

**CHF6001, a novel highly potent and selective phosphodiesterase 4 inhibitor with
robust anti-inflammatory activity and suitable for topical pulmonary
administration**

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ABSTRACT

This study describes the pharmacological characterization of (S)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridine 1-oxide (CHF6001), a novel PDE4 inhibitor, designed for treating pulmonary inflammatory diseases via inhaled administration. CHF6001 was 7- and 923-fold more potent than roflumilast and cilomilast respectively, in inhibiting PDE4 enzymatic activity ($IC_{50}=0.026\pm 0.006$ nM). CHF6001 inhibited PDE4 isoforms A-D with equal potency, showed an elevated high (HARBS) versus low (LARBS) affinity rolipram binding site ratio (>40) and displayed $>20,000$ -fold selectivity versus PDE4 in comparison with a panel of PDEs. CHF6001 effectively inhibited (sub-nanomolar IC_{50} s values) the release of tumor necrosis factor- α (TNF- α) from human peripheral blood mononucleate cells (PBMCs), THP-1 monocyte-derived macrophages and rodent macrophages (RAW 264.7 and NR8383). Moreover, CHF6001 potently inhibited the activation of neutrophils and eosinophils (fMLP-evoked oxidative burst), neutrophil chemotaxis and the release of interferon- γ (IFN- γ) from CD4+ T cells. In all these functional assays, CHF6001, was more potent than previously described PDE4 inhibitors, including roflumilast, UK-500,001 and cilomilast, and comparable to GSK256066. When administered intratracheally to rats as a micronized dry powder, CHF6001 inhibited LPS-induced pulmonary neutrophilia ($ED_{50}=0.205$ μ moles/kg) and leukocytes infiltration ($ED_{50}=0.188$ μ moles/kg) with an efficacy comparable to a high dose of budesonide (1 μ mole/kg, i.t.). In sum, CHF6001 has the potential to be an effective topical treatment for conditions associated with pulmonary inflammation, including asthma and chronic obstructive pulmonary disease (COPD).

INTRODUCTION

Phosphodiesterases (PDEs) form a superfamily of at least 11 intracellular iso-enzymes which are involved in the modulation of signal transduction processes via the degradation of cyclic nucleotides (cAMP and/or cGMP) (Bender and Beavo, 2006). Type 4 cyclic nucleotide phosphodiesterases (PDE4) are a family of cAMP-specific PDEs encoded by four genes (PDE4A, PDE4B, PDE4C, and PDE4D) sharing a highly conserved catalytic domain and abundantly expressed in leukocytes (Srivani et al., 2008). By increasing intracellular cAMP levels, PDE4 inhibitors show a broad spectrum of anti-inflammatory effects in almost all cells of the immune system. In particular PDE4 is a major player in regulating pro-inflammatory cellular functions, such as proliferation and cytokine secretion, chemotaxis, degranulation, antibody IgE release and generation of lipid mediators (Spina, 2003). PDE4 regulates also the function of several structural cells that control lung functions such as airway smooth muscle, airway epithelium, vascular endothelium and airway sensory nerves (Fan Chung, 2006). This, together with a large body of evidence supporting potent anti-inflammatory activity of PDE4 inhibitors in various experimental models, including models of pulmonary inflammation (Hatzelmann et al., 2010), has generated a considerable interest in targeting PDE4 in chronic inflammatory and obstructive airway diseases (Spina, 2003). Clinical investigations of second-generation oral PDE4 inhibitors, e.g., cilomilast, Ariflo (Barnette et al., 1998), and roflumilast, Daxas (Hatzelmann et al., 2001), have demonstrated efficacy in chronic lung diseases such as chronic obstructive pulmonary disease (COPD) and asthma (Press and Banner, 2009). However, the development of these compounds has been hampered by dose limiting adverse events which are mechanism-related, in particular gastrointestinal disturbances such as nausea, diarrhea, abdominal pain, vomiting and dyspepsia (Calvelry et al., 2009). Roflumilast has recently been

approved in EU and USA for once-daily treatment of severe COPD associated with chronic bronchitis and frequent exacerbations as add-on to bronchodilator treatment. However, target-related gastrointestinal side effects may limit roflumilast dosage and thus optimal clinical efficacy (Giembycz, 2010; Page and Spina, 2012). In line with this consideration, the active doses of roflumilast determined in experimental models (Martorana et al., 2005; Hatzelmann et al., 2010) hardly support the daily dose of 0.5 mg approved in COPD patients.

In the respiratory field, a logical way to improve the therapeutic index is to deliver drugs directly into the lung via the inhalation route allowing high topical efficacy coupled with low oral availability and limited systemic exposure. The PDE4 inhibitor UK-500,001 developed for inhaled administration was tested in a double-blind, placebo-controlled, 6-week trial in 209 patients with moderate-to-severe COPD but with disappointing results and the study was stopped following a planned interim analysis for futility (Vestbo et al., 2009). Lack of efficacy in phase II trials in asthma and COPD led to the discontinuation also of AWD 12-281 and tofomilast (Pages et al., 2009). To date, the best in class among inhaled PDE4 inhibitors is GSK256066, which appears to be more potent than all the other PDE4 inhibitors described so far (Nials et al., 2011; Tralau-Stewart et al., 2011), is well tolerated in COPD patients (Watz et al., 2013) and is effective in reducing allergen challenge responses in asthma patients (Singh et al., 2010).

In the present study we report the pharmacological characterization of (S)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridine 1-oxide (CHF6001), a novel highly potent and selective inhaled PDE4 inhibitor which was previously selected and optimized for inhaled delivery (Armani et al., 2013). In addition to defining its profile as PDE4 inhibitor, we extensively studied anti-inflammatory activities of CHF6001 in several *in vitro* cellular models

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(neutrophils, lymphocytes, monocytes, macrophages) in direct comparison with two oral, roflumilast and cilomilast, and two inhaled, GSK256066 and UK500,001, PDE4 inhibitors. Moreover the anti-inflammatory potency and efficacy of CHF6001 was demonstrated *in vivo* in a rat model of endotoxin-induced acute pulmonary inflammation. Some of these data were presented in a poster form at the 2014 American Thoracic Society conference (Moretto et al., 2014).

Materials and Methods

Chemicals

(S)-3,5-dichloro-4-(2-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3-(cyclopropylmethoxy)-4-(methylsulfonamido)benzoyloxy)ethyl)pyridine 1-oxide, CHF6001 (Armani et al., 2014), 6-((3-(dimethylcarbamoyl)phenyl)sulfonyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide, GSK256066 (Tralau-Stewart et al., 2011), 3-(cyclopropylmethoxy)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide, roflumilast (Hatzelmann et al., 2001), (1s,4s)-4-cyano-4-(3-(cyclopentyloxy)-4-methoxyphenyl)cyclohexane-1-carboxylic acid, cilomilast (Pagès et al., 2009), 6-((3-(dimethylcarbamoyl)phenyl)sulfonyl)-4-((3-methoxyphenyl)amino)-8-methylquinoline-3-carboxamide, UK-500,001 (Pagès et al., 2009), were synthesized at Chiesi Farmaceutici S.p.A., Parma, Italy. Unless otherwise stated, all other chemical reagents used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

PDE4 enzymatic assays from U937 extracts. PDE4 activity was determined in U937 human monocytic supernatants cells lysate (ATCC, Manassas, USA). Cells were cultured, harvested and supernatant fraction prepared essentially as previously described (Barnette et al. 1996). All the materials used for cell culture were from Gibco (Monza, Italy). U937 cells were grown at 37°C, 5% CO₂ in RPMI-1640 with GlutaMAX™-I medium supplemented with 10% fetal bovine serum and 100 µg/mL Pen-strep. Cells were harvested and washed twice by centrifugation (150 x g, 8 min) in cold PBS. Washed cells were resuspended in cold Krebs-Ringer-Henseleit buffer at a final concentration 20 x 10⁶ cells /mL and sonicated. After centrifugation at 15,000 x g for 20 min, the supernatants were pooled, divided in aliquots and stored at -80°C. PDE4 activity was determined in cells supernatants by assaying cAMP

disappearance from the incubation mixtures. The concentration of the test compounds ranged between 10^{-12} M and 10^{-6} M. Reactions were stopped by enzyme heat inactivation (2.5 minutes at 100°C) and residual cAMP content was determined using the 'LANCE™cAMP Detection kit' from PerkinElmer (Milan, Italy) following the provider instructions. IC₅₀ values were determined from concentration-response curves by nonlinear regression analysis.

Rolipram Binding Assay. The affinity against HARBS has been evaluated in a radioligand binding assay performed in rat brain membranes, using [3H]-rolipram as radioligand. Fresh rat brains were homogenized in 20 volumes of ice-cold 50 mM Tris-HCl (pH 8.0) buffer containing 1.2 mM MgCl₂ in a polytron PT-10 homogenizer (Brinkman Instruments). The resulting homogenate was centrifuged at 30,000g for 20 min at 4 °C. The pellet was washed by resuspension in 20 volumes of fresh buffer and recovered by centrifugation as before. The final pellet was suspended in Tris buffer (0.5 mg of protein/mL) for binding experiments. Incubation mixtures in duplicates consisted of 0.1 mL of (±) [³H]rolipram (2 nM final), 0.02 mL of inhibitor, and 0.9 mL of membrane preparation (added last). Rolipram (10 μM) was used for nonspecific binding. After 60 min incubation at 4 °C the contents of the incubation tubes were filtered through a Whatman GF/C glass filter. The membranes were washed three times with 3 mL of ice-cold buffer, and radioactivity on the separated filter disks was determined in a liquid scintillation counter. IC₅₀ values were determined from semilog graphs of percent inhibition *versus* concentration.

PDE4 Enzyme Assays. PDE4 assays were performed using recombinant PDE enzymes expressed in a baculoviral system. The radiometric assay method is a modification of the two-step method of Thompson and Appleman (1971) as described by Mackenzie et al., 2010. All the

assays use a substrate concentration below the K_m determined for each enzyme so that $k_i = IC_{50}$. The PDE4 inhibitors were prepared as stocks at a concentration of 40 mM in 100% DMSO and were tested at 11 concentrations (0.5% final DMSO concentration) in duplicate with a starting concentration of 1 μ M and a 1:10 serial dilution against human PDE4A4, PDE4B2, PDE4C2, PDE4D3. Concentration up to 5% DMSO are tolerated in this assay as reported previously (MacKenzie et al., 2010), a notion confirmed also by a preliminary study aimed at evaluating tolerance to DMSO (estimated up to 5%).

PDEs Enzyme Assays. PDE1 was prepared from bovine brain (Gietzen, 1982). PDE2, PDE3 and PDE5 were purified from human platelets (Schudt, 1991). PDE6 was purified from bovine retina (Baehr W, 1979). Recombinant human PDE7A, PDE8A1, PDE9A2, PDE10A2 and PDE11A4 were expressed in a baculovirus/ insect cell system. The radiometric assay method is a modification of the two-step method of Thompson and Appleman (1971). Briefly, the assay mixture contained 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 0.5 mM cAMP or cGMP, and [3H]cAMP or [3H]cGMP (1 μ M/mL), the indicated concentration of the inhibitor and an aliquot of the enzyme solution. CHF6001 was prepared as stock at a concentration of 0.3 mM in 100% DMSO and tested at 5 concentrations (1% final DMSO concentration) in duplicate starting at 30 μ M and a 1:10 serial dilution against human PDEs. PDE1 isoenzyme was assayed in the presence of Ca^{2+} (2 mM) and calmodulin (100 U/ml) using cAMP as substrate. PDE2, PDE3, PDE7A, PDE8A1, PDE10A2 and PDE11A4 were assayed in the presence of cAMP as substrate. PDE5, PDE6 and PDE9A2 were assayed using cGMP as substrate.

PBMCs. Cells were purchased from Lonza (Basel, CH), washed, resuspended in RPMI 1640 medium (w/o Phenol Red) supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and

100 µg/mL streptomycin, and plated in 96-well tissue culture plates at the density of 10^5 cells/well, in an atmosphere of 95% air and 5% CO₂ at 37°C. Cells were treated with different concentrations of PDE4 inhibitors (10^{-15} M- 10^{-8} M, 0.2% final DMSO concentration) 1h before stimulation with lipopolysaccharide (LPS) from *Escherichia coli* (3 ng/mL for 18h). Increasing concentrations of DMSO were tested and no significant effects of DMSO up to 0.4% on cell viability and TNFα release were noticed. Human TNFα in the supernatant was assayed using a paired antibody quantitative ELISA kit (Bender Medsystem, Austria).

Human myeloid leukemia THP-1 cell line. THP-1 cell line was obtained from Sigma (St. Louis, MO) and cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 µg/ml streptomycin, in an atmosphere of 95% air and 5% CO₂ at 37°C. To induce differentiation into adherent macrophages, cells were plated in 48-well plates (2.5×10^5 cells/well) and incubated for 4 days (THP-1) with 50 nM of phorbol 12-myristate 13-acetate, as previously described (Daigneault et al., 2010). Subsequently, cells were treated with different concentration of PDE4 inhibitors (10^{-12} M- 10^{-6} M, 0.2% final DMSO concentration), stimulated with LPS (100 ng/ml final concentration) and incubated for 18 hours in RPMI (w/o Phenol Red) supplemented with 10% FBS. Human TNFα in the supernatant determined by quantitative ELISA (Bender Medsystem, Vienna, Austria).

Murine (RAW264.7) and rat (NR8383) macrophagic cell lines. RAW264.7 and NR8383 cells were purchased from ATCC and cultured in RPMI 1640 medium (w/o Phenol Red) supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 µg/ml streptomycin, in an atmosphere of 95% air and 5% CO₂ at 37°C. RAW264.7 and NR8383 cells were seeded in RPMI (w/o Phenol Red) containing 10% FBS in 48-well tissue culture plates at the density of

7.5×10^4 cells/well and grown for 24 hours at 37°C with 5% CO₂. Subsequently, cells were treated with different concentration of PDE4 inhibitors (10^{-12} M- 10^{-6} M, 0.2% final DMSO concentration), stimulated with LPS (10 ng/ml and 100 ng/ml final concentration for RAW264.7 and NR8383 cells, respectively) and incubated for 18 hours in RPMI (w/o Phenol Red) supplemented with 10% FBS. Murine and rat TNF α in the supernatant were determined by quantitative ELISA kit (Bender Medsystem, Vienna, Austria). As an indirect index of nitric oxide (NO), accumulation of nitrite in the medium production was measured by a colorimetric assay method based on the Griess reaction as previously described (Facchinetti et al., 2004).

CD4+ T Lymphocytes. CD4+ T lymphocytes were purchased from Lonza (Base, CH). CD4+ T lymphocytes were cultured in Lymphocyte Growth Medium supplemented with 10% FBS, albumin, insulin, transferrin and gentamicin. Cells were stimulated via the T-cell receptor CD3 and CD28 co-receptor by using selective mAbs (Hatzelmann and Schudt, 2001). For this purpose, 96-well microtiter plates were prepared on the day before cell plating: each well was incubated with 50 μ l of PBS containing 6 μ g/ml of anti-CD3 mAb (Orthoclone OKT-3; Janssen-Cilag, Neuss, Germany) for about 2.5 h at 37°C; plates were then stored overnight at 4°C and washed three times with PBS (200 μ l) before use. CD4+ T lymphocytes were plated at the density of 10^5 cells/well in 96-well tissue culture plates, pre-coated with the anti-CD3 mAb, and treated with different concentrations of PDE4 inhibitors (10^{-13} M- 10^{-8} M, 0.2% final DMSO concentration). Subsequently, anti-CD28 (clone CD28.2, Coulter-Immunotech Diagnostics, Hamburg, Germany) was added to the final concentration of 3 μ g/ml and the plates were further incubated at 37°C and 5% CO₂ for 72 h. Human IFN γ in the supernatant was assayed using a paired antibody quantitative ELISA kit (Life Technologies, Grand Island, NY).

Human primary peripheral eosinophils. Human primary peripheral eosinophils were purchased from 3H Biomedical (Uppsala, Sweden). Human primary eosinophils were washed, resuspended in RPMI 1640 medium (w/o Phenol Red) supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin and 100 µg/ml streptomycin and plated in 96-well white OptiPlate at the density of 10^5 cells/well. Cells were pretreated with different concentration of PDE4 inhibitors (10^{-13} M- 10^{-8} M, 0.2% final DMSO concentration) for 30 min, primed with cytochalasin B (CB, 5 µM) for 15 minutes and stimulated with fMLP (1 µM) in RPMI (w/o Phenol Red) supplemented with 10% FBS. Following the addition of fMLP, cells were incubated with the chemiluminescence probe L-012 (500 nM) (Wako Chemicals GmbH, Neuss, Germany) and ROS generation was recorded repeatedly over 1 hour (one reading every minute) with a Luminescence reader (Centro LB 960, Berthold Technologies). During luminescence measurement, the plate was rotatively agitated at 37°C. ROS generation was determined by calculating the area under the curve.

Neutrophils oxidative burst. Human neutrophils (PMNL) were purified from diluted “buffy coat” (Blood Bank, University of Milan) centrifuged on a discontinuous Percoll gradient (ρ 1,077-1,098, 4-10°C), followed by red cells lysis (1 volume 0,2% NaCl, balanced with 1 volume 1,6% NaCl and 0.2% saccarose, 4-10°C). Handling in sterile buffer prevented spontaneous activation of PMNL. Purified neutrophils (1×10^6 /ml) were suspended in D-PBS (Sigma-D1408) supplemented with CaCl_2 , MgCl_2 , glucose (0.9, 0.5 e 7.5 mM respectively) and Cytochrome C (50 µM, Sigma-C2506). The cell suspension was dispensed in microplate (200µl /well), gently shaken at 37°C and absorbance read at λ 550-490 by a spectrophotometer (Spectramax190 microplate reader). Neutrophils suspensions (1×10^6 /ml) were then incubated with the direct inhibitor of neutrophil NAD(P)H-oxidase diphenyleneiodonium (DPI), the PDE4 inhibitors

compounds or its vehicle, DMSO, for 10 min at 37°C before challenge with fMLP (1 µM), and ROS production monitored by measuring the variation in absorbance of the reduced cytochrome c: duplicate samples containing SOD (180U/ml) were used as blanks. As PMNL suspension obtained from different donors, significantly differs in response to challenge, results were expressed as percentage of inhibition of ROS release. Each treatment had 4 replicates with cells from the same donor and concentration-response curves were originated using the average values from at least 3 different donors.

Neutrophil chemotaxis. Male C57BL/6J mice were bred in the animal house of the Center for Molecular Biotechnology (University of Turin, Italy) and maintained on a standard diet with tap water *ad libitum* and a 12:00 h light /dark cycle. Mice were sacrificed by cervical dislocation. The femur and the tibia bones from both hind legs were removed and freed of soft tissue attachments, and the extreme distal tip of each extremity was cut off. Saline with 0.1% Fetal Bovine Serum was forced through the bone with a syringe. After dispersing cell clumps, the cell suspension was centrifuged (260 g, 12 min at 4°C) and resuspended in 2 ml of saline-0.1%FBS. Cells were then treated on a three-layer Percoll gradient (72%-64%-52%). Neutrophils were collected from the ring between 72%-64%, counted and concentrated at 2×10^6 cells/ml in RPMI medium. Cells were incubated with the compound at different concentration for 1h at 37°C and then let migrate against C5a 50nM for 2h in the 48 multiwell chamber. The inhibition of the compound was evaluated on the number of the cells still able to migrate towards C5a.

LPS-induced neutrophilia in rat. Non-fasted Wistar rats (150-250g) were weighed, individually identified on the tail with a permanent marker and intra-tracheally dosed with vehicle (lactose), CHF6001 (CHF, 0.01, 0.1, 0.3, 1 µmoles/kg), GSK256066 (1 µmol/kg) or

Budesonide (1 $\mu\text{mol/kg}$), 1 hour prior and 6 hours after LPS exposure. Dry powder formulations were prepared by blending of mainly coarse respiratory grade lactose and micronized test compound. For the challenge, rats were placed into a perspex chamber and exposed to LPS (*salmonella enterica* serotype) or 0.9% saline. The LPS was prepared in a solution of 0.1 mg/mL and aerosolised using a De Vibliss ultrasonic nebuliser 2000, so that 7 mL of the solution was aerosolised during a 30 min exposure period. Compressed air at 6 L min⁻¹ was passed through the nebulizer and the output of the nebulizer was passed into the box containing the rats. 24 hours after LPS challenge the animals were culled by an intraperitoneal injection of 1.0 mL of pentobarbitone sodium (lethobarb). The trachea was then isolated by a midline incision in the neck and separation of the muscle layers. A small incision was made into the trachea and a plastic cannula was inserted and secured in place with a suture. The airway was then lavaged using 2.5 mL of sterile PBS at room temperature. The PBS was left in the airway for 10 seconds before being removed. From this resulting lavage an aliquot (1.5 mL) was centrifuged (1200 rpm for 2 min) and 2 samples (300 μL) of the resulting supernatant were aliquoted and stored at -80°C for cytokine analysis. Total cell counts of the BALF samples were measured using a Neubaur haemocytometer. Results were expressed as cells/mL. Cytospin slides were also prepared by adding a 100 μl aliquot of BALF into cytospin funnels in a Shandon Cytospin4 operated at 1,200 rpm for 2 min at room temperature and stained using a DiffQuik stain system. 100 cells were counted on each slide under a microscope. Cells were classified as neutrophils, eosinophils or mononuclear cells (monocytes, macrophages and lymphocytes) based on morphological criteria. Erythrocytes and epithelial cells were ignored. The number of each cell type were quantified by expressing the cell number as a percentage of the total count.

Statistical analysis. All values are expressed as means \pm SEM of the given number (n) of independent experiments. IC₅₀ values were calculated by the analysis of the sigmoidal dose-response curve (variable slope) elaborated by Graph Pad PRISM4 program. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test for multigroup comparisons.

RESULTS

CHF6001 selection

The structure of CHF6001 is shown in figure 1. It was identified as a potent inhibitor of PDE4 activity (compound 32a) from a series of novel ester derivatives of 1-(S)-(3-(cyclopropylmethoxy)-4-(difluoromethoxy)phenyl)-2-(3,5-dichloropyridin-4-yl) ethanol (Armani et al., 2014). In particular, esters of variously substituted benzoic acids were explored and structural modifications of the benzoic moiety were performed in order to maximize the inhibitory potency. Besides CHF6001, several other novel PDE4 inhibitors with potent anti-inflammatory activity *in vitro* were obtained (Armani et al., 2014).

Activity of CHF6001 and reference compounds in PDE enzyme assays

PDE4 isoenzymes exist in both LARBS and HARBS conformations. CHF6001 enzymatic activity was tested both in a LARBS conformation by utilizing an assay with PDE4 extracts from U937 cells and in HARBS conformation by utilizing rolipram binding assay (see Materials and Methods) in comparison with reference compounds GSK256066, roflumilast, UK 500,001 and cilomilast (Table 1).

The IC₅₀ value for the inhibition of enzymatic activity of PDE4 by CHF6001 in the LARBS assay is 0.026±0.006 nM. The most potent comparator, as expected, resulted GSK256066 (IC₅₀ = 0.025±0.01 nM), whereas the other PDE4 inhibitors tested showed IC₅₀ values ranging between 0.17 and 24 nM (Table 1).

CHF6001 inhibited [³H] rolipram binding in rat brain cytosol (HARBS) with a IC₅₀ of 1.05 nM, giving a HARBS ratio (HARBS IC₅₀/LARBS IC₅₀) of about 40, a value higher than GSK256066

ratio (11), whereas roflumilast, UK 500,001 and cilomilast showed ratios comprised between 2 and 4 (Table 1).

CHF6001 inhibited most of PDE4 splicing variants at sub-nanomolar concentrations (Table 2) and, similarly to GSK256066 and roflumilast, CHF6001 did not show PDE4 subtype selectivity against different PDE isoenzymes. Roflumilast is ten-fold less potent than CHF6001 with respect to PDE4 isoenzymes inhibition, apart from PDE4C, which is inhibited with a slightly lower potency. In contrast, UK 500,001 and cilomilast showed some subtype selectivity for PDE4D (Table 2).

CHF6001 is at least 25,000 fold less potent against the following panel of PDE isoenzymes: PDE1, PDE2, PDE3, PDE5, PDE6, PDE7A, PDE8A1, PDE9A2, PDE10A2 and PDE11A4, therefore showing a marked selectivity for PDE4 (Table 3).

Activity of CHF6001 and reference compounds in human PBMCs, THP-1 monocyte-derived macrophages and CD4⁺ T lymphocytes

We selected two *in vitro* models of LPS-stimulated TNF- α to evaluate the anti-inflammatory potency of CHF6001 in comparison with other known PDE4 inhibitors, including GSK256066, which is the most potent PDE4 inhibitor so far described in the literature, UK500,001, roflumilast (the only clinically approved PDE4 inhibitor) and cilomilast. All the compounds tested inhibited TNF- α release up to a maximum of about 90% in PBMC, an outcome in line with the notion that PDE4 is the predominant isoform expressed in monocytes (Figure 2, upper panel). In PBMCs, basal release of TNF- α (mean \pm s.d.) was of 98.6 \pm 6.1 pg/ml, while upon LPS stimulation became 40146 \pm 2227 pg/ml. The rank order of potency of the compounds (confidence interval values given in parenthesis) is the following: GSK256066, IC₅₀=3 pM (2-4), CHF6001, IC₅₀=28 pM (21-40), roflumilast IC₅₀=1.77 nM (0.45-6.83), UK500,001,

IC₅₀=12.1 nM (2.9-50.1) and cilomilast IC₅₀=165.3 nM (61.8-442.6). These results were well in agreement with the previously reported potencies of GSK256066, roflumilast and cilomilast against TNF- α release in PBMCs (Hatzelmann and Schudt, 2001; Tralau-Stewart et al., 2011). In human THP-1 monocytic-derived macrophages, basal release of TNF- α (mean \pm s.d.) was of 10.3 \pm 5.2 pg/ml, while upon LPS stimulation became 18945 \pm 838 pg/ml. All the compounds tested inhibited TNF- α release up to a maximum of 65-70% (Figure 2, lower panel) with the following potencies: GSK256066, IC₅₀=16 pM (8-35), CHF6001, IC₅₀=43 pM (15-122), UK 500,001, IC₅₀=3.60 nM (1.22-10.45); roflumilast, IC₅₀=4.41 nM (1.0-19.3), cilomilast, IC₅₀=264.0 nM (121.2-575.0). The observed potency ranking in THP-1 monocyte-derived macrophages is similar to that observed in PBMCs with both CHF6001 and GSK256066 displaying a potency in the low picomolar range.

PDE4s are critical regulators in TCR signaling and their inhibition blunts T cell cytokine production (Abrahamsen et al., 2004). To mimic the physiological conditions of T cell activation, we stimulated the CD4⁺ T lymphocytes via the TCR (by plate-bound anti-CD3 antibody) and the CD28 co-receptor (by soluble anti-CD28 antibody). Basal release of IFN- γ (mean \pm s.d.) was of 54 \pm 8.5 pg/ml, while upon LPS stimulation became 3036 \pm 489 pg/ml. All the PDE4 inhibitors tested, including CHF6001, inhibited IFN- γ release up to a maximum of 50-60% (Figure 3) with the following potencies: GSK256066, IC₅₀=3 pM (1-12); CHF6001, IC₅₀=62 pM (13-289); roflumilast, IC₅₀=0.42 nM (0.09-1.87); UK 500,001, IC₅₀=9.4 nM (73-120); cilomilast, IC₅₀=386.7 nM (149.8-998.5). The observed potency ranking in activated CD4⁺ T cells is similar to that observed in PBMCs and THP-1 with both CHF6001 and GSK256066 being active in the low picomolar range.

Activity of CHF6001 and reference compounds in rodent macrophagic cell lines

In two different rodent macrophagic cell lines, namely RAW 264.7 (mouse) and NR8383 (rat), all the PDE4 inhibitors tested inhibited the release of TNF- α and NO evoked by LPS. In RAW 264.7, basal release of TNF- α (mean \pm s.d.) was of 10.3 \pm 5.2 pg/ml, while upon LPS stimulation became 18945 \pm 838 pg/ml. The following inhibitory potencies (confidence interval values given in parenthesis) against TNF- α (Figure 4A) were observed: GSK256066, IC₅₀=0.82 nM (0.3-2.1), CHF6001, IC₅₀=1 nM (0.7-1.5), roflumilast, IC₅₀=54.8 nM (23-130), UK 500,001, IC₅₀=36,60 nM (11.3-181); cilomilast, IC₅₀=830 nM (340-2000). Similar results were obtained in NR8383 cells where the inhibition potencies against TNF- α release (Figure 4C) were the following: GSK256066, IC₅₀=0.08 nM (0.06-0.1), CHF6001, IC₅₀=0.19 nM (0.18-0.2), roflumilast, IC₅₀=8.3 nM (5.5-12.4), UK 500,001, IC₅₀=19.8 nM (3.2-123); cilomilast, IC₅₀=548 nM (353-852). In NR8383, basal release of TNF- α (mean \pm s.d.) was of 1.2 \pm 0.5 pg/ml, while upon LPS stimulation became 1184 \pm 111 pg/ml.

In both NR8383 and RAW 264.7 the potencies of all the tested inhibitors showed slightly higher IC₅₀ values against NO release (Figure 4B and D) but the ranking order observed in the previous assays was maintained.

Activity of CHF6001 and reference compounds in human eosinophils and neutrophils

Since PDE4 inhibitors have been shown to negatively modulate fMLP-stimulated release of reactive oxygen species (ROS) from human eosinophils and neutrophils (Hatzelmann and Shudt, 2001; Press and Banner, 2009), we evaluated the ability of CHF6001 in inhibiting fMLP-induced ROS production. CHF6001 and GSK256066 fully inhibited fMLP-induced ROS generation in human eosinophils, at low picomolar concentrations (Figure 5, upper panel), with IC₅₀s of 5 pM (0.002-0.013) and 5 pM (0.002-0.015) respectively. Similarly, in human neutrophils, CHF6001 and GSK256066 inhibited fMLP-induced ROS generation (Figure 5,

lower panel) with IC_{50} s of 0.55 nM (0.07-4.20) and 0.49 nM (0.17-1.43) respectively. Roflumilast, with an IC_{50} =8.51 nM (1.51-47.88), resulted again less potent than CHF6001 and GSK256066.

Activity of CHF6001 and roflumilast on neutrophilic chemotaxis

Neutrophil-dominant pulmonary inflammation is an important feature of COPD. Since PDE4 inhibitors have a functional role in mediating chemotaxis (Ariga et al., 2004), the ability of CHF6001 in inhibiting neutrophilic chemotaxis was tested in comparison with roflumilast *in vitro* in a Boyden two-chamber chemotactic assay. CHF6001 inhibited of about 60% neutrophilic migration with a subnanomolar potency, IC_{50} =0.093 nM (0.036-0.241). Roflumilast, by showing an IC_{50} =0.937 nM (0.678-1.296), resulted to be less potent than CHF6001 (Figure 6).

CHF6001 inhibits LPS-induced pulmonary leukocytes infiltration in rat

CHF6001 (CHF, 0.01, 0.1, 0.3, 1 μ moles/kg), GSK256066 (GSK, 1 μ moles/kg) or Budesonide (Bude, 1 μ moles/kg) were administered intra-tracheally as micronized dry powder 1 hour before and 6 hours after LPS challenge. Broncho-alveolar lavage fluid (BALF) was collected 24 hours after challenge and total and differential cell counts performed.

When administered 1 hour before and 6 hours after the inflammatory *stimulus*, CHF6001 elicited a dose-dependent inhibition of pulmonary neutrophilia which reached a statistical significance at 0.1 μ moles/kg ($p<0.05$) and was maximal at 1 μ moles/kg (77%) similarly to budesonide at 1 μ moles/kg (77%). CHF6001 elicited also a dose-dependent inhibition of pulmonary white blood cell counts in BALF which reached significance at 0.3 μ moles/kg ($p<0.01$) and was maximal at 1 μ moles/kg (61%), similarly to budesonide at 1 μ moles/kg (62%). Calculated ED_{50} values for

CHF6001 were 0.188 μ moles/kg against total white blood cells and 0.205 μ moles/kg against neutrophilia. Similarly to CHF6001, GSK256066, when tested at 1 μ moles/kg, showed an inhibitory effect on LPS-evoked pulmonary neutrophilia (70% inhibition) and white blood cells infiltration (60% inhibition) (Figure 7).

DISCUSSION

The recent approval of roflumilast as an oral drug for COPD treatment defines the standing of PDE4 as a therapeutic target in respiratory diseases. Nevertheless, this second-generation PDE4 inhibitor is still not without side effects which may compromise optimal dosing and affect patient compliance. Several strategies have been proposed to minimize PDE4 inhibition-related side effects, including designing inhibitors suitable for inhaled delivery that would limit systemic exposure, while increasing the pulmonary anti-inflammatory activity achievable. In the present study we report the pharmacological characterization of a novel highly potent and selective PDE4 inhibitor (CHF6001) optimized for inhaled delivery through a rationale drug-design and screening program (Armani et al., 2014). CHF6001 inhibitory potency was evaluated in enzymatic cell free assays and subsequently in several cell-based assays taking advantage of different immuno-competent cells known to play key roles in asthma and COPD, including monocytes, macrophages, CD4+ T cells, neutrophils and eosinophils. Finally, for testing how *in vitro* anti-inflammatory potency and efficacy translates *in vivo*, CHF6001 was delivered as micronized dry powder in a rat model of endotoxin-induced acute neutrophilia. A further preclinical *in vivo* safety and efficacy profile is presented in a companion paper.

CHF6001 was compared with two oral (roflumilast and cilomilast) and two inhaled (GSK256066 and UK500,001) PDE4 inhibitors already described in detail in the scientific literature. Roflumilast was chosen because is the only PDE4 inhibitor approved in EU and USA for once-daily treatment of severe COPD, cilomilast being the first oral PDE4 that reached preregistration and the inhaled PDE4 inhibitors UK 500,001 and GSK256066 because were both tested in controlled clinical trials of phase II in COPD and asthma respectively. Head to head comparison with the four reference compounds show that CHF6001 is highly potent in

inhibiting all the four PDE4 (A-D) isoforms tested with a potency comparable to that of GSK256066, which is the most potent inhibitor described in the literature so far, and 10 to 100-fold more potent than roflumilast. Indeed, the following ranking could be determined for inhibition of PDE4 isoforms: GSK256066 \geq CHF6001 > roflumilast > UK500,001 > cilomilast. Such ranking was substantially maintained when CHF6001 inhibitory PDE4 activity was determined in U937 human monocytic cells lysate.

Two distinct PDE4 conformers can be distinguished based on their affinity to bind the prototypic inhibitor rolipram: the LARBS conformer, encompassing the catalytic domain of PDE4 and the HARBS conformer, which requires both amino terminal and the catalytic domain and present in the brain but not in peripheral tissues (Barnette et al., 1996; Rocque et al., 1997). Targeting the conformer of PDE4 associated with the LARBS over the HARBS may retain anti-inflammatory activity and have a reduced capacity to cause nausea and emesis (Souness and Rao, 1997). Interestingly CHF6001 results in a HARBS/LARBS ratio of 40, more than ten-fold higher than roflumilast, UK 500,001 and cilomilast, a characteristic suggestive of a potentially wider therapeutic window.

CHF6001 was tested in several functional cellular assays designed to evaluate its anti-inflammatory activities. CHF6001 shows sub-nanomolar potency in inhibiting the release of the clinically relevant pro-inflammatory cytokine TNF- α from PBMCs stimulated with LPS. Macrophage numbers are markedly increased in the lung and in alveolar space of patients with COPD and are preferentially localized to sites of alveolar destruction. Therefore, alveolar macrophages are critical players in the pathophysiology of COPD and a major target for future anti-inflammatory therapy. Indeed, CHF6001 was also tested in THP-1 monocytic-derived macrophages and two macrophagic cell lines, namely RAW 264.7 which is derived from peritoneal mouse macrophages and NR8383 which is derived from rat alveolar macrophages.

Again, the anti-inflammatory effects of CHF6001 were evident in all macrophagic cell lines as TNF- α and NO release were inhibited by CHF6001 with potencies slightly inferior (2 to 10 fold potency difference) to those observed in PBMCs. This probably reflects different PDE4 levels of expression between macrophages and monocytes (Gantner et al., 1997).

CHF6001 was >6000-fold more potent than cilomilast in inhibiting release of IFN- γ in CD4+ T cells which are known to play critical in the initiation and propagation of immune response in asthma (Kumar et al., 2006). CHF6001 was extremely potent (IC₅₀ in the low picomolar range) in inhibiting fMLP-induced eosinophil activation, a finding consistent with the notion that PDE4 is prominently expressed in eosinophils.

Neutrophil-dominant pulmonary inflammation is an important feature of COPD (Watt et al., 2005; Quint and Wedzicha, 2007) and we found that CHF6001 was highly potent in inhibiting fMLP-evoked ROS production from human neutrophils (IC₅₀=0.005 nM) and C5a-induced chemotaxis in mouse neutrophils (IC₅₀=0.093 nM). Overall, these findings suggest that CHF6001 targets neutrophils both through direct inhibition of oxidative burst and chemotaxis, two biological responses known to be scarcely sensitive to glucocorticoids (Kubo et al., 2012). This underlines a therapeutic potential for CHF6001, as well as PDE4 inhibitors, in treating COPD which is often associated with lung neutrophilia.

The high potency of CHF6001 in all the *in vitro* assays utilized in this study translates well in anti-inflammatory efficacy *in vivo* when administered intratracheally as a micronized dry powder. By using an endotoxin rat model of pulmonary neutrophilia, we compared CHF6001 head-to-head with the corticosteroid budesonide and GSK256066 (same formulation and same delivery system). GSK256066 was chosen since has been previously reported to be the most potent PDE4 inhibitor so far described in the literature and it is effective in reducing allergen challenge responses in mild asthmatics (Singh et al., 2010). CHF6001 was highly potent and

efficacious in inhibiting LPS-induced neutrophilia (full inhibitory effect equal to a maximal dose of budesonide or GSK256066). Potency is an important feature for an inhaled compound and UK500,001 failure in phase II clinical development may be, at least in part, a consequence of insufficient potency as suggested by the head-to-head comparisons made in this study. Low systemic exposure is important in determining the therapeutic index of a PDE4 inhibitor being emesis and gastrointestinal disturbances directly associated with the mechanism of action. In a recent safety and tolerability study in healthy volunteers, CHF6001, administered as an inhaled dry powder formulation for 7-day, proved to be well tolerated up to 1.6 mg (Esposito et al., 2013). A complete preclinical *in vivo* safety and activity profiling of CHF6001 is presented in the companion article by Villetti (2014). Given its excellent pharmacological potency and efficacy as a topical anti-inflammatory agent, CHF6001 holds promise as a novel inhaled PDE4 inhibitor for treating lung inflammatory diseases such as asthma and COPD. The testing of CHF6001 in clinical trials is currently ongoing (clinicaltrials.gov).

Authorship Contributions

Participated in the research design: Armani, Rizzi, Amari, Patacchini, Delcanale, Carnini, Hirsch, Civelli, Villetti, Facchinetti

Conducted the experiments: Moretto, Caruso, Bosco, Marchini, Pastore, Buccellati

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Contributed with reagents and analytic tools: Amari, Armani, Ghidini, De Fanti, Capaldi, Carzaniga, Rizzi

Wrote or contributed to the writing of the manuscript: Moretto, Sala, Carnini, Civelli, Villetti, Facchinetti

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Figure legends

Figure 1. Chemical structure of CHF6001.

Figure 2. Inhibitory activity of CHF6001, GSK256066, roflumilast, UK 500,001 and cilomilast on LPS-evoked TNF- α release in human blood mononucleate cells. Concentration-response curve for the inhibition of LPS-induced TNF α in PBMCs (A) and in THP-1 monocyte-derived macrophages cells (B). Each point represents the mean \pm SEM of $n=3$ independent experiments performed in quadruplicate.

Figure 3. Inhibitory activity of CHF6001, GSK256066, roflumilast, UK 500,001 and cilomilast on IFN- γ release in human CD4⁺ T cells. Concentration-response curve for the inhibition of IFN- γ release stimulated by anti CD3/CD28. Each point represents the mean \pm SEM of $n=3$ independent experiments performed in quadruplicate.

Figure 4. Inhibitory activity of CHF6001, GSK256066, roflumilast, UK 500,001 and cilomilast on TNF- α and NO release in mouse (RAW 264.7) and rat (NR8383) macrophagic cell lines. Concentration-response curves for the inhibition of LPS-induced TNF α (A) and NO (B) release in mouse RAW264.7 and TNF α (C) and NO (D) release in rat NR8383 macrophagic cell lines. Each data point represents the mean \pm SEM of $n=3$ independent experiments performed in quadruplicate.

Figure 5. Inhibitory activity of CHF6001 and GSK256066 on ROS production in human eosinophils and neutrophils. Concentration-response curves for CHF6001 and GSK256066 against the inhibition of CB/fMLP-induced ROS in human eosinophils (above) and neutrophils (below, roflumilast is also included). AUCs for chemiluminescence were recorded for 5 min after stimulation with CB 5 μ M and fMLP 1 μ M. Each point represents the mean \pm SEM of $n=3$ independent experiments performed in quadruplicate.

Figure 6. Effects of CHF6001 and roflumilast on mouse neutrophilic chemotaxis. Concentration-response inhibitory curves of CHF6001 and roflumilast *versus* C5a-induced chemotaxis of mouse neutrophils. Each point represents the mean \pm SEM of $n=3$ independent experiments performed in quadruplicate.

Figure 7. Inhibition of neutrophils and total leukocytes cell count in bronchoalveolar lavage fluid in endotoxin (LPS) exposed rats treated with CHF6001. CHF6001 (CHF, 0.01, 0.1, 0.3, 1 μ moles/kg), GSK256066 (GSK, 1 μ moles/kg) or Budesonide (Bude, 1 μ moles/kg) were administered intra-tracheally as micronized dry powder (vehicled in lactose) 1 hour before and 6 hours after LPS challenge. BALF was collected 24 hours after challenge and total and differential cell counts performed. Bars are the counts of neutrophils (left) and total white blood cell (right) in BALF. Treatment groups were compared with LPS/vehicle treated group by using ANOVA followed by Dunnett's t test. Significance $*P<0.05$ and $**P<0.01$ for treatment groups compared to vehicle + LPS control group. Values are expressed as the mean \pm SEM values of each treatment group ($n=8$).

TABLES

Table 1: inhibitory potencies (IC_{50} , nM \pm S.E.M.) of CHF6001 and other reference PDE4 inhibitors against the enzymatic activity of HARBS and LARBS conformers.

(nM)	CHF-6001	GSK256066	Roflumilast	UK 500,001	Cilomilast
LARBS	0.026 \pm 0.006 (n=6)	0.025 \pm 0.010 (n=3)	0.176 \pm 0.095 (n=5)	1.28 \pm 0.168 (n=2)	24.17 \pm 7.83 (n=2)
HARBS	1.05 \pm 0.195 (n=6)	0.274 \pm 0.115 (n=2)	0.784 \pm 0.191 (n=3)	2.84 \pm 0.413 (n=2)	50.9 \pm 10 (n=2)
Ratios	40.6	11.0	4.4	2.2	2.1

Table 2: inhibitory potencies (IC₅₀, nM, C.I.) of CHF6001 and other reference PDE4 inhibitors against a panel of four PDE4 isoforms. The values are the means of triplicate determination in a single experiment.

	CHF6001	GSK256066	Roflumilast	UK 500,001	Cilomilast
PDE4A4 (IC ₅₀ , C.I.)	0.032 nM (0.02-0.05)	0.017 nM (0.008-0.039)	0.35 nM (0.27-0.46)	26.1 (12.9-52.2)	375 (118-1181)
PDE4B2 (IC ₅₀ , C.I.)	0.025 nM (0.01-0.05)	0.015 nM (0.006-0.037)	0.18 nM (0.08-0.44)	22.8 nM (13.2-39.4)	440 nM (269-719)
PDE4C2 (IC ₅₀ , C.I.)	0.061 nM (0.04-0.09)	0.061 nM (0.042-0.088)	2.07 nM (1.44-2.99)	217 nM (93.2-506)	1700 nM (928-3102)
PDE4D3 (IC ₅₀ , C.I.)	0.021 nM (0.01-0.04)	0.015 nM (0.010-0.020)	0.24 nM (0.15-0.38)	0.28 nM (0.13-0.61)	86.2 nM (54.4-136.6)

Table 3: inhibitory potencies (IC_{50} , nM, C.I.) of CHF6001 against different PDE isoforms. The values are the means of triplicate determination in a single experiment. N.d.=not determined.

CHF6001	PDE1	PDE3	PDE5	PDE7A	PDE 2, 6, 8A1, 9A2, 10A2, 11A4
IC_{50}	1.2 μ M	2.6 μ M	1.63 μ M	2.6 μ M	>30 μ M
C.I.	(0.2-7.9)	(0.97-7.2)	(0.03-83)	n.d.	n.d.

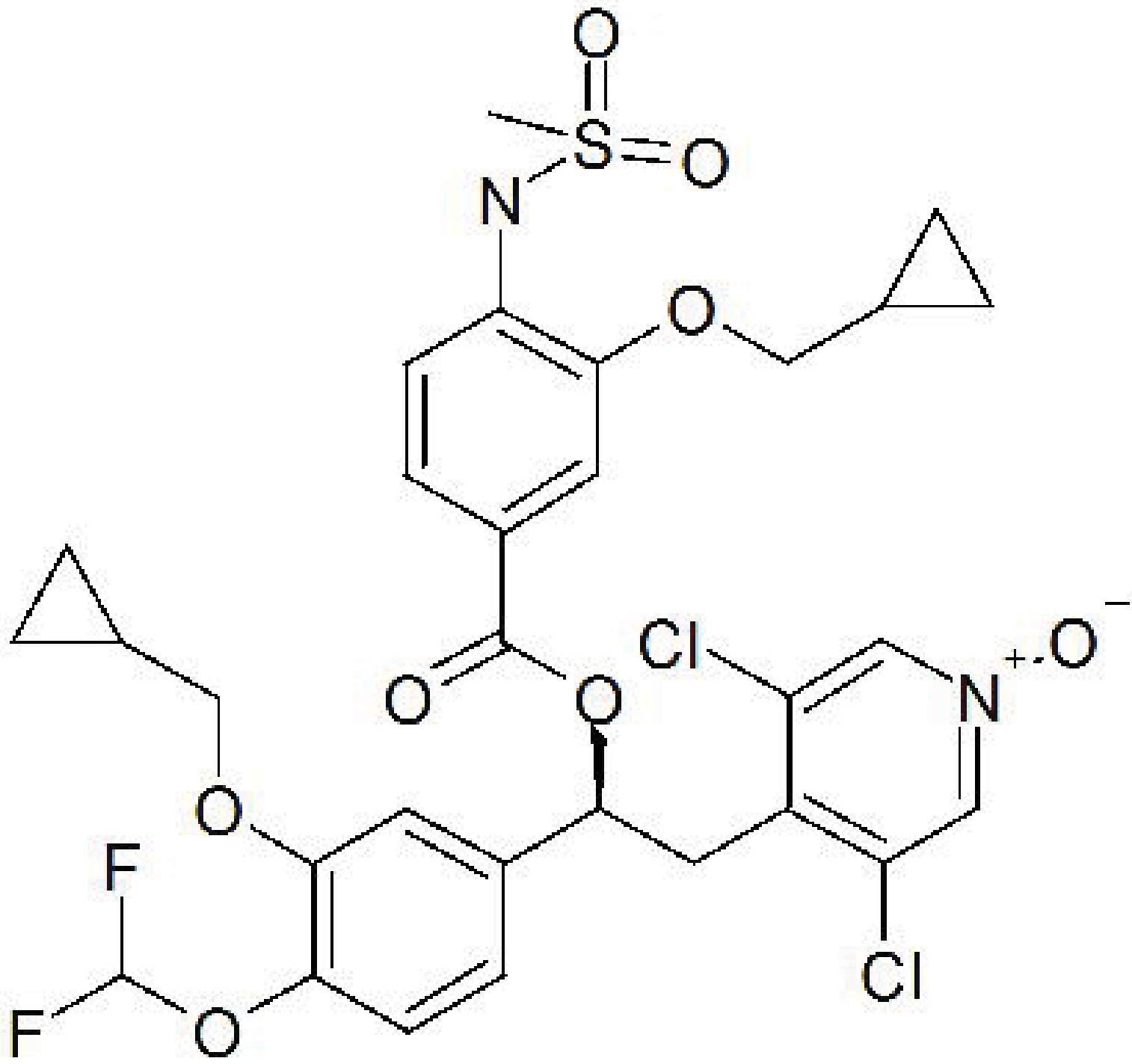


Figure 2

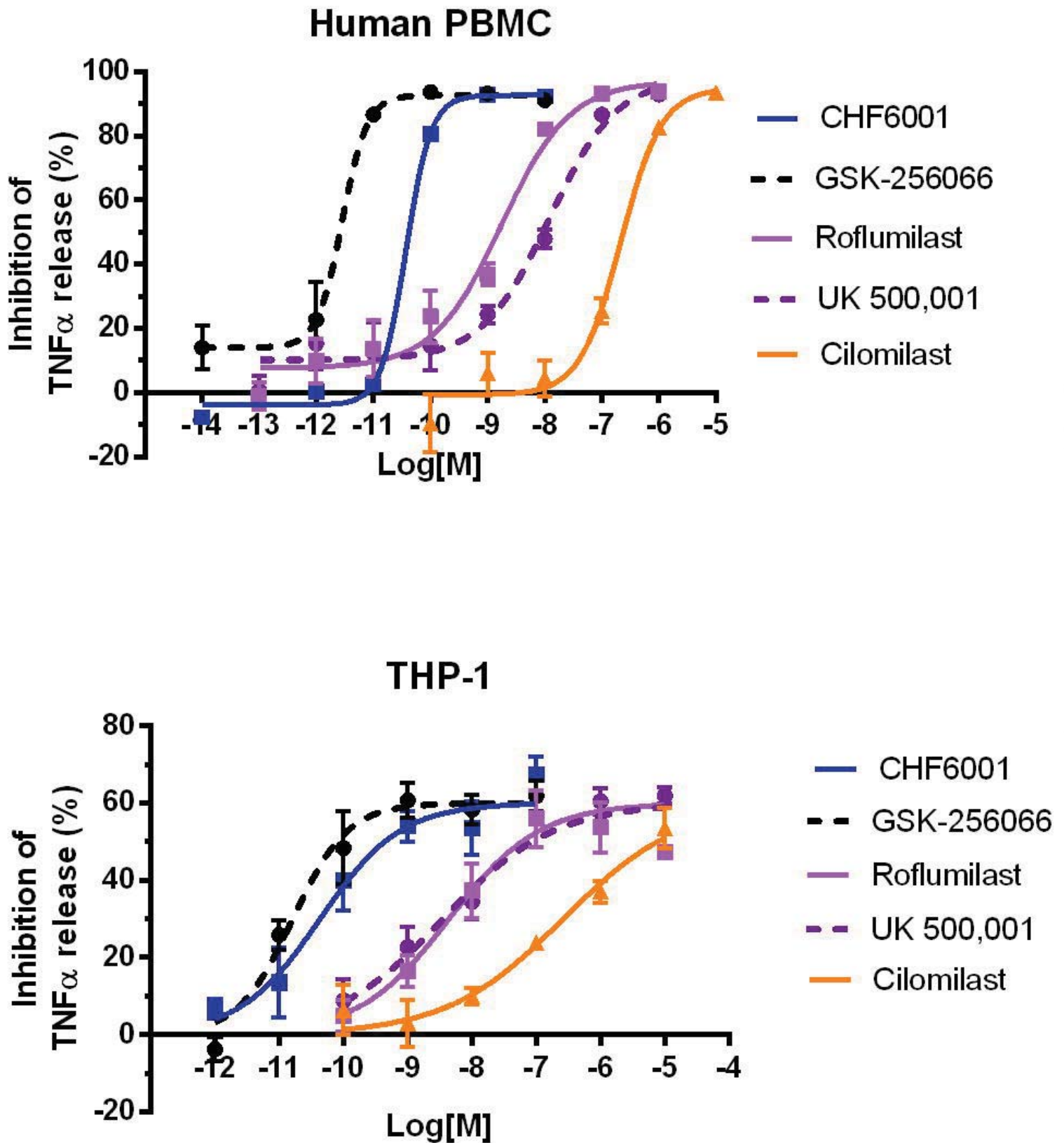


Figure 3

Human CD4+ T cells

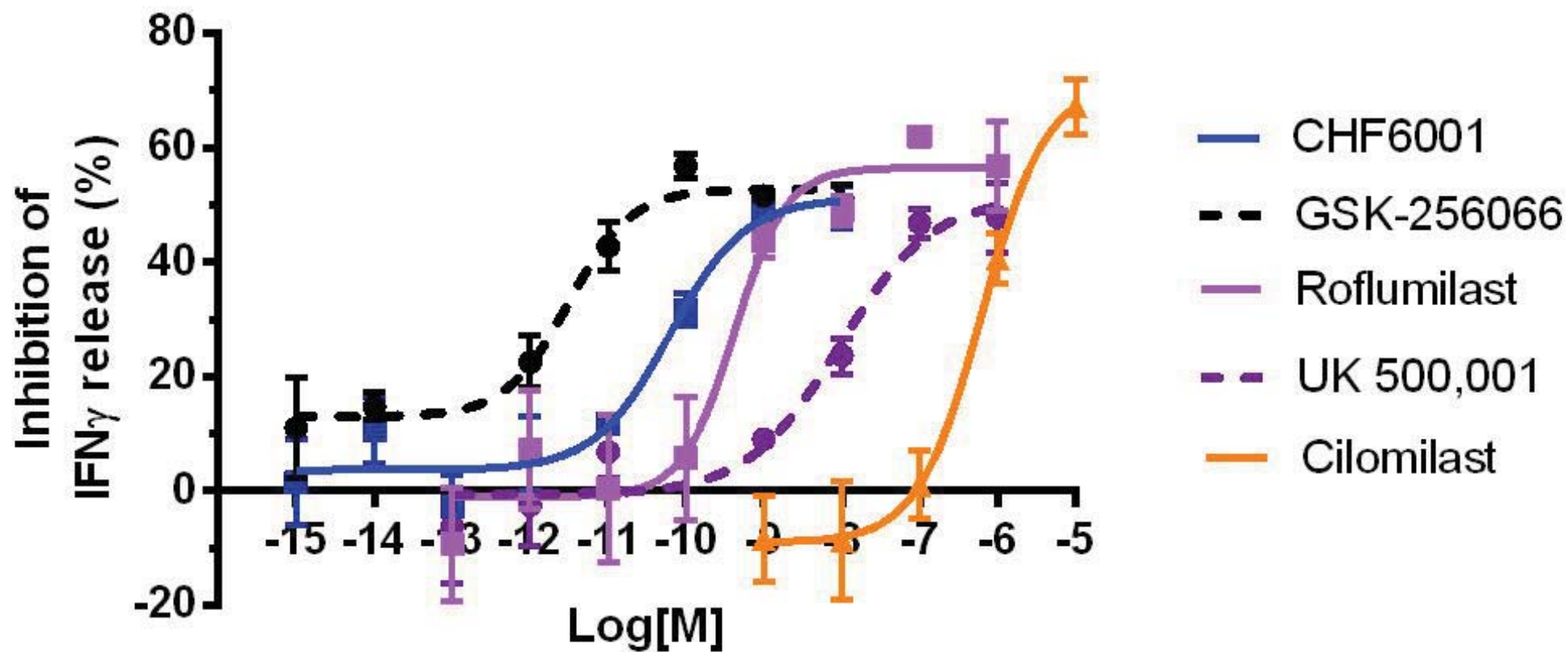


Figure 4

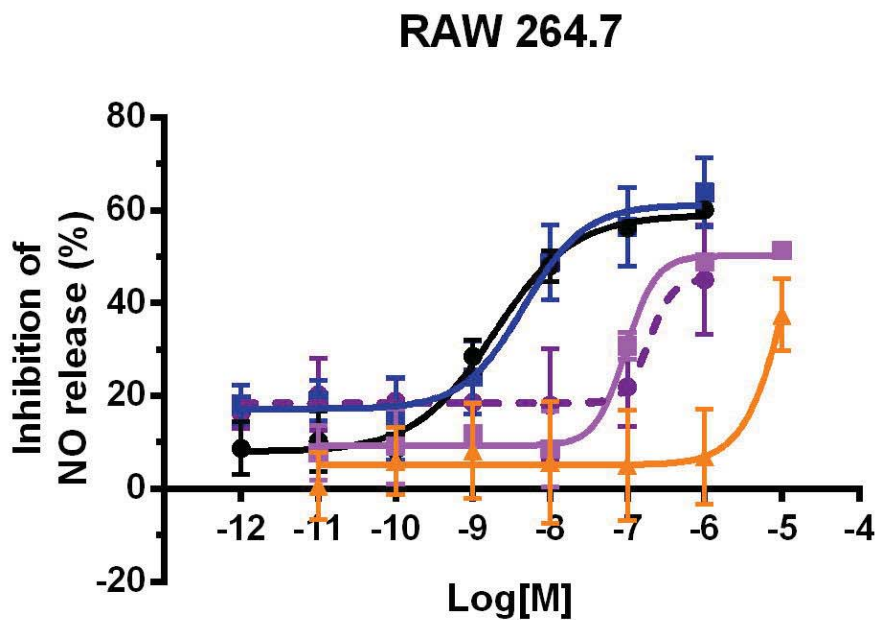
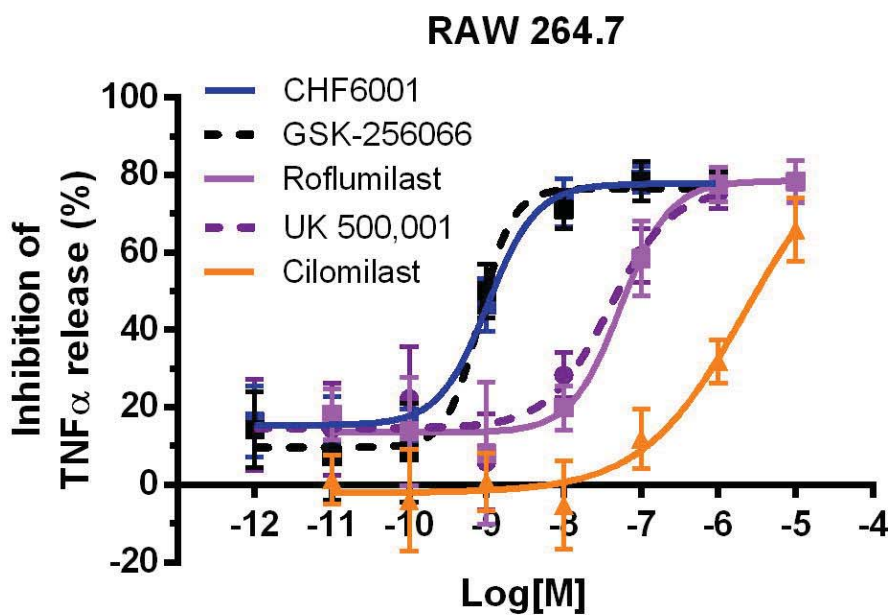
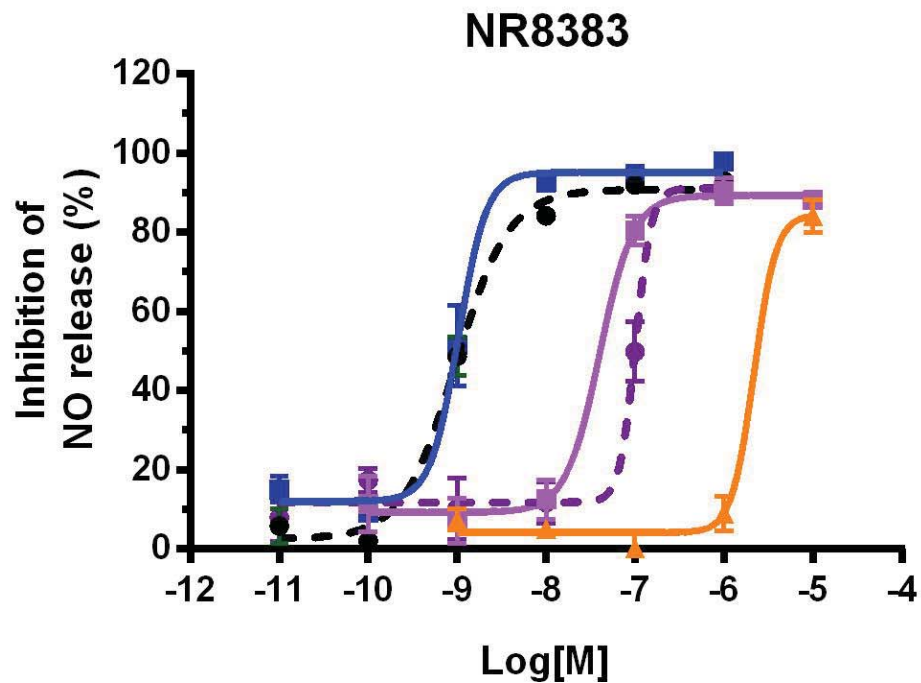
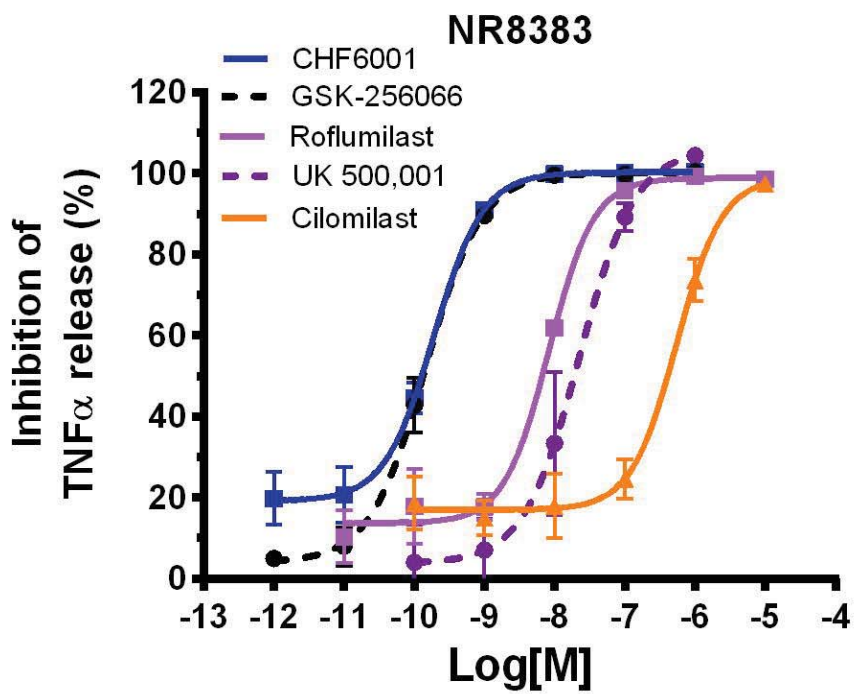


Figure 5

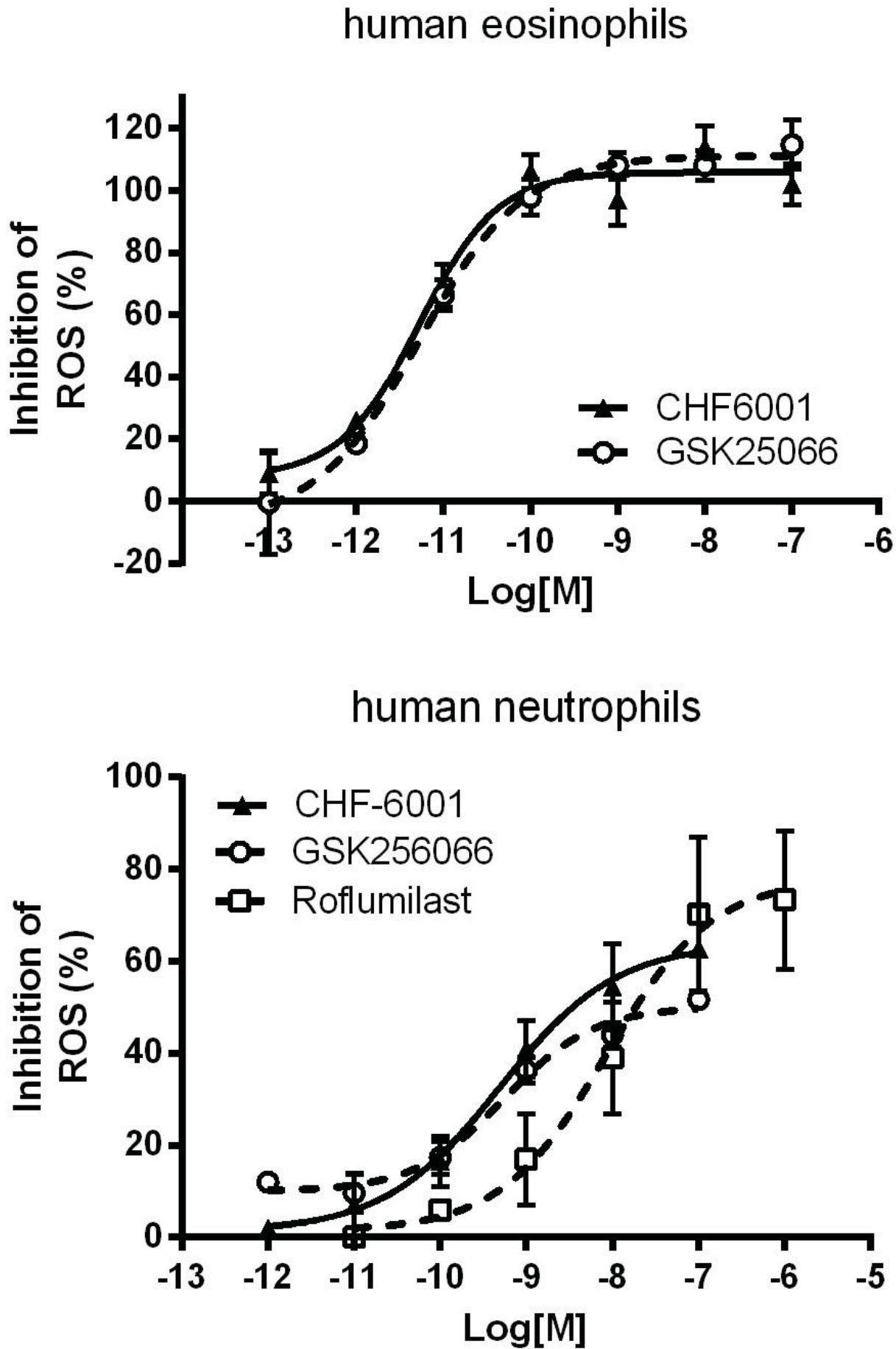


Figure 6

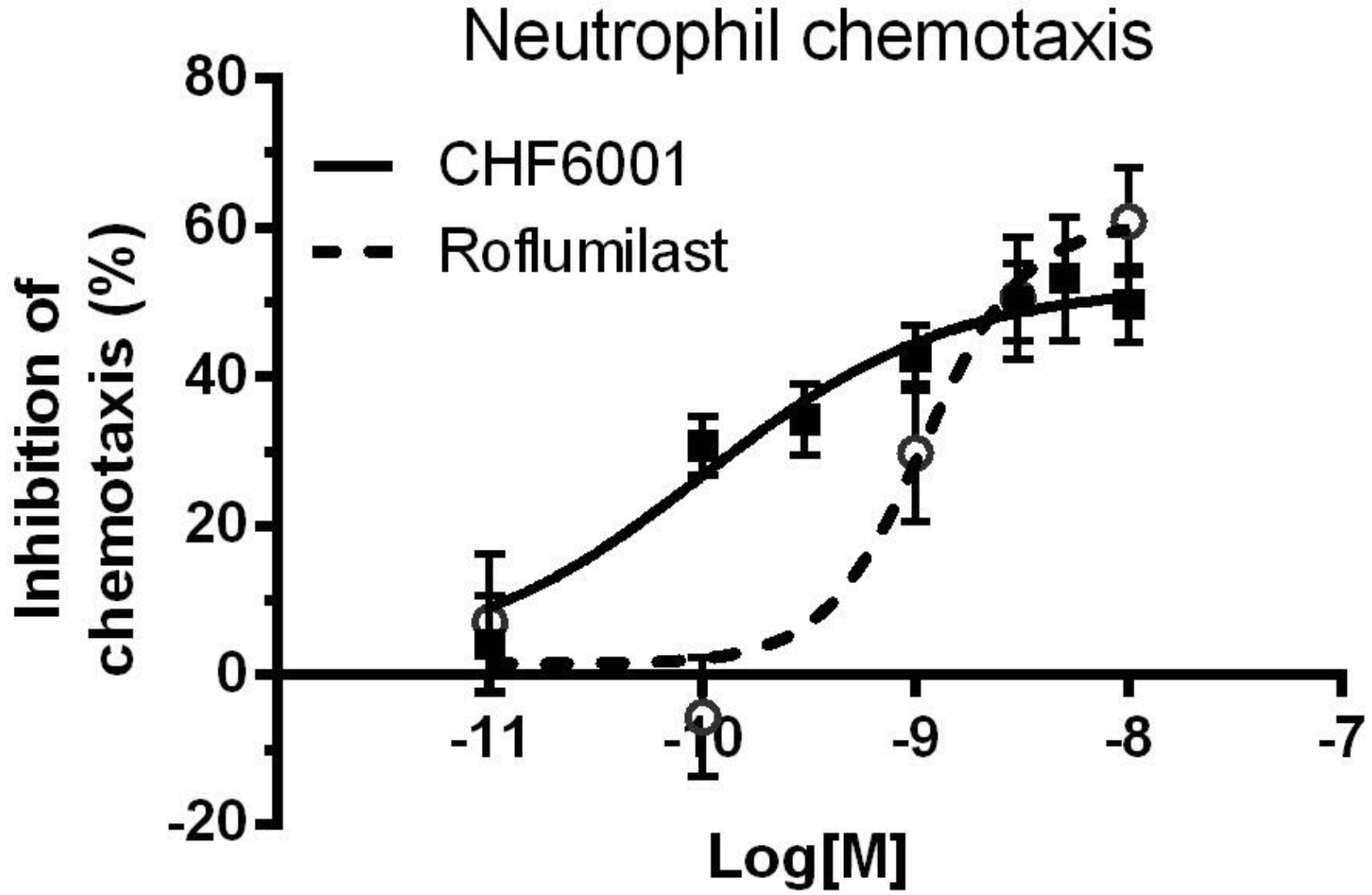


Figure 7

