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CHF6001 II: A Novel Phosphodiesterase 4 Inhibitor, Suitable for Topical Pulmonary Administration—In Vivo Preclinical Pharmacology Profile Defines a Potent Anti-Inflammatory Compound with a Wide Therapeutic Window

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ABSTRACT

CHF6001 [(*S*)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridine 1-oxide] is a novel phosphodiesterase 4 (PDE4) inhibitor designed for use in pulmonary diseases by inhaled administration. Intratracheal administration of CHF6001 to ovalbumin-sensitized Brown-Norway rats suppressed the antigen-induced decline of lung functions (ED_{50} = 0.1 μmol/kg) and antigen-induced eosinophilia (ED_{50} = 0.03 μmol/kg) when administered (0.09 μmol/kg) up to 24 hours before antigen challenge, in agreement with CHF6001-sustained lung concentrations up to 72 hours after intratracheal treatment (mean residence time 26 hours). Intranasal, once daily administration of CHF6001 inhibited neutrophil infiltration observed after 11 days of tobacco smoke exposure in mice, both upon prophylactic (0.15–0.45 μmol/kg per day) or interventional (0.045–0.45 μmol/kg per day) treatment. CHF6001 was ineffective in reversing ketamine/xylazine-induced anesthesia (a surrogate of emesis in rat) up to 5 μmol/kg administered intratracheally, a dose 50- to 150-fold higher than anti-inflammatory ED_{50} observed in rats. When given topically to ferrets, no emesis and nausea were evident up to 10 to 20 μmol/kg, respectively, whereas the PDE4 inhibitor GSK-256066 (6-[3-(dimethylcarbamoyl)phenyl]sulfonyl-4-(3-methoxyanilino)-8-methylquinoline-3-carboxamide) induced nausea at 1 μmol/kg intratracheally. A 14-day inhalation toxicology study in rats showed a no-observed-adverse-effect level dose of 4.4 μmol/kg per day for CHF6001, lower than the 0.015 μmol/kg per day for GSK-256066. CHF6001 was found effective and extremely well tolerated upon topical administration in relevant animal models, and may represent a step forward in PDE4 inhibition for the treatment of asthma and chronic obstructive respiratory disease.

Introduction

Inhibitors of phosphodiesterase 4 (PDE4) are potent anti-inflammatory agents in vivo as a result of significant expression of this PDE isoform in a number of inflammatory and immune cells (Teixeira et al., 1997), where the elevation of intracellular cAMP concentrations inhibits the production of inflammatory mediators (Fan Chung, 2006). Moreover, PDE4 inhibitors regulate the functions of the pulmonary structural cells thought to contribute to the pathogenesis of chronic inflammatory airway diseases, such as airway smooth muscle cells, airway epithelial cells, vascular endothelial cells, and airway sensory nerves (Torphy, 1998; Spina, 2003)

In preclinical in vitro and in vivo models of airway inflammation, PDE4 inhibitors have been shown to suppress the activation and recruitment of inflammatory cells. Clinical investigations of second-generation oral PDE4 inhibitors, such as cilomilast (Ariflo; GlaxoSmithKline, Brentford, UK) (Barnette

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ABBREVIATIONS: BAL, bronchoalveolar lavage; CHF6001, (*S*)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridine 1-oxide; COPD, chronic obstructive respiratory disease; CS, cigarette smoke; FEV200, forced expiratory volume at 200 milliseconds; FVC, forced vital capacity; GSK-256066, 6-[3-(dimethylcarbamoyl)phenyl]sulfonyl-4-(3-methoxyanilino)-8-methylquinoline-3-carboxamide; NOAEL, no-observed-adverse-effect level; OVA, ovalbumin; PDE4, phosphodiesterase 4; PEG, polyethylene glycol; PF-03715455, 1-[5-*tert*-butyl-2-(3-chloro-4-hydroxyphenyl)pyrazol-3-yl]-3-[[2-[[3-[2-(2-hydroxyethylsulfanyl)phenyl]-[1,2,4]triazolo[4,3-*a*]pyridin-6-yl]sulfanyl]phenyl]methyl]urea; TS, tobacco smoke.

et al., 1998), and roflumilast (Daxas; Takeda Pharmaceuticals, Zurich, Switzerland) (Hatzelmann and Schudt, 2001), have demonstrated efficacy in chronic lung diseases, such as chronic obstructive pulmonary disease (COPD) and asthma (Beghe et al., 2013). However, the therapeutic use of this class of compounds is limited by mechanism-related side effects, in particular nausea, diarrhea, abdominal pain, vomiting, and dyspepsia (Giembycz, 2000, 2008). In addition, in preclinical toxicologic studies, mesenteric and organ vasculitis have been observed after chronic dosing, in particular in the rat (Dagues et al., 2007).

Different approaches have been pursued for the development of oral PDE4 inhibitors with improved gastrointestinal tolerability. Because emesis is at least in part a side effect mediated by the central nervous system, new PDE4 inhibitors with low brain penetration have been developed, but this approach has not yielded better tolerated compounds, given that the area postrema, which acts as chemoreceptor trigger zone for emesis, is not protected by the blood-brain barrier and that PDE4 inhibitors also exert direct effects on the gastrointestinal tract (Okuda et al., 2009). Alternative strategies were to selectively target the low-affinity rolipram-binding site conformer of PDE4 over the high-affinity rolipram-binding conformer (Barnette et al., 1996; Rocque et al., 1997) or the isoform PDE4B over the isoform PDE4D (Robichaud et al., 2002b; Lehnart et al., 2005), but both these approaches have been only partially validated (Duplantier et al., 1996).

In an attempt to limit systemic exposure and the associated side effects, novel topically active drugs have been developed to be administered directly into the lung via the inhalation route. This approach has been successfully adopted in respiratory pharmacology, leading to the drugs most widely used for the treatment of pulmonary pathologies such as inhaled corticosteroids and bronchodilators. However, only PDE4 inhibitors specifically designed for topical treatment can be successfully administered by the inhalation route, enabling very potent anti-inflammatory activity in the airways while reducing dose-limiting gastrointestinal side effects.

In the present study, we report the in vivo pharmacologic characterization of CHF6001 [(*S*)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl) pyridine 1-oxide], a novel, extremely potent, and selective PDE4 inhibitor. This compound has been synthesized as part of a drug discovery project aimed at the identification of compounds endowed with pharmacokinetics and physicochemical properties suitable for inhaled dosing (Armani et al., 2014). The in vitro pharmacologic profile of CHF6001 has been extensively characterized in different cellular assays and is presented in our companion article (Moretto et al., 2015).

We studied the in vivo pharmacologic profile of CHF6001, testing its anti-inflammatory efficacy in asthma and COPD animal models both upon prophylactic and therapeutic interventions. The relationships of the anti-inflammatory efficacy, duration of action, and lung/plasma levels of CHF6001 after intratracheal treatment were also investigated. Finally, in vivo studies were performed to determine the safety profile of CHF6001.

Materials and Methods

Chemicals

CHF6001 (mol. wt. 687.54) (Armani et al., 2014), GSK-256066 (6-[3-(dimethylcarbamoyl)phenyl]sulfonyl-4-(3-methoxyanilino)-8-methylquinoline-3-carboxamide; mol. wt. 518.58), roflumilast (mol. wt. 403.21), and budesonide (mol. wt. 430.53) were synthesized at Chiesi Farmaceutici S.p.A., Parma, Italy. Fluticasone furoate (mol. wt. 538.38) was obtained from Pharmabios and PF-03715455 (1-[5-*tert*-butyl-2-(3-chloro-4-hydroxyphenyl)pyrazol-3-yl]-3-[[2-[[3-[2-(2-hydroxyethylsulfanyl)phenyl]-[1,2,4]triazolo[4,3-*a*]pyridin-6-yl]sulfanyl]phenyl]methyl]urea; mol. wt. 700.3) was synthesized by Argenta Discovery Ltd. (Harlow, UK). Test compounds were administered intratracheally either as micronized powder mixed with lactose or as a suspension in 0.2% Tween 80 in saline in a volume of 0.5 ml/kg. In the tobacco smoke–induced inflammation model, test compounds were administered intranasally as a suspension in 0.2% Tween 80 in saline. Roflumilast was suspended in 5% polyethylene glycol 400 (PEG400)/methocel 1% in saline, volume of administration 5 ml/kg, and administered by gavage. Unless otherwise stated, all other chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Animals

All the experimental procedures and conditions were reviewed and approved by the local ethics committees and were performed in full compliance with the international European ethics standards (86/609-EEC), the Italian legislative decree 116/1992, the French National Committee (décret 87/848) for the care and use of laboratory animals, and the Animals (Scientific Procedures) Act of 1986.

Ovalbumin-Induced Lung Eosinophilia in the Rat: Potency and Duration of Action

Adult male Brown-Norway rats (Charles River Laboratories Italy, Calco, Italy) were sensitized by intraperitoneal injection of a suspension containing ovalbumin (OVA; 1 mg/rat) and $Al(OH)_3$ (100 mg/rat) in 1 ml of saline for 3 consecutive days. Then, 2 to 3 weeks later, the animals were exposed to an aerosol of OVA solution (1% in saline) by a nose-only apparatus system for 30 minutes to trigger an influx of inflammatory cells into the airways. Vehicle-control treated animals were exposed to an aerosol of saline using the same general conditions. For the determination of inhibitory potency, test compounds were administered intratracheally as single dose 2 hours before the antigen (OVA) aerosol. In time course studies, CHF6001 (24, 16, 6, and 2 hours before antigen challenge), fluticasone furoate or budesonide (24 hours before antigen challenge only), or vehicle were administered as single intratracheal dose ($ED_{50} \times 3$ or $ED_{50} \times 10$).

For the intratracheal administration of test compounds or vehicle, animals were anesthetized with isoflurane (4% in oxygen), and a laryngoscope was moved forward into the mouth to visualize the trachea and guide the insertion of a fine-tipped dry powder insufflator (PennCentury, Philadelphia, PA) directly into the trachea and located 1–2 mm above the bifurcation. Dry powder formulations were prepared by blending coarse respiratory grade lactose and the micronized test compound to achieve 10 mg/kg body weight total particulate powder. Dry powders were blown into the airways during the spontaneous phase inspiration in an air volume of 3 ml. The difference in weight of the needle before and after the administration was used to calculate the dose administered. Control animals received 10 mg/kg of lactose.

At 24 hours after exposure either to OVA or saline aerosol, animals were sacrificed by an overdose of anesthetic. Bronchoalveolar lavage (BAL) fluid was obtained by gently washing the lungs with 3 aliquots (4 ml each) of solution A (10× Hanks' balanced salt solution, 100 ml; EDTA 100 mM, 100 ml; HEPES 1 mM, 10 ml; distilled water, 790 ml). Routine recovery of BAL fluids did not significantly differ among animals with ~80% of instilled volume recovered (9.5–10.5 ml).

The resulting BAL fluid was centrifuged at 800*g* for 10 minutes at 4°C, and the supernatants were removed and discharged. The pellets were resuspended in a volume of 1.5 ml, and total and differential cell counts were performed within 2 hours using an automated cell counter (Sysmex, Dasit, Cornaredo, Italy).

Forced Maneuvers in OVA-Challenged Brown-Norway Rats

Adult male Brown-Norway rats (Charles River Laboratories Italy) were sensitized to OVA as previously described. On the day of the experiment, 5 hours before the antigen challenge, the animals were intratracheally treated with test compounds as described earlier. Twenty-four hours after the OVA challenge, the rats were anesthetized with an intramuscular injection of a mixture of ketamine (50 mg/kg) and medetomidine (0.33 mg/kg), were fitted with a tracheal cannula connected to a computer-driven breathing valve, and were placed in a forced pulmonary maneuver system (Buxco Research Systems, Wilmington, NC).

After 5 minutes of spontaneous breathing, the computer was programmed to expose the animals to 10 forced breaths: the lungs were inflated, increasing the positive intratracheal pressure up to 10 cm H_2O. At the moment of data acquisition, the lungs were inflated until the airway opening pressure reached 30 cm H_2O. This was immediately followed by a rapid deflation of the lungs, until the intratracheal pressure reached a value of −40 cm H_2O. From the corresponding flow/volume curve, the forced vital capacity (FVC) and forced expiratory volume at 200 milliseconds (FEV200) were measured. This procedure was performed in triplicate, and the results were averaged.

Neutrophilia Induced by Cigarette Smoke Exposure in Mice: Prophylactic and Interventional Treatment

C57Bl6J mice (Charles River Laboratories, Margate, UK) in groups of five were exposed daily to tobacco smoke (TS) (generated using "Marlboro 100" cigarettes) for 5 (baseline groups of the interventional study) or 11 consecutive days in clear polycarbonate chambers (27 cm × 16 cm × 12 cm). To minimize potential problems caused by acute exposure to a high level of TS, the exposure period to TS was increased from 25 minutes at the start of the study (day 1) to a maximum of 45 minutes from day 3 to day 11. One additional group of mice was exposed to air only on a daily basis for equivalent lengths of time as sham controls (no TS-exposure).

All animals received intranasal vehicle or test compounds daily 1 hour before air or TS exposure. Intranasal administration was performed under light anesthesia (isoflurane/O_2) by dripping 50 μl of the test substance onto the nares and allowing the animal to spontaneously aspirate the fluid; the animals were then allowed to recover and returned to their home cage. In both prophylactic and interventional treatments, Tween 80 0.2% in normal saline was used as vehicle. In the prophylactic treatment study, additional groups were treated with vehicle or test compound twice daily, 1 hour before and 6 hours after air or TS exposure. Mice were euthanized either 24 hours after day 5 of air or TS exposure or 24 hours after the final exposure (11-day protocol) to air or TS, by intraperitoneal barbiturate anesthetic overdose.

A BAL was performed using a volume of 0.4 ml of phosphate-buffered saline that was gently instilled and withdrawn 3 times using a 1-ml syringe. Cells from the BAL fluid were separated by centrifugation (6 minutes at 3070*g*), and the supernatant was removed. The resulting cell pellet was resuspended in a known volume of phosphate-buffered saline, and the total cell numbers were calculated by counting a stained (Turks stain) aliquot under a microscope using a hemocytometer. The cell pellet was resuspended to approximately 10^5 cells per ml, and cytospin slides were prepared by centrifugation (8 minutes at 72.26*g;* Shandon Cytospin 3; Thermo Shandon Ltd., Runcorn, Cheshire, UK).

The slides were air-dried and stained using Wright-Giemsa stain, following the manufacturer's instructions. A differential cell count was performed using light microscopy. Approximately 400 cells were counted from each slide. The cells were identified using standard morphometric techniques.

Prophylactic Study. Groups of 10 animals were treated intranasally with either vehicle (Tween 80 in saline), CHF6001 at 0.15 or 0.45 μmol/kg twice daily, CHF6001 at 0.15 or 0.45 μmol/kg once daily, or the inhaled p38 inhibitor PH-03715455 at 0.15 μmol/kg once daily. Treatments were administered either 1 hour before TS exposure or 1 hour before and 6 hours after each TS exposure. An additional control group was exposed to air for 11 consecutive days (sham exposure) and received vehicle twice daily. This group was also sacrificed on day 12, 24 hours after the final exposure. Mice were assumed to weigh 20 g and were dosed at 50 μl/mouse under light anesthesia. This study was performed in parallel with the interventional study described herein. This enabled us to directly compare the prophylactic and therapeutic treatments with CHF6001.

Interventional Study. In this study, one group of 10 mice was subjected to daily TS exposure for 5 consecutive days and was sacrificed on day 6, 24 hours after the final TS exposure. A second group was exposed to air for 5 consecutive days and sacrificed on day 6, 24 hours after the final air exposure. These mice did not receive any treatment and were included in the experimental design to collect baseline data relative to day 6.

Groups of 10 mice received once-daily vehicle, CHF6001 (0.045–0.15–0.45 μmol/kg), or PF-03715455 (0.15 μmol/kg) intranasally on days 6 to 11, 1 hour before air or TS exposure. As for the prophylactic study, mice were assumed to weigh 20 g and were treated with 50 μl/mouse to achieve the required doses.

Pharmacokinetics of CHF6001 in Rats

CHF6001 was administered to Sprague-Dawley rats (250–400 g; Charles River Laboratories Italy) by the intratracheal route as micronized powder as described previously. In a separate study, the CHF6001 and roflumilast dry powders were suspended in 5% PEG400/methocel 1% in saline and were administered by oral gavage (5 ml/kg). Plasma and lung samples were collected at 0.25, 1, 2, 4, 8, 16, 24, 48, and 72 hours after the administration of the test compounds (with $n = 3$ for each sampling time). Animals were sacrificed by overdose of anesthetic (4% isoflurane in 100% O_2).

At the time of sacrifice, blood samples were collected in heparinized tubes. The samples were immediately separated by centrifugation at 1200*g* for 15 minutes.

The lungs were excised and washed with saline 3 times. A portion of lung tissue was homogenized into polypropylene tubes in 3 volumes of a acetonitrile/saline solution (50%:50%; v/v) using an Ultra-Turrax homogenizer (IKA-Werke GmbH, Staufen, Germany) at 3000 rpm for 30 seconds at room temperature.

All biologic samples were then transferred into polypropylene tubes and put in the freezer at −80°C until analysis by electrospray liquid chromatography–tandem mass spectrometry using an Alliance Waters high-pressure liquid chromatography (Waters Corporation, Milford, MA) coupled to a ABI-Sciex API2000 (AB Sciex, Framingham, MA) operated in positive ion mode.

Reversal of Ketamine/Xylazine Anesthesia, a Behavioral Correlate of Emesis in the Rat

Adult male Sprague Dawley rats (Charles River Laboratories Italy) were anesthetized with a combination of ketamine (10, 0.2 ml/kg) and xylazine (10, 1 ml/kg) administered by intramuscular injections in the back hind-limbs. Fifteen minutes later, animals were treated intravenously or intratracheally with various doses of test compounds or with the corresponding vehicle and were subsequently placed in dorsal recumbence. The restoration of the righting reflex—that is, when the animal no longer remained on its back and turned itself spontaneously to the prone position—was used as an end point to determine the duration of anesthesia. The experiment was terminated at 120 minutes after drug administration. The animals that did not restore their righting reflex by this time were given a time equal to the maximum amount of time allowed.

For intravenous administration, CHF6001 and GSK-256066 were dissolved in PEG200 immediately before use, which was injected in the tail vein in a dosing volume of 2 ml/kg; roflumilast was dissolved in 40% saline/60% PEG200.

In a separate series of experiments, CHF6001 was administered intratracheally as dry powders as described earlier.

Emesis and Nausea in Ferrets

Male ferrets (*Mustela putorius furo;* Marshall BioResources, North Rose, NY) weighing 0.98–1.50 kg on the day of the experiment were used in this study. The test compounds, except for roflumilast, were suspended in 0.2% Tween 80 in saline and administered at 1.0 ml/kg intratracheally in animals under gaseous anesthesia (4% isoflurane in 100% O_2). A catheter connected to a propelling device was inserted in the trachea up to a predetermined point (2–3 cm above the carina), and the aqueous suspensions were propelled using two shots of 5-ml compressed air. Roflumilast dry powder was suspended in 5% PEG400/methocel 1% in saline and administered by oral gavage (5 ml/kg).

After recovery from anesthesia, the animals were individually placed in transparent Perspex cages and continuously monitored by a trained technician for 4 hours. During that period, episodes of both retching (abdominal contractions without expulsion of part of the gastrointestinal contents) and vomiting (abdominal contractions with expulsion of part of the gastrointestinal contents) were recorded.

During the same period, nausea-like behavior was also evaluated by studying the occurrence of a set of typical behaviors: licking, gagging, chewing, backward walking, head burying in cage shavings, wet dog shake, mouth clawing and prolonged typical ventral recumbency. For each animal, nausea-like global behavior was expressed as the sum of these different behaviors. The maximal not emetic dose was defined as the maximal dose showing no emesis.

14-Day Inhalation Toxicology Study in the Rat

Male Wistar HsdHan rats (Harlan, Bicester, UK) were assigned to experimental groups upon arrival using a total randomization procedure (n = 6/group). Lactose and the test compounds CHF6001 (0.15, 1.45, and 4.4 μmol/kg per day) and GSK-256066 (0.018, 0.18, and 1.8 μmol/kg per day) were administered to each animal intratracheally once daily for up to 14 days, excluding the day of necropsy, as described previously. All animals were observed daily for signs of ill health or overt toxicity. In addition, each animal was given a detailed physical examination at weekly intervals. Individual body weights and food consumption were recorded for all animals.

Blood samples for clinical pathology analysis were drawn from the lateral caudal vein on day 11 and from the abdominal aorta after an overnight period without food on day 15. Urine samples were collected on day 12 (6-hour period) from all animals. Food and water were removed during collection.

The necropsies were performed after an overnight period without food. Each animal was given an intraperitoneal injection of sodium pentobarbitone. Once a suitable deep anesthesia was established, the animal was exsanguinated by the severing of major blood vessels. A full macroscopic examination was performed under the general supervision of a pathologist, and all lesions were recorded. Tissues designated for histopathologic evaluation were embedded in paraffin wax British Pharmacopoeia, and 5 μm sections were stained with hematoxylin and eosin and examined microscopically.

Statistical Analysis

As tests for normality were positive, statistical analysis was performed on raw data using one-way analysis of variance followed by appropriate tests according to the experimental model (as reported in the corresponding figure legend).

The drug-induced individual changes were calculated comparing the drug-treated with the lactose-treated control animals. For time course studies, the comparison was made with time-matched, vehicle-treated controls. The ED_{50} or ED_{30} values and 95% confidence limits were calculated by log-linear regression analysis based on the individual inhibition data.

All data in the text and figures are expressed as mean ± S.E.M. of n replicates or as mean ± S.D. Statistical analyses were performed using GraphPad Prism, version 4.0 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Effect of CHF6001 and Reference Compounds on OVA-Induced Lung Eosinophilia in Rats

Intratracheal treatment with CHF6001, GSK-256066, fluticasone furoate, or budesonide 2 hours before OVA challenge markedly inhibited (37–90%) eosinophilia. Estimated values of ED_{50} of 0.028, 0.025, 0.044, and 0.039 μmol/kg for CHF6001, GSK-256066, fluticasone furoate, and budesonide, respectively, were calculated from the dose-response curves (Fig. 1A).

The duration of action of CHF6001 was evaluated using a dose 3-times the estimated ED_{50}, administered intratracheally 24, 16, 6, and 2 hours before the OVA challenge. The results obtained showed a statistically significant suppression of the eosinophil influx by dosing of CHF6001 up to 24 hours before the OVA challenge, with inhibition ranging from 60 to 80% (Fig. 1B). A similar suppression was observed upon treatment with fluticasone furoate (0.13 μmol/kg), whereas budesonide at a dose 10-fold the estimated ED_{50} caused only a modest (≈40%) inhibition of eosinophil infiltration when administered 24 hours before the OVA challenge (Fig. 1B).

Effect of CHF6001 and Reference Compounds on Forced Maneuvers in OVA-Challenged Brown-Norway Rats

Forced expiratory maneuvers performed 24 hours after the OVA challenge showed the expected decrease in FVC as well as in FEV200 (Fig. 2, A–F). As previously reported elsewhere, these changes were prevented by treatment with budesonide (0.1–3 μmol/kg) (Celly et al., 2006) (Fig. 2, C and D). Intratracheal treatment with increasing doses of CHF6001 (0.01–1 μmol/kg) resulted in a progressive inhibition of the decrease in FVC and FEV200 induced by the OVA challenge up to a significant reversal observed with 1 μmol/kg (Fig. 2, A and B). GSK-256066 (0.1–1 μmol/kg) and budesonide (0.1–3 μmol/kg) also prevented the changes in lung mechanics, with an efficacy similar to that observed for CHF6001 (Fig. 2, C–F).

Effect of CHF6001 and Reference Compound on Neutrophilia Induced by Cigarette Smoke Exposure in Mice

Prophylactic Study. When compared with the vehicle-treated air-exposed group, mice exposed to TS exhibited a significant increase (fold increase: 9.9) in the total number of cells recovered in BAL at sacrifice on day 12 (24 hours after the last TS exposure). This cellular influx consisted of macrophages (fold increase: 8.5), neutrophils (fold increase: 75.9) and lymphocytes (fold increase: 17.3). Daily intranasal treatment of mice with CHF6001 (0.15–0.45 μmol/kg per day) started at the same time of TS exposure, caused a statistically significant decrease of neutrophil infiltration, similar to that observed with the potent and selective inhaled p38 MAP kinase inhibitor PF-03715455 (Millan et al., 2011). No difference in efficacy was observed when CHF6001 was administered either once or twice daily (Fig. 3A). A statistically significant inhibition of TS-induced increase of macrophages and lymphocytes was also observed (data not shown).

Interventional Study. When compared with the vehicle-treated air-exposed group, TS exposure significantly and time dependently increased the number of macrophages (fold increase: 7.1) and neutrophils (fold increase: 88.3) recovered

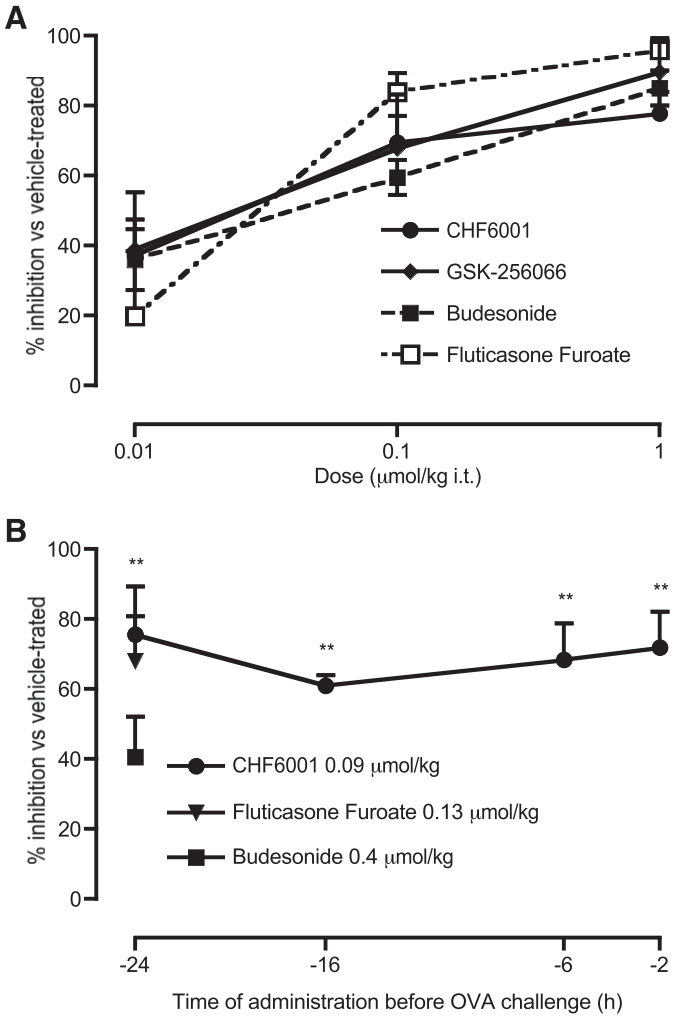

Fig. 1. Effect of CHF6001 and reference compounds on OVA-induced lung eosinophilia in rats. (A) CHF6001, GSK-256066, fluticasone furoate, or budesonide (0.01–1 μmol/kg) were administered intratracheally as micronized dry powder 2 hours before antigen challenge. BAL fluids were collected 24 hours after challenge, and total and differential cell counts were performed. (B) Compounds were administered intratracheally 2, 6, 16, and 24 hours (CHF6001, 0.09 μmol/kg) or 24 hours (fluticasone furoate, 0.13 μmol/kg, or budesonide, 0.4 μmol/kg) before the antigen challenge. BAL fluids were collected 24 hours after the challenge, and total and differential cell counts were performed. Values are expressed as the mean ± S.E.M. (n = 3–4) of the percentage of inhibition of eosinophil infiltration when compared with vehicle + OVA control group. Statistical analysis was performed by one-way analysis of variance followed by Dunnett's test for multiple comparisons versus vehicle-treated animals. GSK-256066, budesonide, and fluticasone furoate (0.01 μmol/kg), not statistically significant versus vehicle-treated animals; CHF6001 (0.01 μmol/kg), $P < 0.05$ versus vehicle-treated animals; GSK-256066 and budesonide (0.1 μmol/kg), $P < 0.05$ versus vehicle-treated animals; CHF6001 and fluticasone furoate (0.1 μmol/kg), $P < 0.01$ versus vehicle-treated animals; CHF6001, GSK-256066, budesonide, and fluticasone furoate (1 μmol/kg), $P < 0.01$ versus vehicle-treated animals. $^{**}P < 0.01$.

via BAL. When administered intranasally to mice already showing a marked neutrophil infiltrate resulting from a 5-day exposure to TS, CHF6001 (0.045–0.45 μmol/kg per day) was able not only to prevent the additional worsening resulting from the final 6 days of TS exposure but also to reverse the infiltration of inflammatory cells already established (Fig. 3B). The efficacy of CHF6001 was again comparable to that of the p38 inhibitor used as reference compound.

Analysis of Lung and Plasma Concentration of CHF6001 and Reference Compound after Intratracheal and Oral Administration in Rats

Intratracheal administration of CHF6001 (0.1–1 μmol/kg) resulted in dose-related, sustained concentrations of the compound in lung tissue, with significant concentrations of CHF6001 observed up to 72 hours after exposure to the highest dose (mean residence time 26 hours), supporting the long-lasting protective effect observed in OVA-challenged Brown-Norway rats and confirming CHF6001 as a compound well suited for topical administration (Fig. 4A). On the contrary, CHF6001 upon intratracheal administration showed a very limited systemic bioavailability, with plasma concentrations several orders of magnitudes lower than lung concentrations (Fig. 4A). Interestingly, oral administration of CHF6001 (1 μmol/kg) resulted in a bioavailability <4%, with plasma concentrations 100- to 1000-fold lower than the concentrations of roflumilast and roflumilast *N*-oxide, suggesting that the potential contribution to systemic bioavailability of the compound ingested upon inhaled administration may be negligible (Fig. 4B).

Effect of CHF6001 and Reference Compounds on Nausea, Emesis, and Its Behavioral Surrogate in Rats, the Reversal of Ketamine/Xylazine Anesthesia

It has been postulated that PDE4 inhibitors may trigger emesis by mimicking the activity of α_2-adrenergic antagonists, and according to this hypothesis PDE4 inhibitors have been shown to dose dependently reverse the hypnotic effect of an α_2-adrenoceptor–mediated anesthetic regimen in rats and ferrets (Robichaud et al., 2001, 2002a). In agreement with these results, intravenous administration of roflumilast (0.1–3 μmol/kg) caused a statistically significant reversal of ketamine/xylazine anesthesia in rats, underlining its ability to affect centrally located PDE4 enzymes, thus increasing cAMP concentrations and counteracting the effect of α_2 agonists (Fig. 5). In contrast, intravenous administration of neither CHF6001 nor GSK-256066 (0.1–3 μmol/kg) affected the duration of the anesthesia in a statistically significant manner. Similarly, intratracheal administration of CHF6001 (1–5 μmol/kg) (Fig. 5) did not affect ketamine/xylazine anesthesia, reflecting its extremely limited ability to cross the blood-brain barrier (as suggested by the low cerebral concentration achieved; data not shown) and, possibly, its ability to preferentially bind low-affinity rolipram binding conformations of the PDE4 enzymes versus high-affinity state (see the companion article, Moretto et al., 2015). This latter form of the enzyme is present in the brain but not in peripheral tissues (Barnette et al., 1996; Rocque et al., 1997), and may contribute to the emetic effect.

Emesis and nausea-like effects were also evaluated in ferrets. Oral administration of roflumilast (1–10 μmol/kg) caused a dose-related and marked increase in the occurrence of emetic episodes, but no effect was associated with the intratracheal administration of CHF6001 (1–10 μmol/kg) (Fig. 6), even in presence of significant systemic exposure (C_{max} 661 pmol/ml at the dose of 10 μmol/kg). Interestingly, at the highest dose tested (10 μmol/kg), GSK-256066 departed from what had been observed with CHF6001, inducing emetic episodes (Fig. 6).

Furthermore, the evaluation of nausea-like effects (described in ferret as licking, gagging, chewing, backward walking, head

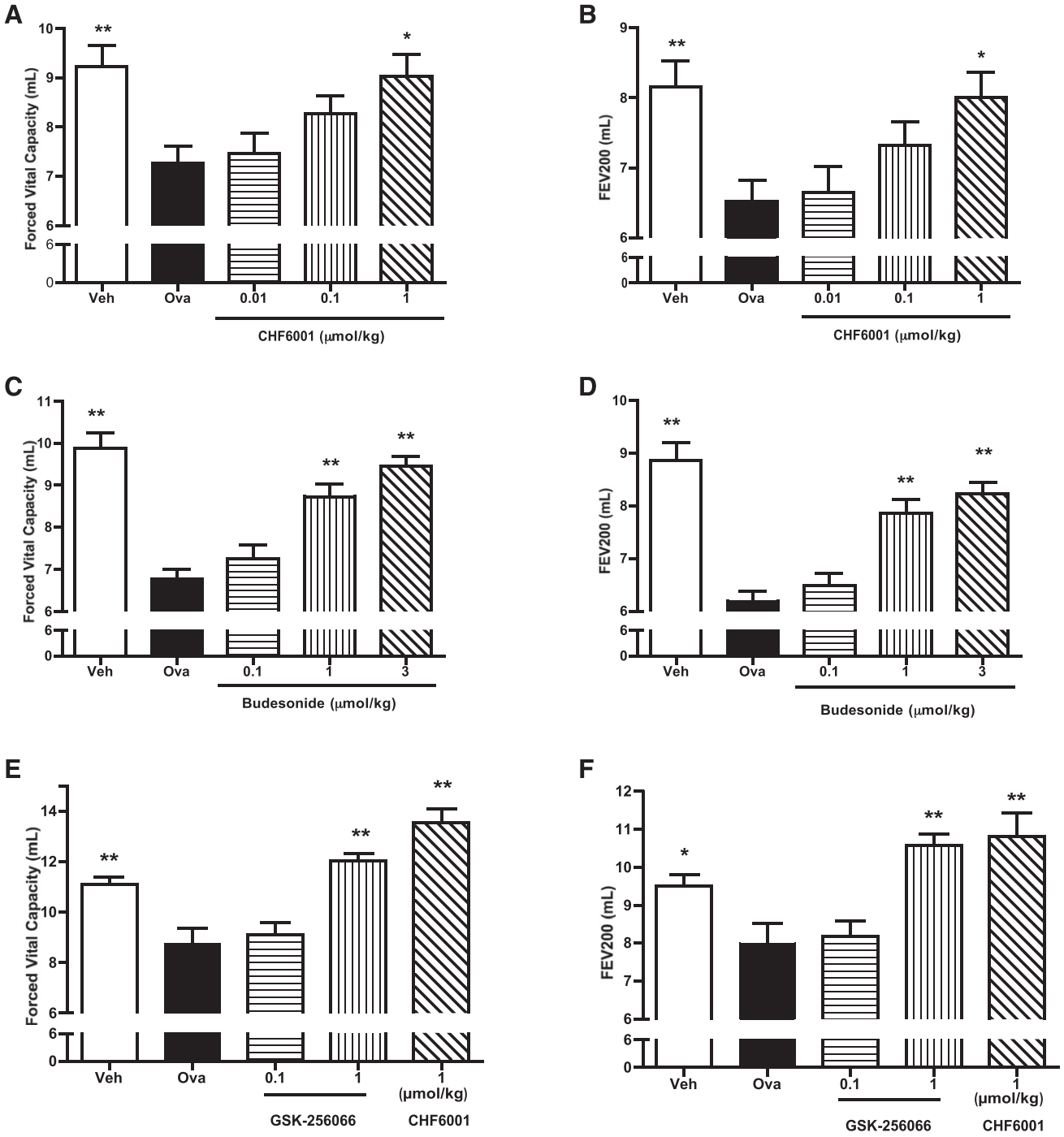

Fig. 2. Effect of CHF6001 and reference compounds on forced maneuvers in OVA-challenged Brown-Norway rats. Animals were treated with dry powders (micronized drugs plus lactose or lactose alone) at different dose levels 5 hours before the antigen challenge. Twenty-four hours after OVA challenge, respiratory parameters were recorded using a forced pulmonary maneuver system as described in *Materials and Methods*. Effects of CHF6001 (0.01–1 μmol/kg) on FVC (A) and FEV200 (B), effects of budesonide (0.1–3 μmol/kg) on FVC (C) and FEV200 (D), and effects of GSK-256066 (0.1–1 μmol/kg) on FVC (E) and FEV200 (F). Values are expressed as mean ± S.E.M. (n = 6–12). Statistical analysis was performed by one-way analysis of variance followed by Dunnett's test for multiple comparisons versus vehicle-treated animals. $^*P < 0.05$ and $^{**}P < 0.01$ for treatment groups compared with vehicle-treated animals.

burying in cage shavings, wet-dog shake, mouth clawing, and prolonged typical ventral recumbence) showed a clearcut difference in tolerability between GSK-256066 and CHF6001: no statistically significant effects were observed for the latter compound up to 20 μmol/kg, but a dose-response increase in nausea-like behavior was evident for GSK-256066 and was statistically significant from the dose of 1 μmol/kg (Fig. 7, right axis). Comparison of the dose-response curves of both compounds relative to the inhibition of eosinophil infiltration in OVA-challenged rats (Fig. 7, left axis) with those relative to nausea-like behaviors (Fig. 7, right axis) showed a wide separation of the therapeutic effect from the adverse effect for

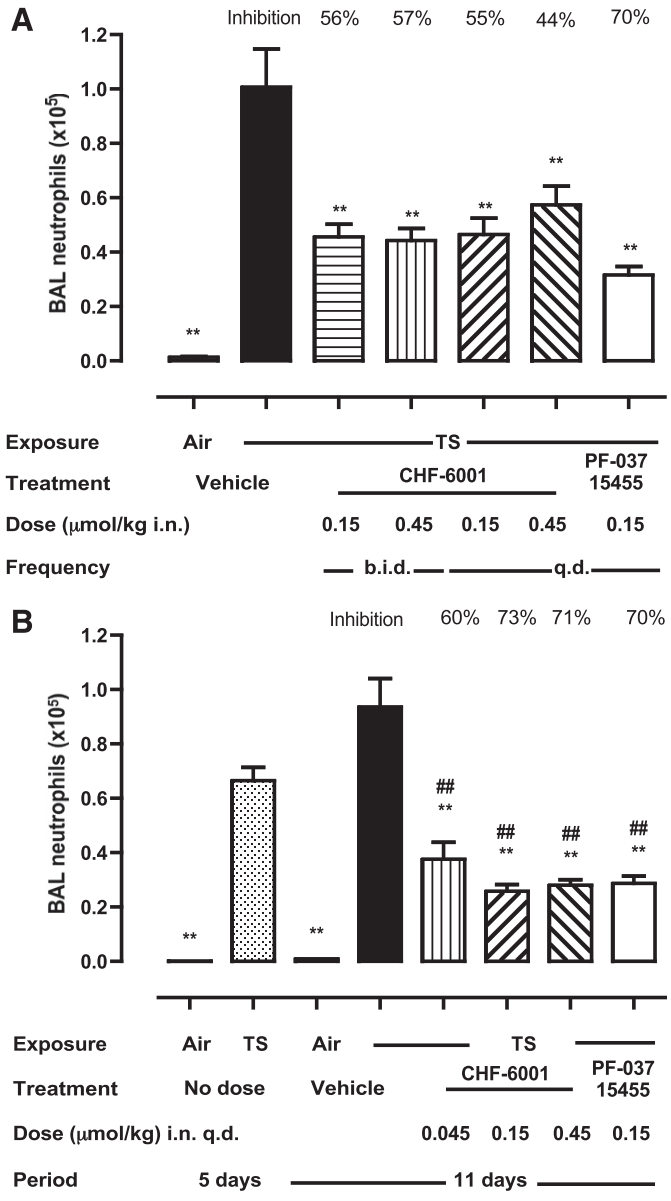

Fig. 3. Effect of CHF6001 and reference compound on neutrophilia induced by TS exposure in mice. (A) CHF6001 (0.15–0.45 μmol/kg each day, 1 hour before TS exposure; or twice a day 1 hour before and 6 hours after TS exposure), or PF-03715455 (0.15 μmol/kg each day, 1 hour before TS exposure) were administered intranasally starting the same day of TS exposure that lasted 11 days. (B) CHF6001 (0.045–0.45 μmol/kg each day, 1 hour before TS exposure), or PF-03715455 (0.15 μmol/kg each day, 1 hour before TS exposure) were administered intranasally on days 6–11 of TS exposure. BAL was collected 24 hours after the last TS exposure, and differential cell counts were performed. Values are expressed as the mean ± S.E.M. (n = 9–10) of the number of neutrophils recovered in BAL. Statistical analysis was performed by one-way analysis of variance followed by Bonferroni correction for multiple comparisons between treatment groups. $^{**}P < 0.01$ for treatment groups compared with vehicle + TS (11 days) control group. $^{\#\#}P < 0.05$ for treatment groups compared with vehicle + TS (5 days) control group.

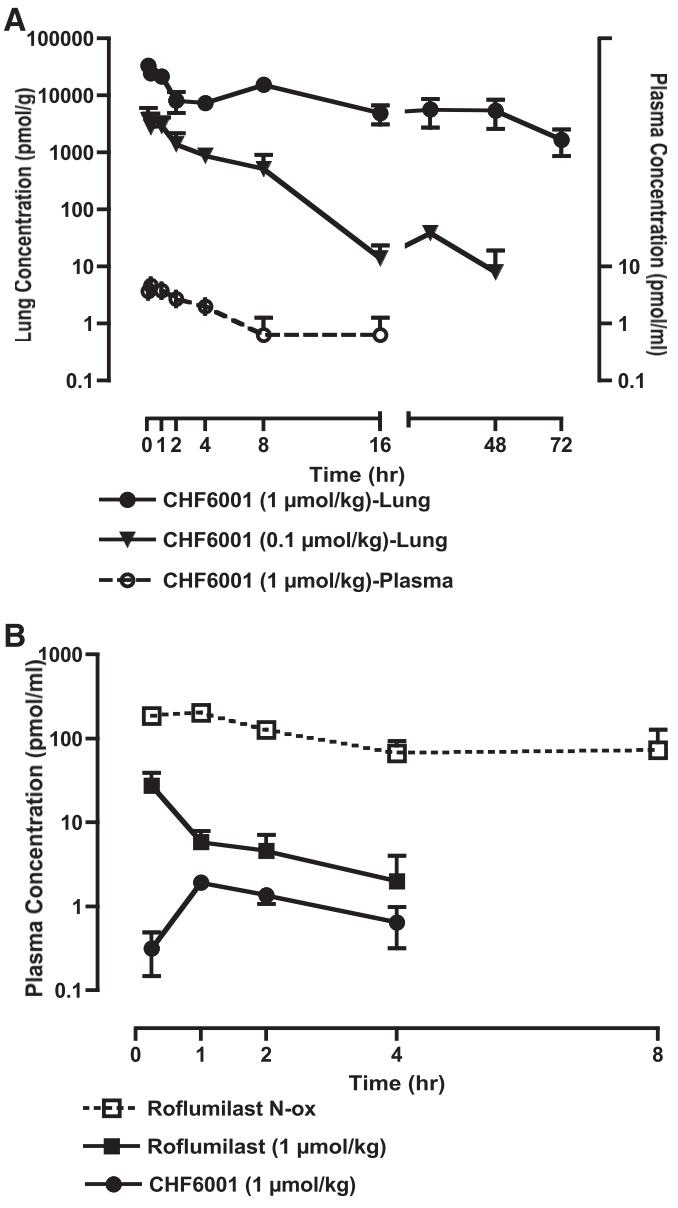

Fig. 4. Analysis of lung and plasma concentration of CHF6001 and reference compound after intratracheal and oral administration in rats. (A) CHF6001 (0.1–1 μmol/kg) was administered intratracheally as micronized dry powder. Lung (left y-axis) and plasma (right y-axis) concentrations were evaluated at 0.25, 1, 2, 4, 8, 16, 24, 48, and 72 hours after the administration of CHF6001. (B) CHF6001 (1 μmol/kg) and roflumilast (1 μmol/kg) were administered orally by gavage. Plasma samples were collected at 0.25, 1, 2, 4, 8, and 1 hours after the administration of test compounds. Concentrations of CHF6001, roflumilast, and the roflumilast active compound roflumilast N-oxide were determined by liquid chromatography–tandem mass spectrometry and expressed as mean ± S.D. of pmol/g of tissue (lung) or pmol/ml (plasma). The limit of quantitation for CHF6001 was 1.16 pmol/g in lung tissue and 0.29 pmol/ml in plasma.

CHF6001 only, suggesting a potentially improved therapeutic index for CHF6001 when compared with GSK-256066. Although no actual therapeutic index can be estimated, as two different species were used for efficacy and tolerability evaluations, it is still worth observing the differences in tolerability between CHF6001 and GSK-256066.

14-Day Intratracheal Toxicology Study of CHF6001 and Reference Compound in the Rat

After 14 days of daily intratracheal administration, CHF6001 was well tolerated at all doses tested, and no effects were observed for clinical signs, clinical chemistry, hematology, or at necropsy. A slight reduction in body weight gain and food consumption was observed at the two highest doses

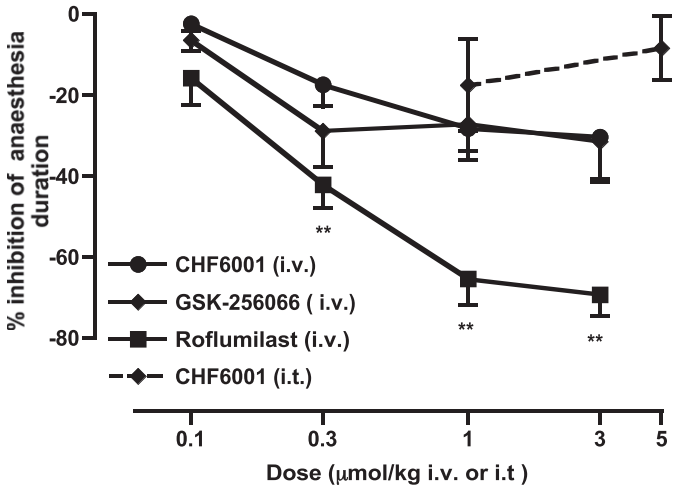

Fig. 5. Reversal of ketamine/xylazine anesthesia by CHF6001 and reference compounds: effect of the intravenous administration of CHF6001 (0.1–3 μmol/kg), GSK-256066 (0.1–3 μmol/kg), and roflumilast (0.1–3 μmol/kg) and the intratracheal administration of CHF6001 (1–5 μmol/kg) on the duration of ketamine/xylazine anesthesia. Rats anesthetized with a combination of ketamine (10, 0.2 ml/kg) and xylazine (10, 1 ml/kg) were treated intravenously or intratracheally with various doses of test compounds or with the corresponding vehicle and were subsequently placed in dorsal recumbency. The restoration of the righting reflex was used as an end point to determine the duration of anesthesia. Values are expressed as the mean ± S.E.M. (n = 3) of the percentage of inhibition of the duration of anesthesia observed in vehicle-treated compounds. Statistical analysis was performed by one-way analysis of variance followed by Dunnett's test for multiple comparisons versus vehicle-treated animals. $^{**}P < 0.01$ for treatment groups compared with vehicle-treated animals.

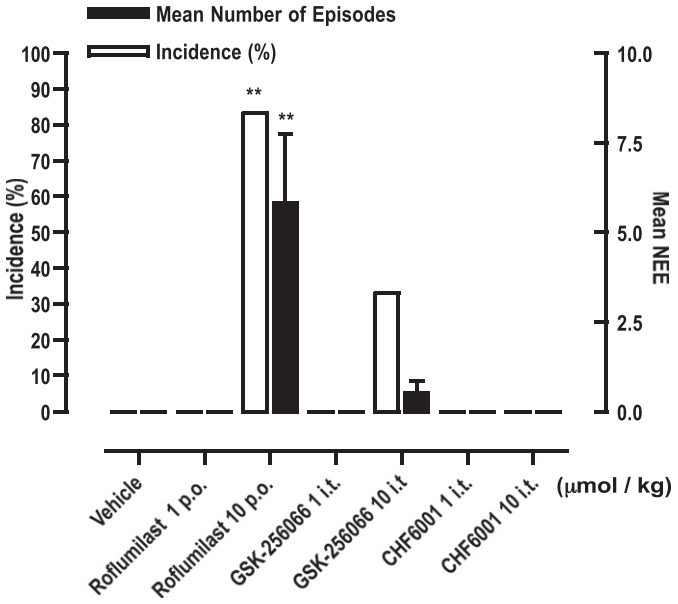

Fig. 6. Induction of emesis by CHF6001 and reference compounds in conscious ferrets. Roflumilast (1–10 μmol/kg p.o.), GSK-256066 (1–10 μmol/kg i.t.), or CHF6001 (1–10 μmol/kg i.t.) were administered, and retching and vomiting were recorded over a period of 4 hours. Data are expressed as the percentage incidence and mean number of episodes ± S.D. Statistical analysis was performed by one-way analysis of variance followed by Fischer's exact test. $^{*}P < 0.05$, $^{**}P < 0.01$ for treatment groups compared with vehicle-treated animals.

tested (1.45 and 4.4 μmol/kg per day). Microscopically, lung (alveolar type 2 cells vacuolization) and heart (infiltration of inflammatory cells) effects were observed in 2 out of 6 animals treated with CHF6001 at 1.45 μmol/kg per day, but these findings were not considered to be of toxicologic relevance; they were likely a result of the rather aggressive nature of the administration procedure combined with the repeated anesthesia. Indeed, at the highest dose tested, these findings did not progress significantly and were still not considered adverse effects.

Animals receiving 0.18 and 1.8 μmol GSK-256066/kg per day showed reduced body weight gain (41% when compared with control group) or significant body weight loss (approximately 6%), respectively. This latter loss correlated with a slight reduction in food consumption. Treatment-related pathologic changes were observed in several tissues. Macroscopic distension and/or thick jejunum, ileum, and/or colon were observed at 1.8 μmol/kg per day, which correlated with histopathologic changes including distension, enteritis, colitis, and/or inflammatory cell infiltrate in the ileum, cecum, colon, and/or rectum. In the duodenum, pallor of the Brunner's gland was observed at 0.018 μmol/kg per day and above. Mesenteritis was observed at 1.8 μmol/kg per day, and portal inflammatory cells in the liver were observed at 0.18 or 1.8 μmol/kg per day. In the trachea, peritracheal inflammation and/or arteritis were observed at 0.018 μmol/kg per day and above, and arteritis in the lung was observed at 1.8 μmol/kg per day (Table 1).

In the parallel toxicokinetic evaluation, 7 out 35 animals receiving GSK-256066 at the dose of 1.8 μmol/kg per day died, and gastrointestinal lesions were considered to be the major responsible cause of death of five animals.

These results highlighted that after 14 days of dosing, the no-observed-adverse-effect level (NOAEL) for CHF6001 was 4.4 μmol/kg per day, while for GSK-256066 the NOAEL was lower than 0.018 μmol/kg per day, providing additional evidence of a significant difference between these two compounds in terms of safety.

Discussion

Our study provides an extensive profile of the in vivo efficacy and safety of CHF6001, a novel, extremely potent PDE4 inhibitor (see the companion article, Moretto et al., 2015) specifically developed for topical administration to the airways as a result of an internal discovery and optimization program (Armani et al., 2014).

CHF6001 was tested by intratracheal or intranasal administration in different animal models of pulmonary inflammation and showed potent anti-inflammatory activities, very similar to that of the extremely potent, topically active PDE4 inhibitor GSK-256066 (Nials et al., 2011). Intratracheal administration of CHF6001 yielded high pulmonary concentrations of the compound up to 48–72 hours after dosing, supporting the observed duration of action of 24 hours, a time point at which GSK-256066 was reported to be inactive (Nials et al., 2011). Systemic bioavailability of CHF6001 upon intratracheal administration was extremely low, and even more so when the drug was given orally, negating the potential systemic absorption of orally ingested drug after mouth deposition during inhaled CHF6001 administration. Finally, CHF6001 was very well tolerated at doses well above the maximally effective dose of 1 μmol/kg. The resulting profile is that of an extremely potent, selective, and topically active PDE4 inhibitor with a superior gastrointestinal tolerability.

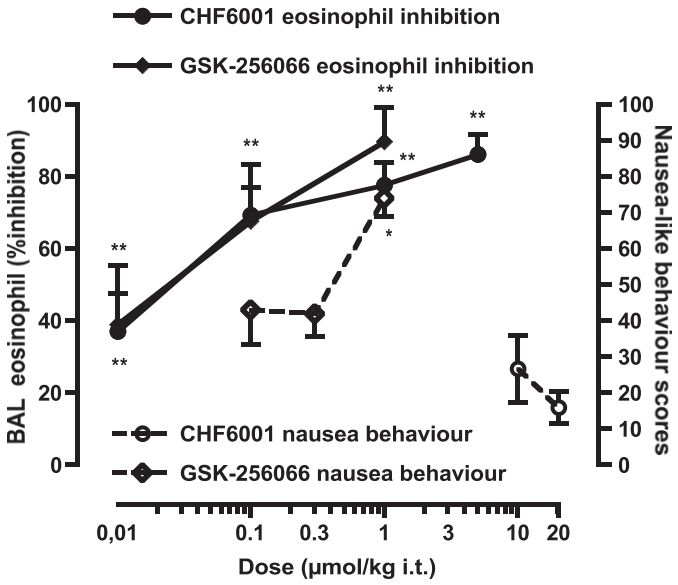

Fig. 7. Inhibition of OVA-induced airways eosinophilia in OVA-sensitized Brown-Norway rats and induction of nausea-like behaviors in conscious ferrets by CHF6001 and GSK-256066. The dose-response inhibition curves of OVA-induced airways eosinophilia (solid lines, left *y*-axis) by CHF6001 (0.01–5 μmol/kg i.t.) and GSK-256066 (0.01–1 μmol/kg i.t.) are compared with the dose-response induction of nausea-like behaviors in conscious ferrets (dashed lines, right *y*-axis) by the same compounds (CHF6001, 10–20 μmol/kg; GSK-256066, 0.1–1 μmol/kg). Nausea-like symptoms were scored by counting the number of typical behaviors, such as licking, gagging, chewing, backward walking, head burying in cage shavings, wet-dog shake, mouth clawing, and prolonged typical ventral recumbency. Values are expressed as the mean ± S.E.M. (n = 3). Statistical analysis was performed by one-way analysis of variance followed by Dunnett's test for multiple comparisons versus vehicle-treated animals (for the inhibition of airway eosinophil infiltration) and Holm-Sidak test (for nausea-like behavior score). $^{*}P < 0.05$, $^{**}P < 0.01$ for treatment groups compared with vehicle-treated animals.

OVA sensitization and challenge in Brown-Norway rats represents a well established model reproducing many features of human allergic asthma (Elwood et al., 1991), including airways eosinophilia (Renzi et al., 1993) and bronchial hyperresponsiveness to metacholine (Bellofiore and Martin, 1988). CHF6001 markedly inhibited the pulmonary eosinophilia observed in OVA-challenged Brown-Norway rats with a potency similar to GSK-256066 and to the inhaled corticosteroids fluticasone furoate and budesonide. Using a dose 3 times the ED_{50}, a significant inhibition of airways eosinophilia was still observed up to 24 hours after the intratracheal administration of CHF6001 and fluticasone furoate, a reference once-daily steroid, whereas the activity of budesonide (at a dose 10-times the ED_{50}) was found to be significantly lower. This result provided support for the potential use of CHF6001 once daily in the clinic.

The inhalation of toxic particles and gases, mostly TS, is the main risk factor for COPD, and mouse models of smoke-induced inflammatory responses have been developed as tools for the evaluation of drugs potentially active in COPD (Miller et al., 2002; Wright and Churg, 2002; Eltom et al., 2013). Neutrophil infiltration is an early feature of acute pulmonary phlogistic response, but an increased number of neutrophils are consistently found in the airways of smokers as well as of COPD subjects, where they may play a central role in many features of COPD (Stockley, 2002). Indeed, orally active PDE4 inhibitors, such as roflumilast, were shown to be able to fully prevent emphysema induced by cigarette smoke (CS) associated with long-term CS exposure in mice (Martorana et al., 2005), and to inhibit CS-dependent pulmonary inflammation (Kubo et al., 2011). CHF6001, administered intranasally, was found to be active in preventing as well as in resolving the inflammatory cell airways infiltration associated with a subacute exposure to CS, an animal experimental model known to be insensitive to steroid treatment (Wan et al., 2010).

Together with the reported efficacy in models of respiratory diseases, a low systemic exposure is important in determining the therapeutic index of a PDE4 inhibitor, as emesis and gastrointestinal disturbances are directly associated with its mechanism of action. Oral administration of CHF6001 did not generate biologically active metabolites (Armani et al., 2014) and resulted in very limited bioavailability (<4%), with plasmatic concentrations orders of magnitude lower than those of roflumilast or its bioactive metabolite roflumilast *N*-oxide upon administration of similar doses (Bundschuh et al., 2001), therefore limiting the potential systemic exposure associated with swallowing of the drug deposited in the oral cavity during the inhalation process and improving the pulmonary selectivity of the compound.

After intratracheal administration, the lung concentrations of CHF6001 were over 1000-fold higher than the plasma levels, and significant concentrations could be measured in lung tissue up to 48–72 hours after dosing, with an estimated mean residence time of 26 hours, well in agreement with the

TABLE 1
Incidence of selected histopathologic findings in the 14-day inhalation toxicologic study in rats

Tissue	Incidence							
	CHF6001				GSK-256066			
	0	0.15	1.45	4.4	0	0.018	0.18	1.8
	μmol/kg per day							
Duodenum								
Brunner's gland apical pallor	0/0	0/0	0/0	0/0	0/6	1/6	3/6	4/6
Mesenteric fat								
Mesenteritis	0/0	0/0	0/0	0/0	0/6	0/6	0/6	4/6
Trachea								
Arteritis	0/0	0/0	0/0	0/0	0/6	1/6	3/6	6/6
Liver								
Portal inflammatory cells	0/0	0/0	0/0	0/0	0/6	1/6	0/6	5/6

results of duration of action obtained in OVA-challenged Brown-Norway rats.

These results indicating a very low systemic exposure and extreme anti-inflammatory potency when administered topically within the airways, taken together with a high high-affinity rolipram-binding conformer/low-affinity rolipram-binding site conformer inhibitory activity ratio (see the companion article, Moretto et al., 2015) and high plasma protein binding (Armani et al., 2014) hold promise for an improved therapeutic index for CHF6001 compared with available comparators. Although the mechanisms mediating the gastrointestinal side effects of PDE4 inhibitors are not conclusively elucidated, they have been shown to be also linked to a noradrenergic pathway (Robichaud et al., 2001), as PDE4 inhibitors appear to functionally mimic the activity of α_2-adrenoceptor antagonists, and emesis in ferrets induced by PDE4 inhibitors is preventable by pretreatment with α_2-adrenoceptor agonists (Robichaud et al., 2001). This was also supported by the reversal of xylazine/ketamine-induced anesthesia observed upon administration of PDE4 inhibitors in rats (Robichaud et al., 1999, 2002a), an effect that has since been proposed to be a functional correlate of PDE4 inhibitor-induced emesis in nonvomiting species. Indeed, intravenous administration of roflumilast significantly reverted xylazine/ketamine-induced anesthesia, whereas CHF6001 and GSK-256066 were devoid of significant effects up to 3 μmol/kg i.v. Upon intratracheal administration, CHF6001 did not affect anesthesia up to 5 μmol/kg, a dose higher than those resulting in maximal anti-inflammatory activity in rats.

A direct estimate of the emetogenic activity in conscious ferrets (Kuss et al., 2003; Nials et al., 2011) was done by evaluating both vomiting and nausea-related behaviors. CHF6001 was completely emetogenic inactive up to the dose of 10 μmol/kg, confirming its low emetogenic potential. Direct comparison with GSK-256066 showed an improved tolerability profile for CHF6001, with GSK-256066 showing nausea-like behaviors and emesis at doses between 1 and 10 μmol/kg. Furthermore, the results of a preliminary toxicologic screening of CHF6001 showed an absence of significant toxic effects after 2 weeks of treatment with intratracheal doses up to 4.4 μmol/kg per day in rats; similar results were obtained in dogs at doses up to 1.15 μmol/kg per day, where no emesis or major pathologic changes typical of this class of compounds—such as disseminated vasculitis and interstitial inflammation often in the gastrointestinal apparatus—were observed (L. Battipaglia, L. Preynat, unpublished observations). In contrast, a NOAEL dose could not be established in the rat for GSK-256066 because even the lowest dose tested (0.018 μmol/kg per day) caused treatment-related pathologic changes. These results indicate that GSK-256066, apart from the effects on emesis, possesses a suboptimal safety profile, possibly leading to a low dose of GSK-256066 being used in phase II clinical trials (87.5 μg/day) as well as to limited, not significant anti-inflammatory activity in COPD patients (Watz et al., 2013).

In line with these observations, in a recent safety and tolerability study in healthy volunteers, CHF6001, administered for 7 days as an inhaled dry powder formulation, proved to be well tolerated up to doses of 1.6 mg/day (Esposito et al., 2013).

Taken together, the potent activity of CHF6001 in a number of preclinical models of pulmonary inflammation and the lack of emesis upon intratracheal administration all point to an improved therapeutic index for this novel, topically active PDE4 inhibitor. GSK-256066 was recently reported to be more potent than all the other PDE4 inhibitors described to date (Tralau-Stewart et al., 2011), and it has been characterized by an improved therapeutic index compared with orally administered compounds. Head-to-head comparison with GSK-256066 in terms of anti-inflammatory activity, emesis, and acute toxicology suggests that CHF6001 may exceed the therapeutic index of the comparator, finally allowing for estimate of the full potential of PDE4 inhibitors as anti-inflammatory in pulmonary diseases.

Given its excellent pharmacologic potency and efficacy as topical anti-inflammatory agent, in conjunction with low emetic potency in ferrets and the excellent toxicologic profile in rat, CHF6001 holds promise as a novel inhaled PDE4 inhibitor for treating lung inflammatory diseases, such as asthma and COPD. In line with its preclinical profile, CHF6001 was shown to be active against allergen challenge in mild asthmatic subjects at the dose of 1.2 mg/day (Singh et al., 2014). At the same dose, an anti-inflammatory effect on sputum biomarkers was also observed after a 4-week treatment in COPD patients (M. Govoni, G. Lucci, A. Nandeuil, unpublished observation). The testing of CHF6001 in phase IIb clinical trials will be carried out in COPD patients.

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Conducted experiments: Bolzoni, Bassani, Caruso, Bergamaschi, Pisano, Puviani, Stellari, Volta, Bertacche.

Contributed new reagents or analytic tools: Mileo, Moretti, Bagnacani, Bertacche.

Performed data analysis: Villetti, Carnini, Battipaglia, Cenacchi.

Wrote or contributed to the writing of the manuscript: Villetti, Carnini, Cenacchi, Sala, Civelli.

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