Sensitivity of Disease Parameters to Flexible Budesonide/Formoterol Treatment in an Allergic Rat Model

Charlotte Brange, Amir Smailagic, Anne-Helene Jansson, Brian Middleton, Anna Miller-Larsson, John D. Taylor, David S. Silberstein, Harbans Lal

Title: Sensitivity of Disease Parameters to Flexible Budesonide/Formoterol Treatment in an Allergic Rat Model

Authors: Charlotte Brange, Amir Smailagic, Anne-Helene Jansson, Brian Middleton, Anna Miller-Larsson, John D. Taylor, David S. Silberstein, Harbans Lal

PII: S1094-5539(08)00104-1
DOI: 10.1016/j.pupt.2008.10.011
Reference: YPUPT 872

To appear in: *Pulmonary Pharmacology & Therapeutics*

Received Date: 12 December 2007
Revised Date: 19 September 2008
Accepted Date: 22 October 2008

Please cite this article as: Brange C, Smailagic A, Jansson A.-H., Middleton B, Miller-Larsson A, Taylor J.D., Silberstein D.S., Lal H. Sensitivity of Disease Parameters to Flexible Budesonide/Formoterol Treatment in an Allergic Rat Model, Pulmonary Pharmacology & Therapeutics (2008), doi: 10.1016/j.pupt.2008.10.011

Cover Page

Type of contribution: Original full-length research paper (revised)

Prepared on 19th September 2008

22 text pages

2 tables

5 figures

Title: Sensitivity of Disease Parameters to Flexible Budesonide/Formoterol Treatment in an Allergic Rat Model

Running Head: Budesonide/Formoterol Dosing

Authors: [1]Charlotte Brange, [1]Amir Smailagic, [1]Anne-Helene Jansson, [2] Brian Middleton, [1]Anna Miller-Larsson, [1]John D. Taylor, [3]David S. Silberstein, [1]Harbans Lal

Author Affiliation: [1]AstraZeneca R&D Lund, Lund, Sweden; [2]AstraZeneca R&D Alderley Park, Macclesfield, UK; [3]AstraZeneca R&D Global, Wilmington, USA

Corresponding author: Charlotte Brange,
AstraZeneca R&D Lund, SE 221 87 LUND, Sweden
charlotte.brange@astrazeneca.com
tel: +46 46 33 6256
fax: +46 46 33 6624

Abstract

Rationale: Clinical studies show that flexible dosing (maintenance and symptom-driven dose adjustments) of budesonide and formoterol (BUD/FORM) improves control of asthma exacerbations as compared to fixed maintenance dosing protocols (maintenance therapy) even when the latter utilize higher BUD/FORM doses. This suggests that dose-response relationships for certain pathobiologic mechanisms in asthma shift over time. Here, we have conducted animal studies to address this issue. **Objectives:** 1) To test in an animal asthma-like model whether it is possible to achieve the same or greater pharmacological control over bronchoconstriction and airway/lung inflammation, and with less total drug used, by flexible BUD/FORM dosing (upward adjustment of doses) in association with allergen challenges. 2) To determine whether the benefit requires adjustment of both drug components. **Methods:** Rats sensitized on day 0 and 7 were challenged intratracheally with ovalbumin on day 14 and 21. On days 13 through 21, rats were treated intratracheally with fixed maintenance or flexible BUD/FORM combinations. On day 22, rats were challenged with methacholine and lungs were harvested for analysis. **Results:** A flexible BUD/FORM dosing regimen (using 3.3 times less total drug than the fixed maintenance high dose regimen), delivered the same or greater reductions of excised lung gas volume (a measure of gas trapped in lung by bronchoconstriction) and lung weight (a measure of inflammatory oedema). When either BUD or FORM alone was increased on days of challenge, the benefit of the flexible dose upward adjustment was lost. **Conclusions:** Flexible dosing of the BUD/FORM combination improves the pharmacological inhibition of allergen-induced bronchoconstriction and an inflammatory oedema in an allergic asthma-like rat model.

Key words: budesonide, formoterol, dose-response, rat, lung oedema, ELGV, maintenance dosing, flexible dosing, adjustable dosing, maintenance and reliever therapy

1. Introduction

Recent clinical studies show that flexible budesonide/formoterol (BUD/FORM) dosing, in the form of symptom-driven dose adjustments, improves control of asthma exacerbations. This benefit is even achieved with lower total drug doses, as compared to fixed doses of BUD/FORM or certain other corticosteroid/β2 agonist combinations [1-3]. The implications of these findings are that the clinical benefit is related to the timing of dose supplements, the adjustment of the dose level for the fixed combination, or both.

In human subjects, it will be difficult to study systematically the pathogenic mechanisms that may respond preferentially to flexible dosing. We were interested to see whether it would be possible to identify beneficial mechanistic effects of flexible BUD/FORM dosing in an animal model. The OVA sensitised Brown Norway rat allergic model was selected, as it is well accepted and a good model of choice to mimic human asthma [4]. In our previous studies using this model we have shown that treatment with BUD/FORM administered in combination was more effective in inhibiting airway hyperresponsiveness and inflammation than BUD and FORM alone [5, 6]. The objectives of present studies in a rat allergen challenge asthma-like model, were to investigate the possible beneficial effect of a BUD/FORM combination flexible dosing with upward adjustment of doses in association with allergen challenges, contra fixed maintenance dosing, on bronchoconstriction and lung inflammatory endpoints, and also to investigate whether any benefit requires adjustment of a single or both drug components.

In the six studies reported here, rats were exposed to a series of allergen sensitizations and challenges followed by provocation with a bronchoconstrictor agent at the end of the experiments. We evaluated the effects of dosing regimens on two different parameters: excised lung gas volume (ELGV), a measure of gas trapped by bronchoconstriction [7, 8] and lung weight, an indicator of oedema and inflammation. The initial two studies

led to the basis for selection of the BUD/FORM doses. Results show that both parameters are sensitive to the BUD/FORM combination treatment. In the subsequent four studies the flexible BUD/FORM dosing was investigated and compared with fixed maintenance dosing.

2. Materials and methods

2.1 Animals

Brown Norway male rats, weighing 200-250 g, were purchased from Charles River Wiga, Sulzfald, Germany, and housed in plastic cages with aspen bedding (5 rats/cage). The animal room was maintained at 22ºC with a daily light-dark cycle (0600-1800 light) and humidity about 50-60% in the animal facility of AstraZeneca R&D Lund (Sweden). Animals were given food and water ad lib. The study was approved by the Malmö/Lund Ethical Committee for Animal Experiments (M216-02) (Sweden).

2.2 Antigen sensitization and challenge

Rats were sensitized by subcutaneous injection of a mixture of ovalbumin (OVA) and aluminium hydroxide (Alum) (100 µg OVA: 100 mg Alum in 1.0 ml PBS per animal) and intraperitoneal injection with 0.5 ml of *B. pertussis* toxin (0.1 mg ml^{-1}) on days 0 and 7. Allergen challenge was done by intratracheal instillation with 25 µg ovalbumin in the volume of 1 ml kg^{-1} body weight on days 14 and 21 after the first sensitization. All intratracheal instillation procedures were performed under light gaseous anaesthesia (4% enfluran gas driven in an airflow of a mix of 1.4 l/min N_2O and 1.2 l/min O_2), and all the groups were treated in similar fashion.

2.3 Measurement of excised lung gas volume (ELGV) and lung weight (LW)

Twenty-four hours after the last OVA challenge (day 22), rats were exposed to aerosol containing 0.2% methacholine in saline for 4 minutes. Ten minutes after the methacholine exposure, rats were terminated by administration of

pentobarbital 50 mg kg^{-1}, intraperitoneally (pentobarbitone 60 mg ml^{-1}, Apoteksbolaget, Sweden). Rats were bled from the abdominal aorta and lungs were dissected out and carefully trimmed of non-pulmonary tissue. The wet lung tissue weight (g) was measured and ELGV (ml of trapped air per lung) was determined at a transpulmonary pressure of 0 cm H_2O by the buoyancy displacement method as previously described [7, 9]. Briefly, a density determination kit and optional density determination software for the balance (P3000, Mettler-Toledo GmbH, Sweden) were used on the basis of the Archimedes' principle that every solid body immersed in a fluid exhibits an apparent loss in weight equal to the volume of the displaced fluid (i.e. the volume of the immersed body) multiplied by fluid density. By setting the system to a zero value, when the liquid density can be excluded, holder weights, tissue weights outside the beaker and tissue buoyancy within the liquid were balanced. ELGV was then determined by the difference between the holder weight and the lung tissue buoyancy in the liquid.

2.4 Drug formulations

The formulation of BUD and FORM were prepared by Department of Product Development, AstraZeneca R&D Lund, Sweden. The vehicle for the drugs consisted of (w/v): sodium chloride (8.5%), EDTA (0.1%), citric acid dried (0.15%), sodium citrate (0.5%), polysorbate 80 (0.2%) in deionized Milli-Q water. Micronised materials of BUD and FORM (formoterol fumarate dihydrate) were used for formulations. BUD formulation was prepared by homogenising BUD in polysorbat 80 and deionized water. The homogenised BUD was then added to the vehicle. BUD/FORM formulations were made by adding FORM to BUD suspension for concentrations of 5.72 mg/ml and 200 mg/ml for FORM and BUD respectively, and then diluted with vehicle to the desired concentrations. All formulations were adjusted to pH 5.0, protected from light, and stored in the refrigerator up to 3 months. The intratracheal instillation volumes were 1 ml kg^{-1} body weight in all animals.

2.5 Experimental design

The BUD/FORM ratio (all experiments except two groups shown in Fig. 5) was fixed to the ratio 35:1 on the basis of the human therapeutic formulation [10, 11] and dose ranging studies previously conducted in rats [5, 6].

Rats were allocated randomly into experimental groups. By way of introduction, two dose-response studies were designed. The intermittent dosing protocol (Fig.1 A) employed intratracheal treatment with BUD/FORM; 30/0.86 μg kg^{-1}, 100/2.9 μg kg^{-1} and 300/8.6 μg kg^{-1} administered two hours prior to allergen challenge on days 14 and 21 (n=8 animals per group).The maintenance dosing protocol (Fig. 1 B) employed intratracheal treatment with BUD/FORM; 1.0/0.029 μg kg^{-1} (low), 3.0/0.086 μg kg^{-1} (medium), 10/0.29 μg kg^{-1} (high) administered on all days 14 through 21 (n=8 animals per group).

The dose-response studies for intermittent and maintenance protocols led to the selection of suboptimal doses (a low dose, which will have a minimal or no effect of it's own) for the intermittent treatment (carried out only on the days of OVA challenge) and for the maintenance treatment (carried out each day around and between days of OVA challenge). The combination of these two treatment strategies were then used to design studies with a flexible dosing protocol (Fig. 1 C). Four studies (a - d) were conducted that compared flexible dosing protocol with fixed maintenance protocols at two dose levels (Table 1). In the flexible treatment group, animals received a 1.0/0.029 μg kg^{-1} dose of BUD/FORM on days 13 through 21, except on days of challenge (days 14 and 21) when they received a ten-fold higher dose of BUD/FORM=10/0.29 μg kg^{-1}. The total dose of BUD/FORM during these 9 days of treatment was 27/0.77 μg kg^{-1}. This flexible treatment was compared with the fixed maintenance treatment at the same total drug load divided in 9 equal portions and given as a fixed mean dose of 3.0/0.086 μg kg^{-1} BUD/FORM per day on days 13 through 21. The flexible treatment was also compared with the fixed maintenance high dose group that received 10/0.29 μg kg^{-1} at all 9 days from 13 to 21 (at the total dose BUD/FORM=90/2.6 μg kg^{-1}, i.e. 3.3 times greater than for the fixed mean or flexible dosing groups). In these series of four studies, drug treatment on days of challenge (14 and 21) was given within 30 minutes after the challenge. In each experiment, negative and positive

controls were included as well as two or three of the BUD/FORM treated groups (n=10-12 animals per group). With this layout each dosing variant was tested in 2-4 separate experiments (Table 1).

In one of the flexible dosing experiments (d) the effect of flexible treatment was examined in additional groups by varying the single components of BUD or FORM alone at days of challenge. In one of the groups only BUD was adjusted to the higher dose of 10 μg kg^{-1} combined with the low dose of FORM 0.029 μg kg^{-1}. In another group only FORM was adjusted to 0.29 μg kg^{-1} and combined with the low dose BUD 1.0 μg kg^{-1}.

2.6 Data analysis

Data from the maintenance and intermittent treatments in the two dose finding studies are presented as means ± standard error of the mean (SEM). Comparisons were made for each group against the positive control using a one-sided Dunnett's test in a one-way ANOVA allowing for group effects only.

In the flexible dosing studies, data from all four experiments with different setup of groups were used (excluding the negative control group). The description of which groups were included in respective study (a-d) is included in Table 1. The analysis was done using two-way ANOVA, allowing for study and group effects and their interaction. The purpose was to pool together the inter animal variability from all groups to get a stable estimate of the underlying variability, and to adjust for overall study level differences. Each comparison between groups is based on studies where both groups were present using a t-test based on the pooled inter animal variability. This is why, in the flexible dosing group comparison with the fixed dosing groups (Fig. 4), the difference in means does not match the difference between the two mean differences from the vehicle control. The Sidak (pairwise comparisons) adjustment to p-values for multiple comparisons to allow for the number of comparisons was made. One-sided comparisons were made for vehicle control *vs*. treatment groups and two-sided comparisons were made between the treatment groups.

3. Results

3.1 Effect of OVA challenge

Results showed that ELGV measured 10 minutes after exposure to methacholine was significantly higher in the OVA challenged rats compared with saline challenged rats (in all six studies ELGV increased between 1.4 and 1.6 folds; $p<0.01$). The lungs were weighed at the same time point as ELGV was measured. Lung weight was not affected by the methacholine challenge, therefore, the total lung weight increase was seen as an effect of the OVA challenge. Lung weight was significantly higher in the OVA challenged rats *vs*. saline control rats (in all six studies lung weight increased between 1.5 and 1.7 folds; $p<0.01$).

3.2 Dose finding studies

The initial dose finding studies showed that both ELGV and lung weight responded to the BUD/FORM combination in a dose-dependent fashion. The intermittent treatment data are shown in Fig. 2A (ELGV) and Fig. 2B (lung weight). In OVA challenged rats, BUD/FORM (30/0.86, 100/2.9, 300/8.6 μg kg^{-1}) treatment produced a dose-dependent inhibition of ELGV (18%, 66%, 58%, respectively) and lung weight (35%, 56%, 63%, respectively).

Results from the fixed maintenance treatment study (conducted with 30-fold lower doses than in the intermittent study) are shown in Fig. 3A (ELGV) and Fig. 3B (lung weight). In OVA challenged rats, BUD/FORM (1.0/0.29, 3.0/0.086, 10/0.29 μg kg^{-1}) treatment produced a dose-dependent inhibition of ELGV (0%, 52%, 92%, respectively) and lung weight (27%, 52%, 54%, respectively).

The doses of BUD/FORM used in these dose-finding studies appeared to define a part of the dose-response curve. These allowed to select the dose range to study possible improvements by flexible dosing protocols. For the intermittent dose (to be given on days of OVA challenge), BUD/FORM

dose=10/0.29 μg kg^{-1} was chosen. This was a dose below the lowest intermittent dose tested (BUD/FORM=30/0.86μg kg^{-1}) since the lowest tested dose showed a good and highly significant efficacy on lung weight and it also tended to decrease ELGV (Fig. 2). For the maintenance dose (to be given each day around and between days of OVA challenge), BUD/FORM dose=1.0/0.029 μg kg^{-1} was chosen since this was a threshold dose with a minimal effect on lung weight and no effect on ELGV (Fig. 3).

3.3 Flexible treatment

The experimental design for flexible treatment is shown in Fig.1. The sensitized and OVA challenged animal groups were all treated identically except for the doses of BUD/FORM (or vehicle) administered from days 13 to 21. Results showed that the flexible BUD/FORM treatment delivered the most effective inhibition of ELGV and lung weight (Table 2 and Fig. 4). The comparison of flexible dosing to the fixed maintenance high dosing shows that at least the same pharmacological control was achieved with flexible dosing at a 3.3 times lower total dose than for fixed dosing. The comparison to the fixed mean dose shows that at a given total dose level, significantly greater control was achieved by flexible variation for the lung weight parameter ($p<0.01$; Fig. 4).

In order to determine whether flexible dosing of both BUD and FORM contribute to the enhanced pharmacological control, the effect was tested by varying the single components at days of challenge (Fig. 5). Results show that neither the flexible dosing of BUD alone (flexible BUD, low FORM) or FORM alone (flexible FORM, low BUD) delivered the benefit of flexible dosing. Thus, concomitant adjustment of both components was required.

Discussion

The goal of asthma therapy is to achieve and maintain control of symptoms with the minimum required medication. Clinical studies show that flexible dosing of BUD/FORM improves control of asthma exacerbations as compared to fixed maintenance therapy, and that this is achieved by flexible therapy even at total lower doses [1-3]. This suggests that dose-response relationships for certain pathobiologic mechanisms in asthma shift over time. It is difficult to study systematically pathogenic mechanisms that may respond preferentially to flexible dosing, therefore we have addressed this issue in an allergic asthma-like rat model applying BUD/FORM flexible dosing with upward adjustment of doses in association with allergen challenge. For this purpose, flexible dosing protocol was designed (based on dose-response studies) using a "low" maintenance BUD/FORM dose (1.0/0.029 $\mu g\ kg^{-1}$) and a "high" intermittent dose (10/0.29 $\mu g\ kg^{-1}$) on days of allergen provocations.

The data reported here show that in rats subjected to periodic allergen exposure and bronchoconstrictor provocation, it is possible to achieve a more effective control of inflammation and bronchoconctriction using a flexible BUD/FORM dosing strategy as compared to a fixed maintenance-dose daily treatment at the same total dose. Furthermore, the same control can be reached by flexible dosing strategy at a 3.3 fold lower dose as compared to the fixed dosing strategy. Moreover, results show that a flexible dosing by adjusting only one of the drug components is not sufficient to produce the same benefit.

Our results show that flexible dosing strategy improved the pharmacological control of lung weight and ELGV parameters that were both significantly increased in OVA challenged rats, and were sensitive to BUD/FORM intermittent or fixed maintenance treatment in a dose-response manner. The lung weight values reflect lung inflammatory oedema while the values for ELGV reflect the severity of bronchoconstriction, mucus plugging, and also to a certain extent, oedema [8]. We have also observed that the inflammatory

markers in bronchoalveolar lavage (BAL) fluid, *i.e*. eosinophils and IL-1β, were reduced in a similar manner under all dosing conditions of BUD/FORM (data not shown). However, the doses selected for flexible treatment were based on dose-response relationship for ELGV and lung weight, and were too high for a possible benefit of flexible dosing on eosinophils and IL-1β in BAL fluid (since these parameters appeared to be more sensitive to BUD/FORM treatment than ELGV and lung weight). It is noteworthy that in a recent clinical study, sputum eosinophils were reduced by approximately 25% (p=0.05) after 6 months of BUD/FORM flexible therapy (maintenance and reliever therapy) as compared to 5% decrease (p=0.97) by conventional best practice therapy (based on maintenance dosing) [12].

The significant increase of ELGV after methacholine aerosol in OVA challenged rats (as compared to saline control rats) is indicative of increased airway contractility. This is in agreement with studies showing the enhanced bronchoconstrictor responses to methacholine in rodent models of allergic asthma [13, 14]. We have chosen ELGV method since it is a rapid, quantitative, sensitive and reproducible technique that does not interfere with other experimental results. The ELGV measurements have been shown to reflect the severity of bronchoconstriction [8]. Before the ELGV measurements, we have exposed the animals to methacholine (0.2% aerosol), which was selected as a threshold concentration for saline control rats, but at this concentration methacholine produces a marked bronchoconstriction in OVA challenged rats (in-house unpublished observations). Furthermore, in the present and previous studies we have observed that rats (OVA challenged) after methacholine exposure at this dose showed various degree of noisy breathing, hunched body position, reduced mobility, and had cold paws and tail. However, we have not systemically followed these signs, and possible effects of drug treatments on rat behaviour after methacholine exposure were not obvious to an investigator since group labels were blinded.

The Brown Norway rat model mimics the allergic human asthma in several respects, i.e. Brown Norway rats (genetically Th2 predisposed) produce high levels of IgE in response to active immunization and develop both early and late airway constriction and inflammatory responses after inhalation of allergen [4, 15]. Inflammatory mediators may contribute to the change in contractility of airway smooth muscles (either directly or indirectly), and structural changes, such as airway wall remodelling, oedema and mucus secretion, may also amplify the airway narrowing during airway smooth muscle contraction [16]. The inhaled or intratracheally administered BUD has been shown to inhibit both airway/lung inflammation and increased airway contractility in allergic Brown Norway rat model [5, 6, 16-18]. Similarly, FORM alone reduced inflammation and airway hyperreactivity in this model although BUD/FORM combination was more effective than either drug alone [5, 6].

In this study, we have used a combination of BUD and FORM to assess the benefits of flexible dosing on two different key components of asthma: inflammation and bronchoconstriction. The complementary effects between corticosteroids and β2-adrenoceptor agonists targeting mainly inflammation and bronchoconstriction, respectively, have been appreciated for many years. However, there is also substantial evidence of cooperative or synergistic effects as a feature of the drug classes [19-21] and for the BUD/FORM combination specifically [22]. There is evidence that the β2-adrenoceptor agonists support the corticosteroid signal and that corticosteroids support the β2-adrenergic signal. Corticosteroid therapy increases expression of the β2-adrenoceptor in rats and humans [23, 24]. In human cell systems, β2-adrenoceptor agonists synergistically enhance corticosteroid-dependent transcription [25], and corticosteroids protect the airway smooth muscle cells from degradation of the β2-adrenergic signal upon exposure to cytokines [26]. In human studies, systemic or inhaled steroids rapidly reverse desensitization of the β2-adrenergic signalling pathway [27, 28]. For these reasons, it is not surprising to observe that the benefit of flexible dosing required concurrent increase of both BUD and FORM. The cooperative effects may also explain the efficacy of the low BUD/FORM dose during the maintenance interval at concentrations that would be sub-optimal for either component alone [29].

There are limits to extrapolate from an animal model to human disease, however the parallels between the laboratory animal and the clinical studies of flexible BUD/FORM dosing are striking and worth serious consideration. When there are multiple allergen exposures and provocations over time, it appears possible to optimize pharmacologic control of asthma indices and to minimize total drug exposure by adopting a strategy of flexible dosing.

In conclusion, the findings in this allergic asthma-like rat model show the benefit of a flexible BUD/FORM treatment, where a low dose maintenance therapy combined with an increased dose during allergen-exposure days is more effective in reducing airway inflammatory and constrictive responses than a fixed mean dose each day. In addition, the study also shows that it is important that both the drug components in the combined treatment are upregulated on days of allergen-exposure. We believe that an allergic OVA rat model presented in this study can be a useful pre-clinical model to investigate existing and novel corticosteroid and β2-adrenoceptor agonist combinations.

References

[1] Fitzgerald JM, Sears MR, Boulet L-P, Becker AB, McIvor AR, Ernst P, Smiljanic-Georgijev N, Lee JSM. Adjustable maintenance dosing with budesonide/formoterol reduces asthma exacerbations compared with traditional fixed dosing: A five-month multicentre Canadian study. Can Respir J 2003;10:427-34.

[2] Vogelmeier C, D'Urzo A, Jaspal M, Merino JM, Johansson G, Boutet S. Symbicort for both maintenance and relief reduces exacerbations compared with a titration of Seretide (Advair) in patients with asthma: a real-life study. Proc Am Thorac Soc 2005;2:A628.

[3] Kuna P, Peters MJ, Manjra AI, Jorup C, Naya IP, Martínez-Jimenez NE, Buhl R. Effect of budesonide/formoterol maintenance and reliever therapy on asthma exacerbations. Int J Clin Pract 2007;61:725-36.

[4] Pauluhn J, Ulrich M. Experimental approaches to evaluate respiratory allergy in animal models. Exp Tox Pathol 2005;56:203-34.

[5] Brange C, Smailagic A, Jansson AH, Eriksson C, Wang XD. Effects of intermittent therapy with budesonide/formoterol combination on airway hyperresponsiveness and inflammation in an allergic rat model. In: Abstract Book XXIII EAACI Congress 2004:218, Abs 722.

[6] Brange C, Smailagic A, Eriksson C, Wang XD. Effects of budesonide/formoterol maintenance therapy on airway hyperresponsiveness and inflammation in an allergic rat model. Eur Respir J 2004; 24 (suppl 48) 351s.

[7] Jansson A-H, Eriksson C, Wang XD. Lung inflammatory responses and hyperinflation induced by an intratrachael exposure to lipopolysaccharide in rats. Lung 2004;182:163-71.

[8] Stengel PW, Yiamouyiannis CA, Obenchain RL, Cockerham SL, Silbaugh SA. Methacholine-induced pulmonary gas trapping in guinea pigs, hamsters, mice, and rats. J App Physiol 1995;79:2148-53.

[9] Jansson A-H, Smailagic A, Dutius Andersson A-M, Zackrisson C, Fehniger TE, Stevens TR, Wang XD. Evaluation of excised lung gas volume measurements in animals with genetic or induced emphysema. Respir Physiol Neurob 2006;150:240-50.

[10] Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, Ullman A. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and corticosteroids establishing therapy (FACET) international study group. New Eng J Med 1997;337:1405-11.

[11] O'Byrne PM, Barnes PJ, Rodriguez-Roisin R, Runnerstrom E, Sandstrom T, Svensson K, Tattersfield A. Low dose inhaled budesonide and formoterol in mild persistent asthma. The OPTIMA randomized trial. Am J Respir Crit Care Med 2001;164:1392-7.

[12] Sears MR, Boulet L-P, Laviolette M, Fitzgerald JM, Bai TR, Kaplan A, Smiljanic-Georgijev N, Lee JS-M. Budesonide/formoterol maintenance and reliever therapy: impact on airway inflammation in asthma. Eur Respir J 2008;31:982-9.

[13] Kamachi A, Nasuhara Y, Nishimura M, Takahashi T, Homma Y, Ohtsuka Y, Munakata M. Dissociation between airway responsiveness to methacholine and responsiveness to antigen. Eur Respir J 2002;19:76-83.

[14] Zosky G, von Garnier C, Stumbles P, Holt P, Sly P, Turner D. The pattern of methacholine responsiveness in mice is dependent on antigen challenge dose. Respir Res 2004;5:15.

[15] Hylkema MN, Hoekstra MO, Luinge M, Timens W. The strength of the OVA-induced airway inflammation in rats is strain dependent. Clin Exper Immunol 2002;129:390-6.

[16] Fernandes DJ, Mitchell RW, Lakser O, Dowell M, Stewart AG, Solway J. Do inflammatory mediators influence the contribution of airway smooth muscle contraction to airway hyperresponsiveness in asthma? J App Physiol 2003;95:844-53.

[17] Huang T-J, Eynott P, Salmon M, Nicklin PL, Chung KF. Effect of topical immunomodulators on acute allergic inflammation and bronchial hyperresponsiveness in sensitised rats. Eur J Pharmacol 2002;437:187-94.

[18] Xu L, Olivenstein R, Martin JG, Powell WS. Inhaled budesonide inhibits OVA-induced airway narrowing, inflammation, and cys-LT synthesis in BN rats. J App Physiol 2000;89:1852-8.

[19] Besse JC, Bass AD. Potentiation by hydrocortisone of responses to catecholamines in vascular smooth muscle. J Pharmacol Exp Ther 1966;154:224-38.

[20] Eickelberg O, Roth M, Lorx R, Bruce V, Rudiger J, Johnson M, Block L-H. Ligand-independent activation of the glucocorticoid receptor by beta2-adrenergic receptor agonists in primary human lung fibroblasts and vascular smooth muscle cells. J Biol Chem 1999;274:1005-10.

[21] Samuelson WM, Davies AO. Hydrocortisone-induced reversal of beta-adrenergic receptor uncoupling. Am Rev Respir Dis 1984;130:1023-6.

(22] Roth M, Johnson PR, Rudiger JJ, King GG, Ge Q, Burgess JK, Anderson G, Tamm M, Black JL. Interaction between glucocorticoids and beta2 agonists on bronchial airway smooth muscle cells through synchronised cellular signalling. Lancet 2002;360:1293-9.

[23] Mak JCW, Nishikawa M, Shirasaki H, Miyayasu K, Barnes PJ. Protective effects of a glucocorticoid on downregulation of pulmonary beta2-adrenergic receptors in vivo. J Clin Invest 1995;96:99-106.

[24] Baraniuk J, Ali M, Brody D, Maniscalco J, Gaumond E, Fitzgerald T, Wong G, Yuta A, Mak J, Barnes P, Bascom R, Troost T. Glucocorticoids

induce beta2-adrenergic receptor function in human nasal mucosa. Am J Respir Crit Care Med. 1997;155:704-10.

[25] Kaur M, Chivers JE, Giembycz MA, Newton R. Long-acting beta2-adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human airway epithelial and smooth muscle cells. Mol Pharmacol 2008;73:203-14.

[26] Moore PE, Laporte JD, Gonzalez S, Moller W, Heyder J, Panettieri RA, Jr., Shore SA. Glucocorticoids ablate IL-1beta-induced beta-adrenergic hyperresponsiveness in human airway smooth muscle cells. Am J Physiol 1999;277:L932-42.

[27] Tan KS, Grove A, McLean A, Gnosspelius Y, Hall IP, Lipworth BJ. Systemic corticosteroid rapidly reverses bronchodilator subsensitivity induced by formoterol in asthmatic patients. Am J Respir Crit Care Med1997;156:28-35.

[28] Aziz I, Lipworth BJ. A bolus of inhaled budesonide rapidly reverses airway subsensitivity and beta2-adrenoceptor down-regulation after regular inhaled formoterol. Chest 1999;115:623-8.

[29] Miller-Larsson A and Selroos O. Steroid sparing strategies. Long acting inhaled β2-agonists. In: Adcock IM and Chung KF (ed.). Overcoming steroid insensitivity in respiratory disease. John Wiley & Sons, Ltd, 2008: 163-185.

Figure Captions

Fig. 1. Experimental design

Fig. 2. Intermittent BUD/FORM treatment

Fig. 3. Fixed maintenance BUD/FORM treatment

Fig. 4. Comparisons of BUD/FORM flexible and fixed dosing

Fig. 5. Flexing only one of the BUD/FORM components

Figure legends

Fig. 1. Experimental design:
Rats were intraperitoneally sensitized (S) with OVA or saline (vehicle control) on day 0 and 7. Intratracheal (*i.t.*) challenge (Ch) with ovalbumin or saline was performed on day 14 and 21. (A) Intermittent BUD/FORM treatment; only on day 14 and 21 close in time to challenge. This protocol was used in the first dose finding study. (B) Fixed maintenance BUD/FORM treatment; with treatment on days 14 through 21 tested at two dose levels (see, Experimental design). (C) Flexible dosing; treatment with combination of low maintenance dose of BUD/FORM or vehicle on day 13 and days 15 through 20 and the intermittent treatment with higher dose of BUD/FORM or vehicle performed (*i.t.*) on days 14 and 21. Doses administered at various days are specified in Table 1. Small arrows represent low maintenance doses and large arrows represent high intermittent doses of BUD/FORM.

Fig. 2. Intermittent treatment (BUD/FORM administered on days of challenge). OVA challenge caused a significant increase in ELGV and lung weight ($p<0.01$** Veh/PBS compared to Veh/OVA). BUD/FORM treatment (B/F; 30/0.86; 100/2.9; 300/8.6 μg kg^{-1}) caused a dose-dependent inhibition of OVA-induced increases in ELGV (A) and lung weight (B) ($p<0.05$*, $p<0.01$**, $p<0.001$*** compared to Veh/OVA, n=8 per group). Data are shown as mean ± SEM. Statistical comparisons made to see whether all other groups are significantly lower than vehicle treated OVA challenged group (2nd bar in A and B). Abbreviations: OVA, ovalbumin; Veh, vehicle; B, BUD; F, FORM; PBS, phosphate buffered saline.

Fig. 3. Fixed maintenance treatment (BUD/FORM administered on days 14 through 21). OVA challenge caused a significant increase in ELGV and lung weight ($p<0.01$** Veh/PBS compared to Veh/OVA). BUD/FORM maintenance treatment (1.0/0.029; 3.0/0.086; 10/0.29 μg kg^{-1}) produced a dose-dependent inhibition of OVA-induced increases in ELGV (A) and lung weight (B)

(*p<0.05, p<0.01** compared to Veh/OVA, n=8 per group). Data are shown as mean ± SEM. Statistical comparisons made to see whether all other groups are significantly lower than vehicle treated OVA challenged group (2nd bar in A and B). Abbreviations: OVA, ovalbumin; Veh, vehicle; B, BUD; F, FORM; PBS, phosphate buffered saline

Fig. 4. Mean difference in inhibitory efficacy; comparisons of flexible and fixed maintenance BUD/FORM dosing treatments. Data in the first three bars from bottom show that mean differences from vehicle for treatments Flex (flexible dose), Fix M (fixed mean dose) and Fix H (fixed high dose) are statistically significant for both ELGV (A) and lung weight (B) parameters (p<0.05*, p<0.01**, p<0.001***). Mean difference between vehicle treatment and drug flexible dosing (1st bar from bottom) is more pronounced than between vehicle treatment and drug fixed dosing (fixed mean dose and fixed high dose; 2nd and 3rd bars from bottom in A, respectively), and for the lung weight the difference is significantly greater for flexible treatment compared to fixed mean dose (p<0.01* 4th bar from bottom in B). Data are shown as mean difference ± SEM (n = 8-12 per group). Length of the bars represents the benefits of different treatments. Abbreviation; Veh, vehicle.

Fig. 5. Effect of flexing only one of the BUD/FORM components.
Flexible BUD/FORM dosing treatment (Flex BHFH, 3rd bar in A and B) in sensitized and OVA challenged rats significantly inhibited changes in ELGV and lung weight when compared to OVA exposed and vehicle treated rats (2nd bar in A and B). When only one of the components, FORM or BUD, was adjusted flexibly (5th and 6th bars, respectively), this benefit of flexible adjustment essentially disappeared for ELGV (A) and was lost partially for lung weight (B). Data are shown as mean ± SEM (n = 8-12 per group). All statistical comparisons are performed against Flex BHFH group (p<0.05*, p<0.01**) Abbreviations; OVA, ovalbumin; Veh, vehicle; BHFH, flexible dosing with BUD high/FORM high (10/0.29 μg kg^{-1} on days of challenge and

1.0/0.029 μg kg^{-1} on other days); Flex BLFH, flexible BUD low/FORM high (1.0/0.29 μg kg^{-1} on days of challenge and 1.0/0.029 μg kg^{-1} on other days) ; Flex BHFL, flexible BUD high/FORM low (10/0.029 μg kg^{-1} on days of challenge and 1.0/0.029 μg kg^{-1} on other days); PBS, phosphate buffered saline.

Table 1
Group description with doses for flexible/fixed dosing

Group	Included in Study nr	S/Ch	Treatment	Doses (μg kg^{-1})	Days	Total dose (μg kg^{-1})
PBS/Veh	a – d	+/-	Vehicle	0	13 – 21	0
OVA/Veh	a – d	+/+	Vehicle	0	13 – 21	0
Fixed Mean	a – c	+/+	BUD/FORM	3.0/0.086	13 – 21	27/0.77
Fixed High	c - d	+/+	BUD/FORM	10/0.29	13 – 21	90/2.6
Flexible	a - d	+/+	BUD/FORM	1.0/0.029 +10/0.29	13, 15 – 20 14, 21	27/0.77

Comparison of fixed and flexible dosing protocols. The table shows the drug treatment administered in the different groups of animals. Two groups were treated with fixed doses (one with high and one with mean dose) and one group was treated with flexible dosing. The total dose of BUD/FORM administered over the 9 days course of treatment is shown in right hand column. The fixed mean daily dose is calculated from the total dose used in the flexible dosing during 9 days of treatment divided by 9 (n= 10-12 animals/group). Abbreviations: OVA, ovalbumin; S, sensitization with ovalbumin/alum/toxin; Ch, challenge with ovalbumin or vehicle.

Table 2. Data on comparisons in inhibitory efficacy of flexible and fixed dosing for ELGV and lung weight values

Variable	Comparison treated groups	No of rats (No of studies)	Difference Mean	(SEM)	Sidak p-value
ELGV	Veh – Flex	90 (4)	0.428	(0.065)	<0.001
	Veh – Fix M	65 (3)	0.238	(0.076)	<0.01
	Veh – Fix H	46 (2)	0.249	(0.091)	<0.05
	Fix M – Flex	65 (3)	0.175	(0.077)	
	Fix H – Flex	46 (2)	0.168	(0.091)	
Lung Weight	Veh – Flex	89 (4)	0.489	(0.041)	<0.001
	Veh – Fix M	65 (3)	0.364	(0.047)	<0.001
	Veh – Fix H	46 (2)	0.361	(0.056)	<0.001
	Fix M – Flex	65 (3)	0.166	(0.047)	<0.01[1)]
	Fix H – Flex	45 (2)	0.067	(0.057)	

[1)] SidaK 2-sided p-value

The mean differences (with SEM) in ELGV (ml air/lung) and lung weight (g) values after different treatment protocols are shown. Flexible BUD/FORM dose (Flex), fixed medium dose (Fix M) and fixed high dose (Fix H) groups are tested against the vehicle (Veh) group (values after active treatments are subtracted from values after vehicle treatment). Similarly, values after flexible BUD/FORM dose are tested against values after fixed mean dose and fixed high dose groups. All three treatment regimes significantly inhibited OVA-induced changes in ELGV and lung weight when compared to vehicle treatment. The changes in lung weight are significantly further decreased with flexible BUD/FORM treatment when compared to fixed medium dose.

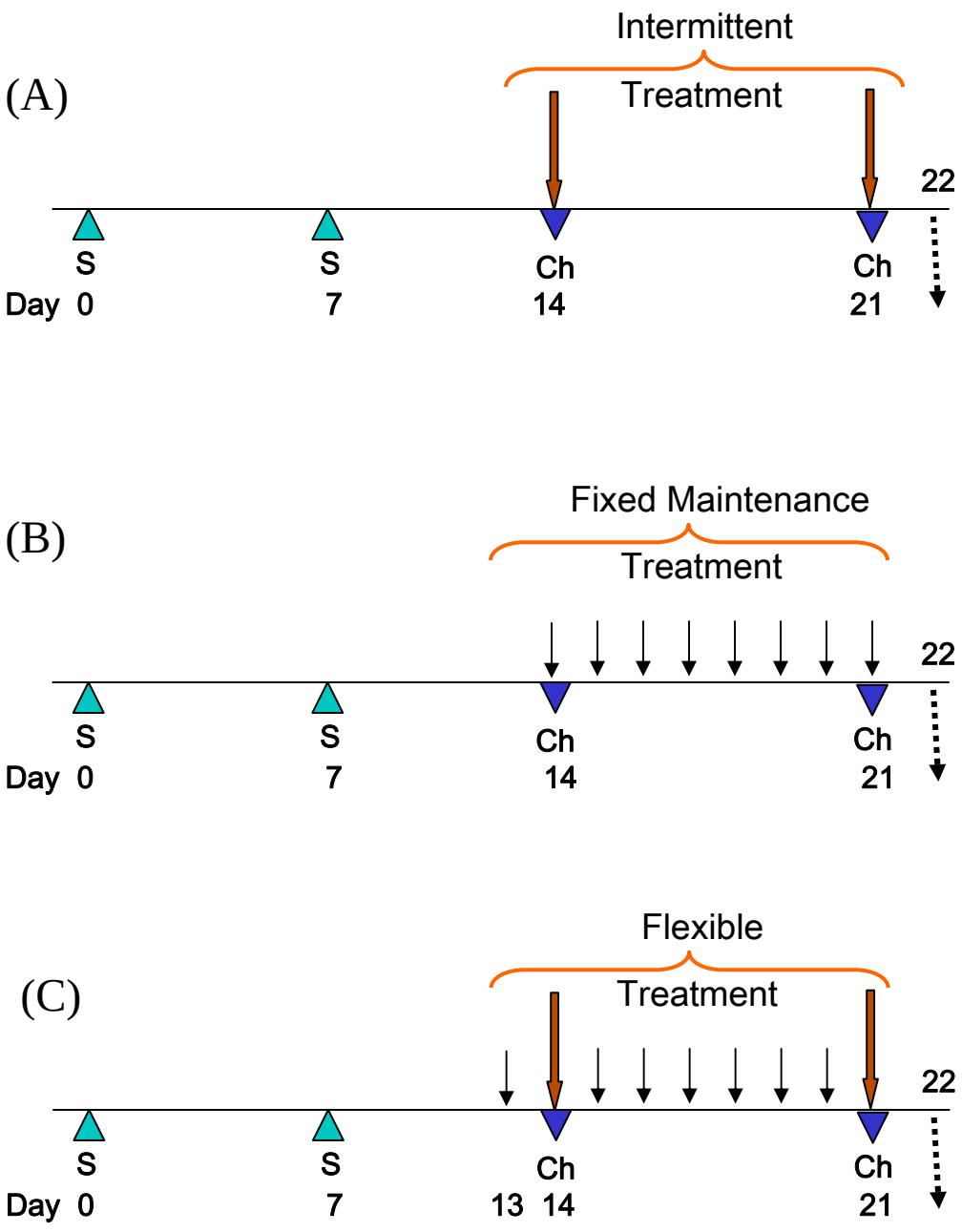
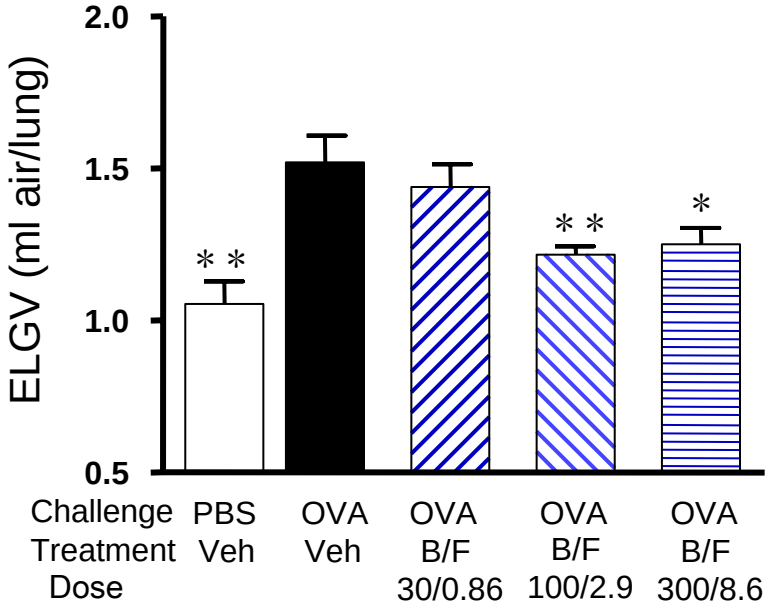
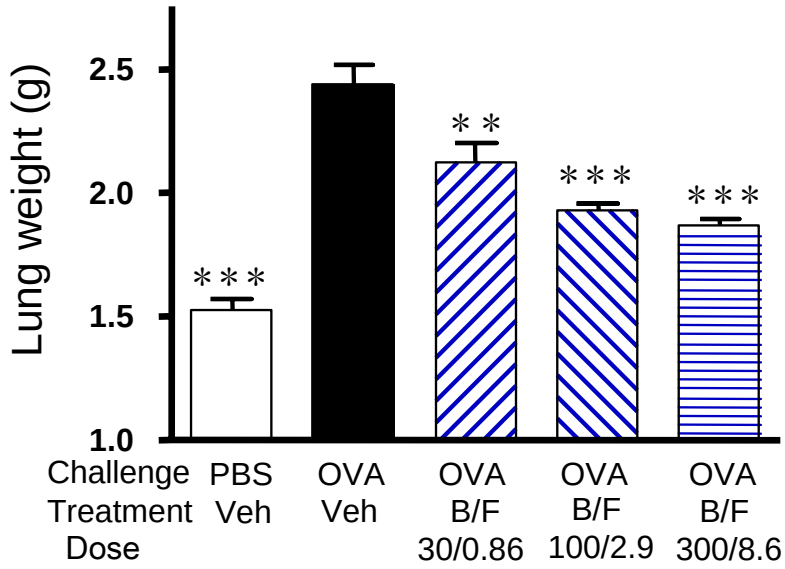


Fig. 1

Charlotte Brange



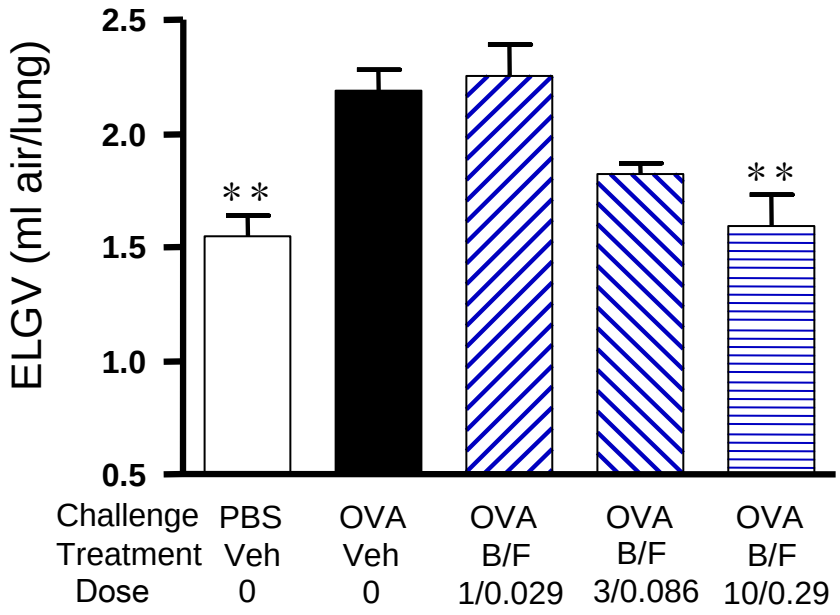
(A)



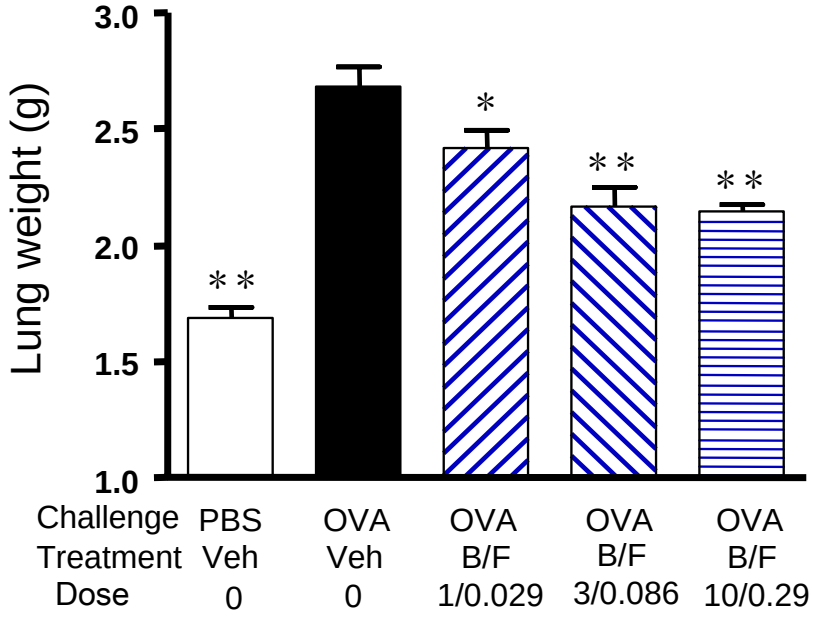
(B)

Fig. 2

Charlotte Brange



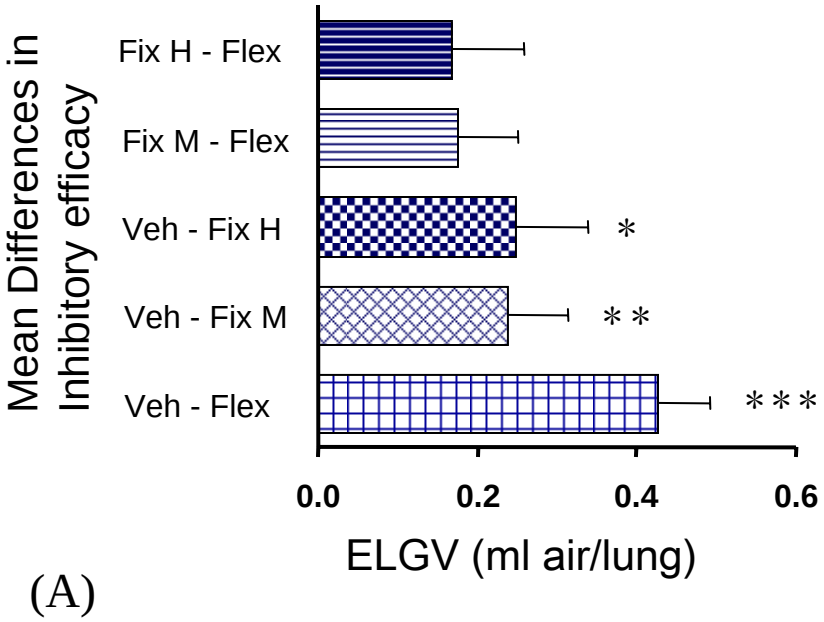
(A)



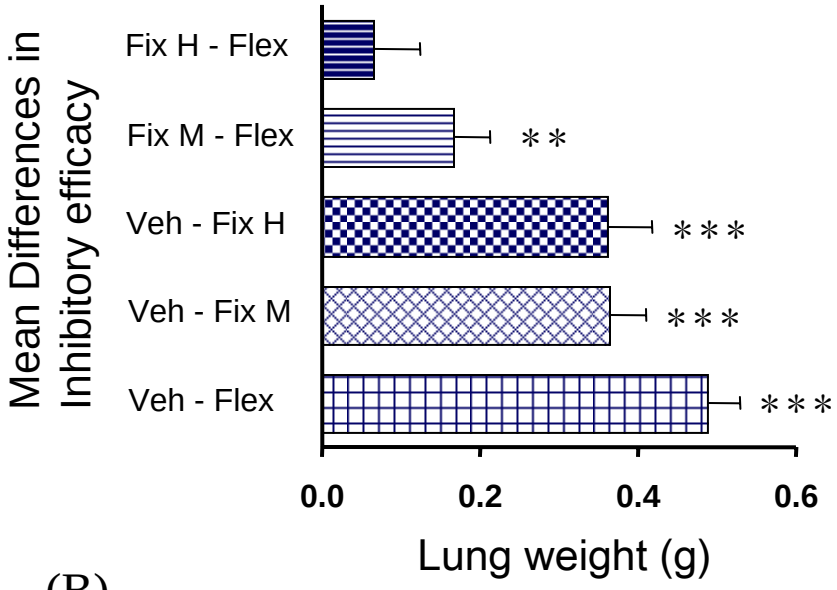
(B)

Fig. 3

Charlotte Brange



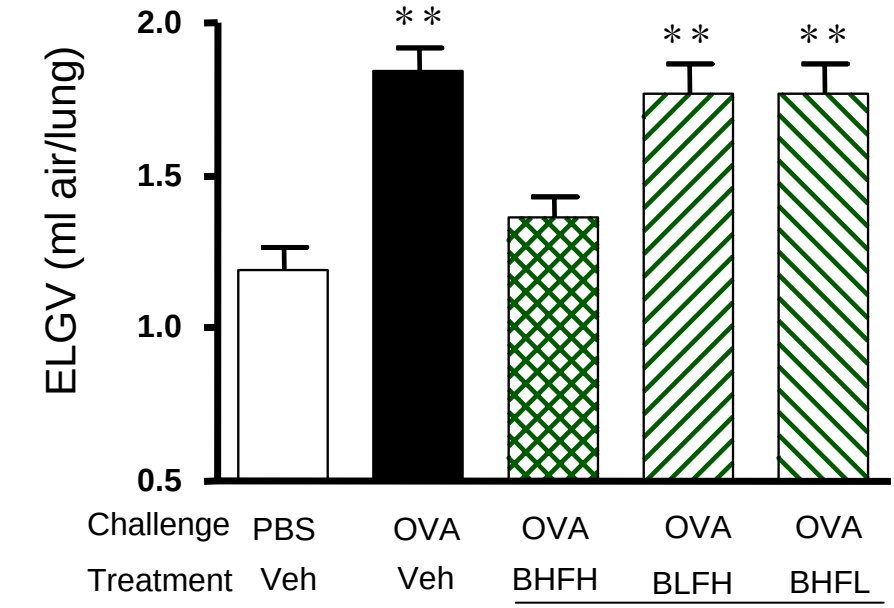
(A)



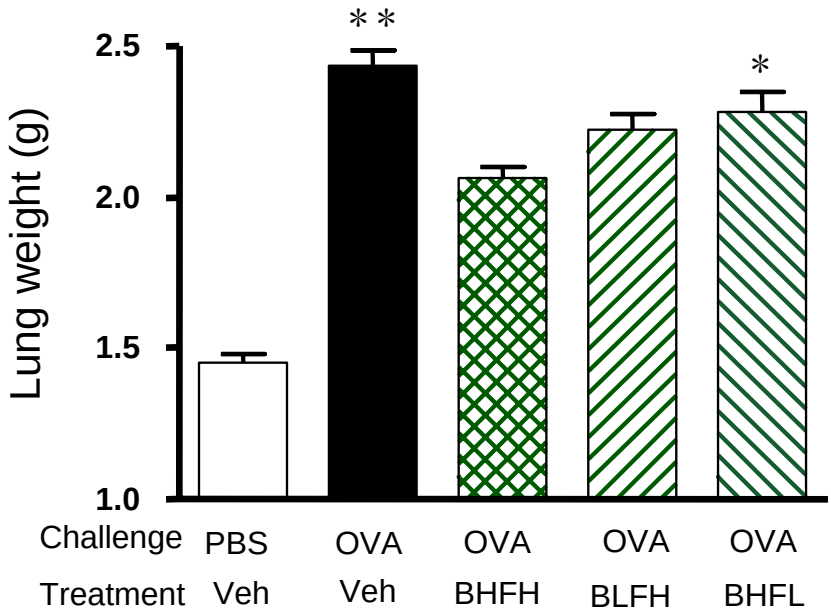
(B)

Fig. 4

Charlotte Brange



(A)



(B)

Fig. 5

Charlotte Brange