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# Evaluating the Suitability of Using Rat Models for Preclinical Efficacy and Side Effects with Inhaled Corticosteroids Nanosuspension Formulations

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**Abstract** Inhaled corticosteroids (ICS) are often prescribed as first-line therapy for patients with asthma Despite their efficacy and improved safety profile compared with oral corticosteroids, the potential for systemic side effects continues to cause concern. In order to reduce the potential for systemic side effects, the pharmaceutical industry has begun efforts to generate new drugs with pulmonary-targeted topical efficacy. One of the major challenges of this approach is to differentiate both efficacy and side effects (pulmonary vs. systemic) in a preclinical animal model. In this study, fluticasone and ciclesonide were used as tool compounds to explore the possibility of demonstrating both efficacy and side effects in a rat model using pulmonary delivery via intratracheal (IT) instillation with nanosuspension formulations. The inhibition of neutrophil infiltration into bronchoalveolar lavage fluid (BALF) and cytokine (TNFα) production were utilized to assess pulmonary efficacy, while adrenal and thymus involution as well as plasma corticosterone suppression was measured to assess systemic side effects. Based on neutrophil infiltration and cytokine production data, the ED50s for ciclesonide and fluticasone were calculated to be 0.1 and 0.03 mg, respectively. At the ED50, the average adrenal involution was 7.6 ± 5.3% for ciclesonide versus 16.6 ± 5.1% for fluticasone, while the average thymus involution was 41.0 ± 4.3% for ciclesonide versus 59.5 ± 5.8% for fluticasone. However, the differentiation became less significant when the dose was pushed to the EDmax (0.3 mg for ciclesonide, 0.1 mg for fluticasone). Overall, the efficacy and side effect profiles of the two compounds exhibited differentiation at low to mid doses (0.03–0.1 mg ciclesonide, 0.01–0.03 mg fluticasone), while this differentiation diminished at the maximum efficacious dose (0.3 mg ciclesonide, 0.1 mg fluticasone), likely due to overdosing in this model. We conclude that the rat LPS model using IT administration of nanosuspensions of ICS is a useful tool to demonstrate pulmonary-targeted efficacy and to differentiate the side effects. However, it is only suitable at sub-maximum efficacious levels.

**Keywords** Inhale · Glucocorticoids · Inflammation · Nanosuspension · Safety · In vivo

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## Introduction

Pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) and asthma, are complex human airway diseases, which affect millions of people worldwide. Despite their complexity, it is well understood that human airway diseases are often associated with local (lung) inflammation. The incidence of pulmonary diseases appears to be growing worldwide. For example, according to a report from the US Centers for Disease Control and Prevention (CDC), greater than 6% of total American population suffered from asthma in 2004, up from a little over 3% in 1980. For patients with asthma, inhaled corticosteroids (ICS) are often prescribed as first-line therapy to control symptoms, improve lung function, and reduce morbidity and mortality [1]. Among these patients with asthma, 5–10% are characterized as having severe disease that do not adequately respond to current therapeutic options, in part because of side effects associated with

elevated doses and/or a plateau in dose response. Treatment options for severe asthma are oral steroids (e.g., prednisone) or a high dose of an ICS. However, long-term use of oral steroids or high-dose ICS therapy has the potential to cause a number of severe side effects, including impaired growth in children, decreased bone mineral density, cataracts, skin thinning and bruising, altered glucose metabolism, and hypothalamic–pituitary–adrenal (HPA) axis suppression [1–5]. Numerous studies have demonstrated that the side effects of glucocorticoid therapy for human airway diseases are related to systemic exposure. Most importantly, side effects are mediated by the glucocorticoid receptor in both the lung and systemic tissues [6–9]. Because of this, pulmonary targeting, such as inhaled delivery, is believed to provide an advantage over systemically administered compound (IV or oral) because the same degree of efficacy may be achieved using a lower dose of inhaled drug. However, despite the success of using an inhaler for pulmonary administration, similar side effects still remain for ICS, especially when the doses are escalated. This raises a question with regard to what portion of the efficacy observed with inhaled ICS is related to local pulmonary exposure and what portion of the efficacy is from systemic exposure. Thus, improved discernment of pulmonary vs. systemic efficacy remains a key element to the development of new drugs with better safety profiles.

One key concept for reducing systemic side effects via pulmonary drug delivery is to select drug candidates with prolonged pulmonary efficacy and minimal systemic exposure [7, 9]. It is believed that a drug with durable, pulmonary-targeted activity, and low systemic exposure would have a theoretical advantage over currently marketed therapies. Pharmacokinetic/pharmacodynamic (PK/PD) modeling suggested that pulmonary targeting might be achievable via modification of the pharmacokinetic profile [10]. Pharmacokinetic (PK) parameters such as long lung retention, high lung deposition, high receptor binding, and high lipophilicity have been sought to improve or maintain the pulmonary-targeted efficacy [11]. In addition, appropriate physicochemical properties (i.e., dissolution rate, solid state form), particle size, and formulation can be utilized to further optimize the PK profile. Drugs with the aforementioned profile should provide the benefit of greater pulmonary exposures with reduced systemic exposure, ultimately resulting in an improved therapeutic index, assuming that efficacy is not driven by systemic drug exposure [10, 12, 13].

Despite an understanding of what is needed, a major hurdle for pulmonary drug discovery is to assess therapeutic index (topic effects vs. systemic effects) with an appropriate preclinical animal model(s). To date, appropriate animal models have not been fully characterized in the literature. In an attempt to characterize pulmonary vs. systemic side effects preclinically, we chose the acute lipopolysaccharide (LPS)-induced inflammation model in rats as an efficacy model and also to set doses for multiple-dose side effect studies. This model utilizes the recruitment and activation of neutrophils into bronchial alveolar lavage fluid (BALF) as the efficacy endpoint. This model was selected for the study because it provides two distinctive advantages. First, this acute animal model has been well studied by researchers and used to mimic human pulmonary inflammation [14–18]. Secondly, compared with other animal models such as the mouse ovalbumin model, the rat LPS model offers the advantage of serial blood sampling and more precise delivery of drug into the lung via intratracheal dosing. We chose two ICS compounds to evaluate in this rat LPS model—fluticasone propionate and ciclesonide.

Fluticasone is a highly potent anti-inflammatory drug that is the most commonly prescribed inhaled glucocorticoid. It is one of the available ICS with a good combination of PK and PD properties. It has high receptor binding affinity, high clearance ($\sim$liver blood flow), poor bioavailability (<1%), high protein binding, and it has been used effectively at low to medium doses to treat patients with mild and moderate asthma. However, fluticasone is associated with adverse systemic effects at high doses and is therefore administered twice daily.

Ciclesonide has been reported to have similar efficacy to fluticasone but fewer side effects due to its special drug design. Ciclesonide is a prodrug that is converted to an active metabolite, desisobutyryl-ciclesonide (des-CIC), in pulmonary airways by endogenous esterases. This onsite activation reduces oropharyngeal exposure and subsequent side effects. In radioligand binding assays, des-CIC and fluticasone exhibited similar high-affinity binding to the glucocorticoid receptor, whereas ciclesonide exhibited 100-fold less binding affinity than fluticasone [19]. Furthermore, des-CIC undergoes reversible esterification to fatty acid conjugates in the lung. These conjugates slowly re-release des-CIC and act to greatly enhance lung retention which should provide more topical efficacy and less systemic side effects. Once in the systemic circulation, des-CIC is rapidly metabolized by P-450 enzymes, mainly CYP3A4 [19]. It has been claimed that ciclesonide has a better safety profile compared to other ICS. For example, Belvisi et al. [19] showed that in preclinical models of antigen-induced airway eosinophilia and Sephadex-induced lung edema using Brown Norway rats, ciclesonide showed comparable efficacy with fluticasone, although ciclesonide was 7–9-fold less potent in terms of ED50. In a subsequent 7-day side effect study with Sprague–Dawley rats, ciclesonide was 44-fold less potent at inducing adrenal involution, sixfold less potent at inducing thymus involution, and 22-fold less potent at decreasing bone growth than fluticasone [19].

The goal of the present study is to evaluate whether efficacy and side effect profiles can be differentiated preclinically in conjunction with systemic exposure by utilizing nanosuspension formulation. These findings will help to determine whether a simple and robust preclinical model can be established that is useful for screening of new ICS drug candidates. A nanosuspension drug delivery formulation was used to administer fluticasone and ciclesonide intratracheally (IT) to rats. Recently, utilization of nano drug delivery for both efficacy and safety evaluation has drawn lots of attentions from researchers, and its advantages were widely accepted by industry [20–26]. In our study, the acute LPS rat model was used to establish the dose–response curves for efficacy. Based on these data, doses were picked for 6-day repeat dose studies for the evaluation of the side effects. Adrenal and thymus involution as well as lung and heart tissue receptor occupancy was measured to assess side effects. Corticosterone levels in whole blood were measured for biomarker evaluation [19].

## Materials and Methods

### Materials

LPS (*E. coli* O111: B4) was purchased from Sigma–Aldrich (St Louis, MO) and prepared in phosphate-buffered saline solution (PBS). Fluticasone propionate was purchased from Sequoia Research Products (Oxford, UK) while ciclesonide was prepared in house. Microtainer tubes with lithium heparin for blood collection were purchased from Becton–Dickinson Biosciences (Franklin Lakes, NJ). Ninety-six-well polypropylene plates were purchased from Corning Inc.(Corning, NY). The Pari Proneb ultra compression nebulizer system was purchased from Pari Co. (Midlothian, VA), and Hamilton dosing needles (IT) and syringes were purchased from Hamilton Co. (Reno, NV). The ammonium chloride buffer was purchased from Stem Cell Technologies (Vancouver, BC). The FACS Calibur flow cytometer was purchased from Becton–Dickinson Biosciences (San Jose, CA) while the coupled 96-well sampler that determined the absolute cell counts (cells/μL) was from Cytek Development (Freemont, CA). The cytometry-based cell count was validated against a Beckman Coulter Z2 cell counter (Miami, FL). All analysis was done using FlowJo flow cytometry software from Treestar (Ashland OR). The electroplated 96-well plates custom coated with anti-rat TNFα antibody, Read buffer T (150 μL/well, 2×), and the Sector Imager 6000 were purchased from Meso Scale Discovery (Gaithersburg, MD). Rat recombinant TNFα standards were purchased from Linco Research (St. Charles, MO). Tris wash buffer was purchased from BioRad (Hercules, CA). HPLC-grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI), and reagent-grade formic acid and sodium hydroxide were obtained from EM Science (Gibbstown. N J). The HPLC system used for formulation potency check was an Agilent HP 1100 HPLC equipped with diode array (DAD) and variable wavelength UV (VWD) detectors and a quarternary solvent delivery system (Palo Alto, CA). An Applied Biosystems Sciex API 4000 mass spectrometer (Foster City, CA) coupled with HPLC was used for plasma drug analysis and quantification. Powder X-ray diffraction (PXRD) was done on a Bruker D-8 Advance diffractometer for all the solid state work to confirm no form changes. A scintillation counter was used for detection. In house fabricated aluminum inserts or inserts with a Hasteloy sintered filter (0.45 μm) pressed in the center and held in Bruker plastic sample cup holders were utilized for all analyses. The water purification system was a Millipore milli-Q system. All other chemicals were obtained from Sigma–Aldrich (St. Louis, MO) and were used without further purification.

### Formulation

To make a nanosuspension formulation of fluticasone, a bench scale wet milling (micronization) device was used [20, 21] with an appropriate amount of glass beads. Tween 80 (0.5%, w/w) in phosphate-buffered saline (pH 7.4) was added in a scintillation vial. The mixture was stirred at 1200 rpm for a period of 24 h with occasional shaking. The stock formulation was then harvested, and potency was checked by HPLC/DAD, and solid state was checked by powder X-ray diffraction (PXRD). Thermal gravimetric analysis with simultaneous differential thermal analysis (TGA/SDTA) was done on a Mettler TGA/SDTA851e. Particle size distribution was determined on a Beckman Coulter LS 230 particle size analyzer using the small-volume accessory (Miami, FL). A PIDS obscuration water optical model was employed. Particle size distribution was computed by the software using Mie scattering theory. Potency, homogeneity, chemical stability, and solid-state stability were performed following the same procedure listed previously. Control samples were prepared by following the same milling procedure and using vehicle only to serve as baseline for nanosuspension.

### In Vivo Studies

The Pfizer Institutional Animal Care and Use Committee (IACUC) reviewed and approved the animal use in these studies. The Association for Assessment and Accreditation of Laboratory Animal Care International fully accredits the Pfizer animal care and use program.

*Intratracheal (IT) Dose Administration*

Male Sprague–Dawley rats (300–400 g, Charles Rivers Laboratories, Wilmington, MA) were anesthetized with 4–5% isoflurane anesthesia for dose administration. Intratracheal (IT) dose administration was performed using an otoscope to view vocal cords and trachea. A Hamilton dosing needle was inserted through the larynx into the trachea, and a Hamilton syringe (250 μL) was used to inject 100 μL dosing volume directly into the trachea. Using this technique, virtually 100% of the dosing solution is delivered directly to the lung.

*LPS Aerosol Challenge*

One hour post-dosing (fluticasone or vehicle control), rats were placed into a chamber (12 × 12 × 16 inch; 3 outlet holes, one inlet, fabricated at Pfizer St. Louis) and connected to a Pari Proneb Ultra compression nebulizer. The nebulizer cup was filled with 5 mL of a 1 mg/mL solution of LPS dissolved in pH 7.4 phosphate-buffered saline (PBS) or PBS alone for control. Total exposure time in the chamber was 30 min. After active aerosolization for 15 min, the nebulizer was then turned off, the chamber inlet and outlets were plugged, and the rats remained in the chamber for another 15 min to breathe the remaining aerosolized solution. In order to equalize variability, 10–12 rats representing each study group were challenged in the aerosolizing chamber at a time.

*BALF Collection and Differential Cell Counts*

Four hours after aerosol challenge, rats were terminally anesthetized with an intraperitoneal (IP) injection of 100 mg/kg pentobarbital and bled via the vena cava. With rats in the supine position, the throat and trachea were incised and a cannula (14ga) was inserted into the trachea. The cannula was tied to the trachea with suture, and 2.5 mL of 2.6 mM EDTA in PBS was instilled into the lungs. The lavage was recovered immediately after instillation. This was repeated three additional times for a combined 4 instillations totaling 10 mL. The total fluid recovered per subject varied between 6–8 mL. BALF was collected into a 15-mL conical tube on ice. Ninety-six-well polypropylene plates containing 200 μL of BALF cells were centrifuged at 1800 RPM at 5°C for 3 min. The supernatant was removed by inversion and blotting. The plates were gently vortexed and resuspended in 200 μL of ammonium chloride buffer to lyse red blood cells, and the plates were incubated for 5–10 min at room temperature. Plates were centrifuged, supernatant removed, and blotted as earlier. Following vortexing, cells were re-suspended in 180 μL/well of flow cytometry buffer ($Ca^{+2}$/$Mg^{+2}$ free Dulbecco's PBS, 0.1% bovine serum albumin), then 0.1% sodium azide was added, and the final volume was brought to 200 μL. An aliquot of 70 μL was diluted to 280 μL (1:4) with flow cytometry buffer prior to analysis if running immediately or 2% formaldehyde if stored overnight at 4°C. Cell suspensions were analyzed by flow cytometry on a FACS Calibur flow cytometer. Monocytes were identified based on elevated autofluorescence at 525 nM using FL1 detector, forward and 90° light scatter. The remaining cells were identified as lymphocytes or granulocytes based on forward and 90° scatter. All analyses were done using FlowJo flow cytometry software. This approach was initially validated against microscope-counted differentials prepared using a cytospin centrifuge (Shandon, Waltham, MA).

*Six-Day Repeat Dose Side Effect Study*

Male Sprague–Dawley rats were randomized into the following 7 treatment groups (10 subjects per group)—vehicle, ciclesonide (30, 100, and 300 μg/day), and fluticasone (10, 30 and 100 μg/day). For each drug dosing group, animals were dosed IT once daily with 100 μL nanosuspension formulations to deliver the desired doses for a period of 6 days (total 6 doses). For the vehicle control group, animal were dosed IT s.i.d with 100 μL with vehicle control (described in formulation section) for the same period. Animals were terminally anesthetized on day 6 at 2 h following the final dose. Blood samples were collected for the determination of drug and corticosterone levels by LC/MS/MS. Adrenal, thymus, heart, and lung from each animal were extracted following a standard protocol. The adrenal and thymus were used for the measurement of organ involution, while heart and lung tissues were used to assess the GR receptor occupancy.

*Blood Collection and Ex Vivo LPS Challenge Studies (Ex vivo Whole Blood Assay)*

Whole blood samples from the repeat dose side effect studies were collected in microtainer tubes containing lithium heparin via the vena cava prior to BALF collection or at the same time interval [21]. Blood for PK analysis was collected in microtainer plasma separator tubes containing lithium heparin via retro-orbital bleeds. For the ex vivo LPS challenge study, whole blood from each animal was plated in triplicate in 96-well plates (175 μL/well) and stimulated with LPS (10 μg/mL) for 16 h. Post stimulation, plasma was collected following centrifugation to measure TNF-α production. For the cytokine measurement, TNFα levels were determined using electroplated 96-well plates custom coated with anti-rat TNFα antibody. Plates were shaken at room temperature for 5 min, left to rest overnight

at 4°C, and then washed three times with 100 μL/well of Tris wash buffer (BioRad, Hercules, CA). Sulfo-tagged cytokine detection antibody (20 μL/well) was added at 1 μg/mL, the plates sealed, incubated at room temperature with gentle shaking for 60 min, and then washed three times as above. Read buffer T was then added, and the cytokine levels were quantified using a Sector Imager 6000. The 100% control value was defined in the presence of LPS stimulation and the 0% control reflected basal release. Data were combined from at least 2 independent experiments. Results are expressed as a mean ± S.E.M.

### Pharmacokinetic Sample Analysis

For all samples, plasma concentrations of fluticasone, ciclesonide, des-ciclesonide, and corticosterone were determined by LC/MS/MS on a Sciex API 4000 mass spectrometer in positive electrospray mode and MRM transitions. The analysis system comprised a triple-quadrupole mass spectrometer (API4000, Applied Biosystems, Foster City, CA) with an atmospheric pressure electrospray ionization source (MDS SCIEX, Concord, Ontario, Canada) and 2 pumps with a controller (LC-10ADvp, Shimadzu, Columbia, MD). A 10-μL sample of homogenized tissue or plasma was injected onto an Altima-C18 column (2.1 × 50 mm; 3.0 μm; Alltech, Deerfield, IL) and eluted by a mobile phase with initial conditions of 10% solvent B for 1 min followed by a gradient of 10% solvent B to 100% solvent B over 2 min (solvent A: 95% $H_2O$–5% acetonitrile with 0.1% formic acid; solvent B: 100% acetonitrile with 0.1% formic acid); 100% solvent B then was held for 1 min, followed by an immediate return to initial conditions and maintained for 1 min, with a flow rate of 0.4 mL/min. Using the positive-ion mode, protonated molecules were formed by an ion-spray voltage of 5000 V, source temperature of 400°, and an entrance potential of 10 eV. The declustering potential, collision energy, collision cell exit potential, and MRM mass transition for each key analyte are listed in Table 1. The peak areas of all the analytes, standards, blanks, and internal standard were quantified using Analyst 1.4.1 (MDS SCIEX, Ontario, Canada). For sample preparation in general, 50 μL of plasma was extracted with 150 μL of acetonitrile containing 0.05 μM of the internal standard (made in house). Non-compartmental Pharmacokinetic analysis was performed using Watson 7.2 Bioanalytical LIMS system by Thermo Electron Corporation (Waltham, MA). Limit of detection (LOD) was 0.00015 μg/mL and limit of quantification (LOQ) was 0.0006 μg/mL for fluticasone, ciclesonide, and corticosterone and LOQ for the des-ciclesonide was 0.0000381 μg/mL.

## Results and Discussion

### Formulation and Particle Size

The micronization of fluticasone and ciclesonide was successfully achieved. For fluticasone, the particle size of the bulk material was reduced from a D50 of 35 μm to 0.24 μm (Fig. 1). For ciclesonide, the particle size of the bulk material was reduced from a D50 of 56 μm to 0.22 μm (Fig. 2). The smaller particle size allows the nanosuspension to achieve much better content uniformity. This is especially critical for lower doses since most of the doses will be only a few hundred micrograms in a very limited dosing volume. The solid form of each of the micronized materials was examined by PXRD to ensure the crystallinity post micronization process, which assured the quality of material used for further studies. Control samples (milled vehicle) were clean without glass shards

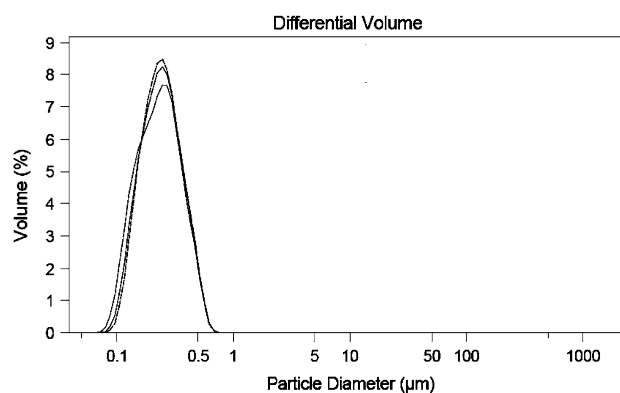


**Fig. 1** Fluticasone nano particles

**Table 1** Mass spectrometer parameters in quantitation of ICS

| | Declustering potential (ev) | Collision energy (ev) | Collision cell exit potential (ev) | Q1/Q3 transion (m/z) |
|---|---|---|---|---|
| Fluticasone | 91 | 17 | 16 | 500.18/313.04 |
| Ciclesonide | 96 | 23 | 24 | 541.26/323.20 |
| Des-CIC | 86 | 21 | 24 | 471.20/323.03 |
| Corticosterone | 81 | 25 | 10 | 347.55/135.81 |

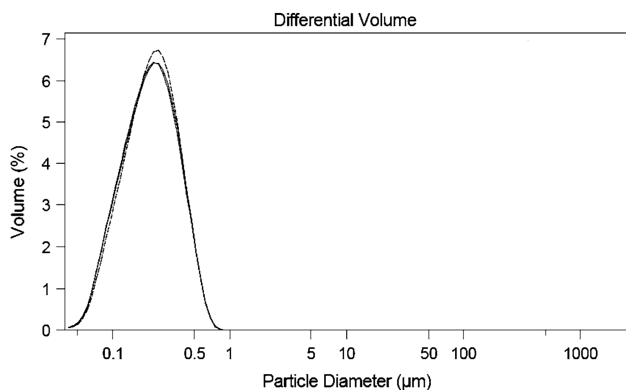


**Fig. 2** Ciclesonide nano particles

observed. The control sample was dosed intratracheally to serve as the vehicle baseline for the nanosuspension. No differences were observed between in vivo and ex vivo values when control samples or direct vehicle was used.

## Preclinical Model and Efficacy

In the efficacy study, nanosuspension formulations were used to deliver both fluticasone and ciclesonide (3, 10, 30, 100, and 300 µg) prior to LPS challenge in Sprague–Dawley rats ($N \geq 6$). The doses were chosen to cover the expected full dose–response range and were based on both literature and preliminary in house data. The efficacy was measured as the inhibition of neutrophil infiltration or TNFα production in BALF following LPS challenge (Fig. 3). Dose-dependent inhibition was observed with the maximum effect at 100 µg for fluticasone and 300 µg for ciclesonide, respectively. The $ED_{50}$ of the fluticasone in the LPS assay was 30 µg, while the $ED_{50}$ of the ciclesonide was 100 µg which was about threefold less potent than fluticasone. This finding was not surprising since similar results have been reported using a Brown Norway rat model of antigen-induced airway inflammation with fluticasone and ciclesonide [19]. In that study, antigen-induced influx of eosinophils in airway BALF and lung tissue were inhibited by IT-administered ciclesonide and fluticasone in a dose-dependent manner. ED50s were calculated as 0.068 mg/kg for fluticasone and 0.49 mg/kg for ciclesonide, which are comparable to our results when expressed as dose relative to body weight. In a separate study using the same preclinical model, ciclesonide exhibited an ED50 of 0.5 mg/kg in the inhibition of accumulation of eosinophils in BALF [27]. These results demonstrated that the acute rat LPS challenge model is a fast and reliable assay and therefore useful in a preclinical setting to assess the efficacy of inhaled glucocorticoids in vivo.

## Preclinical Model and Side Effects

The efficacy data were used to set doses for the 6-day repeat dose side effect study in rats. For fluticasone, the compound was dosed at 10, 30, and 100 µg/rat/day (IT, once daily). Similarly, ciclesonide doses were set at 30, 100, and 300 µg/rat/day (IT, once daily). The results from the repeat dose studies were used to compare the side effect profiles of the two drugs. With respect to the effects on the adrenal gland at equally efficacious doses (ED50), ciclesonide had less severe side effects than fluticasone. For example, at the ED50 dose (30 µg for fluticasone versus 100 µg for ciclesonide), the average adrenal involution for ciclesonide was 7.6 ± 5.3% versus 16.6 ± 5.1% for fluticasone (Fig. 4). However, the differentiation became non-significant when the dose was pushed to the EDmax (100 µg for fluticasone and 300 µg for ciclesonide). Similar results were observed for thymus involution (Fig. 5). At the ED50 dose, the average thymus involution was 41.0 ± 4.3% for the ciclesonide group (30 µg) versus

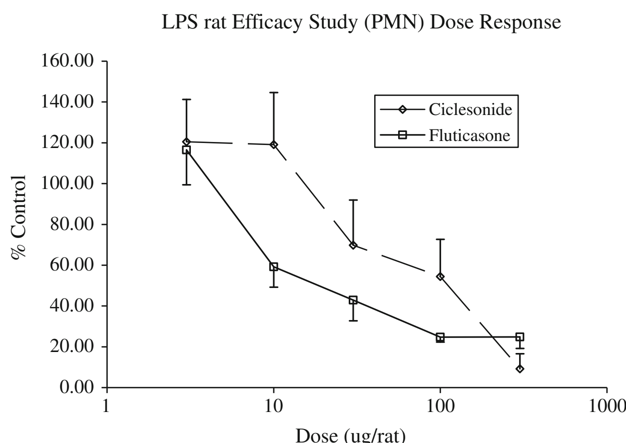


**Fig. 3** LPS rat efficacy data ($N > 6$ for each group and presented with *error bar* as standard deviation)

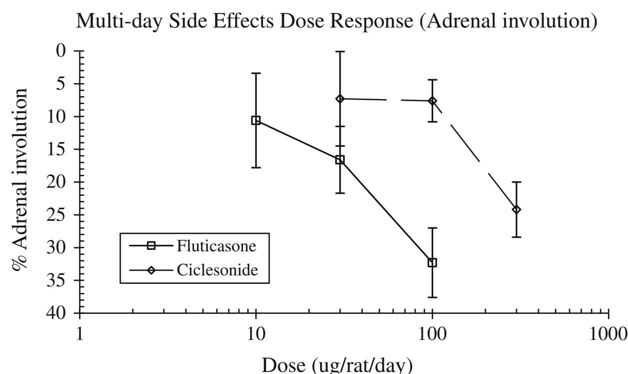


**Fig. 4** Multi-day side effects (adrenal involution) dose response ($N > 6$ for each group and presented with *error bar* as standard deviation)

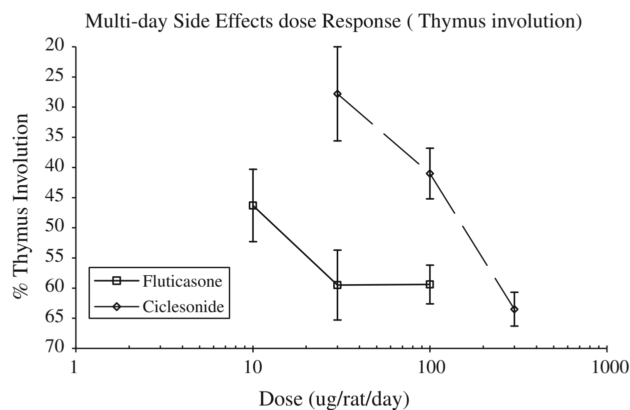


**Fig. 5** Multi-day side effects (thymus involution) dose response ($N > 6$ for each group and presented with *error bar* as standard deviation)

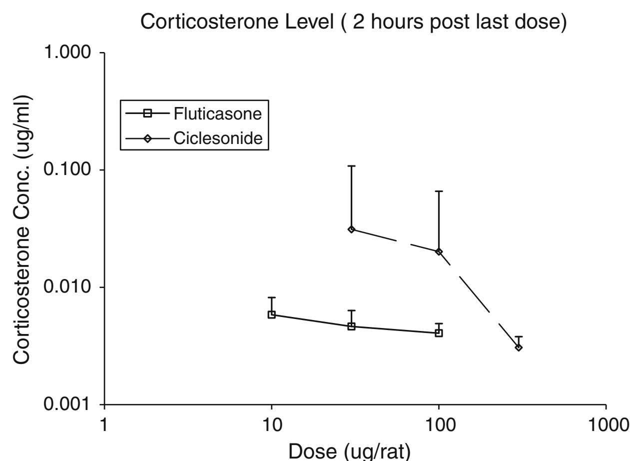


**Fig. 6** Multi-day side effects study ($N = 10$ for each group, presented with *error bar* as standard deviation) Corticosterone level (2 h post the last dose)

59.5 ± 5.8% for the fluticasone group (100 μg), while at the EDmax the effect on thymus weight for both compounds was similar.

These results are in accord with published data from a 7-day study in Sprague–Dawley rats (IT administration) designed to assess side effects with fluticasone and ciclesonide [19]. In this study, both compounds produced dose-dependent reductions in adrenal and thymus weights. Fluticasone was approximately 44-fold more potent at inducing adrenal involution and sixfold more potent at inducing thymus involution compared to ciclesonide on the basis of dose. However, a more relevant comparison takes into account the anti-inflammatory potency of each compound. In this study, the $ED_{50}$s for the inhibition of eosinophil infiltration into BALF were 0.095 and 0.75 mg/kg for fluticasone and ciclesonide, respectively. At doses producing approximately equivalent efficacy, separation could still be observed in the effects on adrenal weight, whereas at the highest dose no separation was apparent.

In the current study, decreases in plasma corticosterone levels (indicative of HPA axis suppression) with the two compounds correlated with the effects on organ involution (Fig. 6). At the $ED_{50}$ doses, animals treated with 30 μg fluticasone had an approximately 10-fold lower level of plasma corticosterone than did animals treated with 100 μg ciclesonide, while at the EDmax doses corticosterone levels were similar. Corticosterone levels were measured 2 h following the last dose.

Leung et al. [28] has reported that fluticasone significantly suppressed plasma corticosterone levels at 0.1 mg/kg, compared to ciclesonide which did not change corticosterone levels when dosed in a range of 0.01 to 0.1 mg/kg/day in a 28-day allergen-induced rat airway inflammation model. At these doses, ciclesonide did not have any effect on HPA axis suppression.

Plasma exposure data obtained from the current study were in good agreement with in house historical data (Fig. 7). Dose-dependent increases in plasma concentration were observed for fluticasone, ciclesonide, and its metabolite, des-CIC. In general, the side effect profile for fluticasone in this study correlated well with systemic exposure. For ciclesonide, however, the side effect profile had a better correlation to the systemic exposure level of des-CIC. This finding is not a surprise, since des-CIC is more potent than the parent drug, ciclesonide. Interestingly, while comparing the exposure of des-CIC at the ED50 dose of ciclesonide (100 μg/rat/day) versus fluticasone at its ED50 dose (30 μg/rat/day), we observed that the level of des-CIC in the systemic circulation was much higher than fluticasone, in spite of the fact that ciclesonide had a lower side effect profile. However, this differentiation disappeared when the EDmax doses were analyzed. At the EDmax doses, the plasma concentrations of fluticasone and des-CIC were very similar and no differentiation between the side effect profiles of the two drugs was observed at the high doses. The efficacy margin based on the ratio of ED50 between ciclesonide and fluticasone is approximately threefold (100 μg/30 μg), while the side effect profile based on the systemic exposure is greater than sixfold (plasmas exposure of des-CIC at Ed50 dose was 0.00375 μg/mL and fluticasone was 0.00056 μg/mL). Considering that des-CIC and fluticasone exhibited similar high-affinity binding to the glucocorticoid receptor [19], this differentiation of side effects is very significant. One hypothesis is that the differentiation at the ED50 doses may be influenced, in part, by the difference in plasma protein binding of ciclesonide and des-CIC (>99%) [29] versus fluticasone (90%) that may result in lower free drug concentrations and subsequent better side effect profile. This

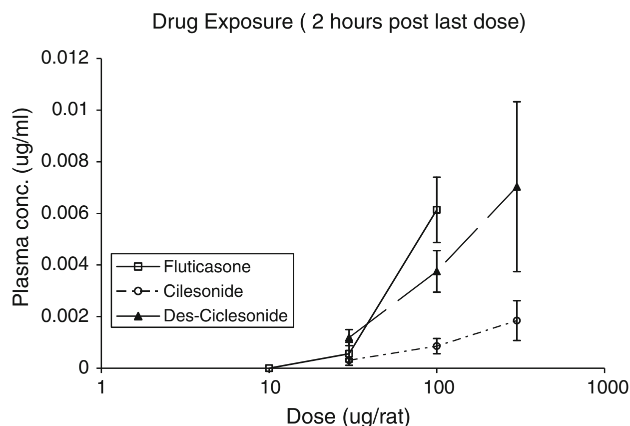


**Fig. 7** Multi-day side effects study ($N = 10$ for each group, presented with *error bar* as standard deviation) plasma drug exposures (2 h post the last dose)

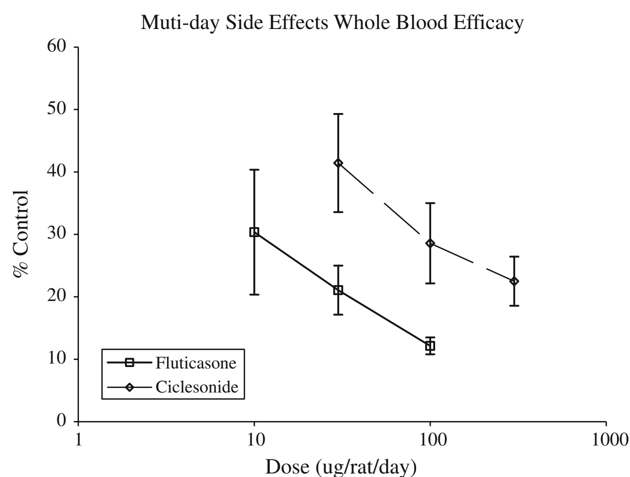


**Fig. 8** Multi-day side effects study ($N = 10$ for each group, presented with error bar as standard deviation) whole blood assay

advantage disappeared when higher doses were given where the drug concentration increased to saturate the glucocorticoid receptor. The systemic effect was measured by TNFα reduction in whole blood as a surrogate marker following ex vivo LPS challenge (hereafter referred to as ex vivo WB). The finding was repeatable when the ex vivo WB assay results (Fig. 8) were compared with side effect profile where clear differentiation was found at lower doses.

It is also possible that the differentiation at lower doses is driven by PK differences in the lung where des-CIC was designed to conjugate with fatty acids. These conjugates in turn slowly re-release des-CIC in pulmonary tissue with the overall effect being enhanced lung retention of the active metabolite. This might provide improved efficacy in the lung whereas the systemic side effects would be primarily driven by free drug concentrations in plasma.

Based on our data, we conclude that the IT-dosed rat LPS-induced inflammation model, in combination with fluticasone and ciclesonide nanosuspensions, is a useful tool to demonstrate pulmonary-targeted efficacy and to differentiate the side effects. The differentiation in side effect profiles, however, was only observed at sub-maximum doses. At higher, fully efficacious doses, the side effect profiles could not be differentiated between the two drugs. There are several possible causes of why the model failed to show differentiation at higher doses. First of all, it is believed that the glucocorticoid receptors in lung and systemic tissues are responsible for therapeutic benefit and side effects, respectively. Likely, the inhibition of neutrophil infiltration in response to LPS challenge is not sensitive enough (PMN assay), which requires higher drug concentration than needed in clinical to achieve effectiveness [20]. As a result, the effective IT dose preclinically results in a higher systemic exposure which explains the systemic inhibition (whole blood assay). Furthermore, the in vivo PK/PD of fluticasone and des-CIC may be different which could make impacts on the read out. The second possibility is that because the rat LPS model is an acute animal model with stimulated inflammation, a relative large amount of drug is needed to offset this robust inflammatory response in order to achieve full efficacy in this animal model (EDmax). It is highly likely that both drugs were "over dosed" at EDmax compare with actual human dose associated with side effects [30] which resulted in no differentiation in the side effect profile. Another possibility is that the duration of the side effect study was not sufficient to give a full picture of long-term usages of ICS. A longer study may be necessary to further understand the differentiation observed at ED50 doses. Overall, we conclude that determining the individual and concomitant effects of inhaled steroids has proven to be very challenging in preclinical models. This combination may only be suitable to test drug candidates for side effect profiles at partially efficacious levels but not the maximum efficacious levels. At higher dose, this combination failed to demonstrate differentiation of safety and efficacy at ED max which may limits the usage and potential. Extra caution should be taken when using ED50's from rat LPS model for efficacy and safety evaluation for inhale ICS with regard to the outcome, most importantly, the translatability to human.

## Conclusion

One of the most important qualifiers in searching for better drug candidates for inhalation is to reduce systemic side

effects while maintaining efficacy. It is believed that a drug with durable, lung-targeted activity and low systemic exposure would have a theoretical advantage over current therapies with improved therapeutic index, allowing for better pulmonary-targeting effect and minimized systemic effects.

In drug discovery, it is very important to demonstrate preclinical pulmonary-targeted efficacy vs. safety in animal model. A popular and convenient acute rat LPS model was tested using fluticasone and ciclesonide as model compounds with nanosuspension formulation. The results from the LPS rat study were used for a rat multi-day side effect study to evaluate whether differentiation of side effects can be observed. We conclude that the efficacy and side effect profile could be differentiate only at low doses. However, this differentiation diminishes at the maximum efficacious dose. Combination of fluticasone and ciclesonide nanosuspensions and the rat LPS model could be utilized to differentiate pulmonary-targeted efficacy and systemic side effects. However, it is only suitable at sub-maximum efficacious levels. Further investigations into finding a suitable in vivo model and tool compound (i.e., non-steroid) should be pursued. Differentiation of mode of action is important for designing non-steroid drug therapies and such efforts cannot be overemphasized.

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