range of allergen sources. These properties support short sublingual holding times for the freeze-dried SLIT-tablets and offer an administration form that may be particularly well suited for pediatric use where adherence to prolonged sublingual holding times can be challenging.

Acknowledgments

This work was funded by ALK-Abelló A/S, Hørsholm, Denmark, who took part in the design of the study, data collection, data analysis, data interpretation, provided study materials, and funded medical writing services.

K. Ohashi-Doi, S. Lawton, and K. Lund contributed with experimental design and data analyses. T. Yamamoto. L. Verhoog, and H. Matsuhashara contributed with experimental data acquisition and data analyses. M. Lindholm provided statistical data analyses. All authors contributed to the preparation and review of the
manuscript and approved the content of the final article and the decision to submit.

Conflicts of Interest

T. Yamamoto, K. Ohashi-Doi, and H. Matsuhara are employees of Torii Pharmaceutical Co. Ltd. S. Lawton and M. Lindholm are employees of ALK-Abelló A/S. K. Lund is consultant to ALK-Abelló A/S and Torii Pharmaceutical Co. Ltd. S. Lawton and K. Lund are holders of ALK-Abelló A/S. The authors have indicated that they have no other conflicts of interest regarding the content of this article.

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