Bronchodilator Activity of (3R)-3-[[[(3-fluorophenyl)\[(3,4,5trifluorophenyl)methyl]amino] carbonyl]oxy]-1-[2-oxo-2-(2-thienyl)ethyl]-1-azoniabicyclo[2.2.2]octane bromide (CHF5407) a Potent, Long-Acting and Selective Muscarinic M₃ Receptor Antagonist


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Running Title: bronchodilator activity of CHF5407
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**Abbreviations:**  
AUC, area under the curve; CHF5407, ((3R)-3-[[[(3-fluorophenyl)(3,4,5-trifluorophenyl) methyl] amino]carbonyl]oxy]-1-[2-oxo-2-(2-thienyl)ethyl]-1-azoniabicyclo[2.2.2]octane); Cl, total body clearance; Cmax, maximum plasma concentration; COPD, Chronic Obstructive Pulmonary Disease; CV, cardiovascular; DMSO, dimethyl sulfoxide; dP/dt max, maximum rate of LVP rise; F, bioavailability; HPLC, high-performance liquid chromatography; i.t., intratracheal; HR, heart rate; LIOP, Lung Inflation Overflow Pressure; LVP, left ventricular pressure; MAP, Mean arterial pressure; Penh, Enhanced Pause; Tmax, time to reach maximal concentration; Vz, volume of distribution; EFS, electrical field stimulation;

**Section:** Gastrointestinal, Hepatic, Pulmonary and Renal
ABSTRACT

The novel quaternary ammonium salt, CHF5407, showed subnanomolar affinities for human muscarinic M1, M2 and M3 receptors, and dissociated very slowly (t1/2 = 166 min from hM3 receptors (t1/2 = 166 min) with a large part of the receptorial complex (54%) remaining undissociated at 32h from radioligand washout. In contrast, [3H]-CHF5407 dissociated quickly from hM2 receptors (t1/2=31 min), whereas [3H]-tiotropium dissociated slowly from both hM3 (t1/2=163 min) and hM2 receptor (t1/2=297 min). In the guinea-pig isolated trachea and human isolated bronchus, CHF5407 produced a potent (pIC50=9.0-9.6) and long-lasting (up to 24h) inhibition of M3-receptor mediated contractile responses to carbachol. In the guinea-pig electrically-driven left atrium, the M2 receptor-mediated inhibitory response to carbachol was recovered more quickly in CHF5407-pretreated, than in tiotropium-pretreated preparations. CHF5407, administered intratracheally (i.t.) to anaesthetized guinea pigs, potently inhibited acetylcholine (Ach)-induced bronchoconstriction with an ED50 value of 0.15 nmoles/kg. The effect was sustained over a period of 24 h, with a residual 57% inhibition 48 h after antagonist administration at 1 nmoles/kg. In conscious guinea pigs, inhaled CHF5407 inhibited Ach-induced bronchoconstriction for at least 24 h as did tiotropium at similar dosages. Cardiovascular parameters in anaesthetised guinea pigs were not significantly changed by CHF5407, up to 100 nmoles/kg i.v. and up to 1000 nmoles/kg i.t. In conclusion, CHF5407 shows a prolonged antibronchospastic activity both in vitro and in vivo, due to a very slow dissociation from M3 receptors. In contrast, CHF5407 is markedly short-acting at M2 receptors, a behaviour not shared by tiotropium.
Introduction

Chronic obstructive pulmonary disease (COPD) is a syndrome caused predominantly by chronic cigarette smoking, and characterized by progressive airflow limitation. Chronic bronchitis and pulmonary emphysema are the two major pathological events occurring in COPD (Barnes, 2000). Bronchoconstriction and mucus hypersecretion are additional pathological features occurring in COPD. In COPD, bronchial smooth muscle tone is increased mostly by parasympathetic nerve (hyper)activity. Acetylcholine (ACh) released from parasympathetic nerves, activates postjunctional muscarinic M3 receptors present on airway smooth muscle cells and submucosal glands, to produce bronchoconstriction and mucus secretion, respectively (Barnes, 2004b). ACh also feeds back onto prejunctional muscarinic M2 receptors to inhibit further acetylcholine release (Haddad et al. and Rousell, 1998) (Barnes, 2004b). In addition, ample evidence has been accumulating for a role of ACh released from pulmonary cells which can activate various muscarinic receptor subtypes expressed by non-neuronal cells (Racké et al., 2006). This is the case of pulmonary fibroblasts, in which muscarinic-mediated activation (mainly of the M2 subtype) lead to increase in proliferation (Matthiesen et al., 2006) and collagen synthesis (Haag et al., 2008). Nowadays, the only effective treatment in COPD is represented by bronchodilators that, by decreasing smooth muscle tone, facilitate expiratory flow thus leading to enhanced lung emptying. No other therapy is currently available to halt the decline in lung function and the parenchymal loss associated with COPD (Barnes and Stockley, 2005) (Krishna et al., 2004). Anticholinergic drugs have been found more effective bronchodilators in COPD than β2 receptor agonists which, in contrast, are very
effective in asthma (Barnes, 2004a). Thus, anticholinergics are recommended in all current guidelines as appropriate treatment for first-line maintenance therapy of COPD, including mild disease (Rabe et al., 2007). Anticholinergic drugs most used in COPD are ammonium quaternary salts like ipratropium bromide (Gross, 1988), oxitropium bromide (Skorodin et al., 1986) and tiotropium bromide (Disse et al., 1993). In particular, the introduction of tiotropium bromide in COPD therapy has represented a breakthrough in the pharmacological management of the disease. The major advantage of tiotropium over ipratropium and other used anticholinergics is its very slow dissociation from M3 muscarinic receptors (Disse et al., 1993) (Haddad et al., 1994) (Villetti et al., 2006). This latter characteristic of tiotropium is thought to account for its long lasting bronchodilator activity, that allows COPD patients to follow an once-a-day administration regimen with this drug (Littner et al., 2000) (Gross, 2004). It is worth mentioning that neither tiotropium nor the other anticholinergic drugs used in COPD possess higher potency at M3 vs. any of the other muscarinic receptor subtypes (Barnes, 2004b). A drawback arising from poor selectivity of the available antimuscarinic drugs is that they may enhance the release of ACh at pulmonary level, as the negative feedback played by ACh at M2 autoreceptors is blocked by the antagonist. This has been demonstrated with ipratropium bromide in human isolated airways (Patel et al., 1995). In theory, the higher ACh pulmonary level may render the antimuscarinic therapy less effective, as ACh competes with the antagonist for smooth muscle M3 receptors. This phenomenon might also contribute to the paradoxical bronchoconstriction sometimes observed with inhaled ipratropium bromide (Connolly, 1982). However, tiotropium was claimed to possess a functional/kinetic selectivity for M3/ M1 over M2 receptors, as apparently it dissociates more quickly from this latter receptor subtype than from the
first two (Disse et al., 1993) (Takahashi et al., 1994). Various clinical trials have consistently shown tiotropium to have a greater impact than ipratropium on clinically important outcome measures (Vincken et al., 2002; Oostenbrink et al., 2004). Moreover, recent evidence suggests that the combination of tiotropium with the long-acting β2-adrenoceptor agonists formoterol provides additive beneficial effects for COPD patients (van Noord et al., 2006; Rabe et al. 2007). These observations have prompted great efforts for the identification of new long-lasting anticholinergic agents. Some compounds, namely LAS-34273, LAS-35201, GSK656398, GSK233705 and NVA-237 (glycopyrrolate), are now under development with the hopes of achieving quicker onset of action and improved safety profile in comparison to tiotropium (Hanania and Donohue, 2007).

Here we present a pharmacological characterization of a novel quaternary ammonium salt bearing M3 antimuscarinic activity: CHF5407 (Fig. 1). We found that CHF5407 is as effective and long-lasting as tiotropium as bronchodilator. Moreover CHF5407, but not tiotropium, showed a very short-lasting activity at M2 receptors.
Methods

Radioligand binding studies

Cell lines. Chinese hamster ovary (CHO-K1) clone cells expressing the human M1, M2 or M3- receptors (Swissprot P11229, P08172, P20309, respectively; Euroscreen, Brussels, Belgium) were grown at 37°C and 5% CO₂ in Ham’s F12 medium supplemented with 10% calf serum, 400 μg/ml of active G418, 100 U/ml Penicillin, 100 μg/ml Streptomycin and 2.5 μg/ml Fungizone (Gibco, San Giuliano Milanese, Italy).

Membrane preparation. Cells were harvested in Ca++/Mg++ free phosphate-buffered saline and collected by centrifugation at 1500 rpm for 3 min. The pellets were resuspended in ice-cold buffer A (15 mM Tris-HCl pH 7.4; 2 mM MgCl₂, 0.3 mM EDTA, 1 mM EGTA) and homogenized with a tissuemizer (Dispergerate; PBI International, Milan, Italy) (setting 5 for 15 s). The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000 g for 20 min at 4 °C, separated by a washing step in buffer A. The pellets obtained were finally resuspended in buffer B (75 mM Tris HCl, pH 7.4, 12.5mM MgCl₂, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose), and aliquots were stored at −80 °C. The day of experiment, frozen membranes were resuspended in buffer C (50 mM Tris-HCl pH 7.4, 2.5 mM MgCl₂, 1 mM EDTA). Membrane protein concentration was determined using the ‘Quant-iT Protein Assay Kit’ (Molecular Probes, San Giuliano Milanese, Italy). Final protein concentration were 1-2 μg/ml, 10-15 μg/ml and 15-25 μg/ml for hM1, hM2 and hM3.
receptor assay respectively. The non specific binding was determined in the presence of cold N-methyl scopolamine 10 μM.

**Receptorial binding assays.** The non-selective muscarinic radioligand [3H]-N-methyl scopolamine (NMS, PerkinElmer, Milan, Italy; SA 81 μCi/nmole) was used to label the hM1, hM2, and hM3 binding sites. The non specific binding was determined in the presence of cold N-methyl scopolamine 10 μM. Specific binding of [3H]-N-methyl scopolamine to hM1, hM2 or hM3 muscarinic receptors was saturable and best described by interaction of the radioligand with a single population of high affinity binding sites. [3H]-N-methyl scopolamine affinities at hM1, hM2 and hM3 receptors was (K_D, nM): 0.081 ± 0.015, 0.24 ± 0.01 and 0.016 ± 0.02, n=4 each, respectively. Saturation and competition binding experiments were performed in duplicate. Competition experiments were performed at radioligand concentration of 0.1-0.3 nM. Samples (final volume 0.75 ml) were incubated at room temperature for 120 min for hM1, 60 min for hM2 and 90 min for hM3 binding assay. The reaction was terminated by rapid filtration through GF/B Unifilter plates and two washes (0.75 ml) with cold buffer C using a Packard Filtermate Harvester (Milan Italy). After the addition of the scintillation cocktail Microscint-20 (PerkinElmer, Milan, Italy) radioactivity on the filters was measured by a microplate scintillation counter TopCount NXT (PerkinElmer, Milan Italy). In addition, the binding affinity of CHF5407 for 66 different neurotransmitter/hormone receptors and ion channels was measured, according to established methods, by MDS Pharma Services (Taiwan Ltd. Pharmacology Laboratories, 158 Li-Teh Road, Peitou, Taipei, Taiwan 112, R. O. C).
[3H]-tiotropium and [3H]-CHF-5407 binding studies. Experiments were performed in polipropilene tubes in triplicate. The non-specific binding was determined in the presence of cold N-methyl scopolamine 10 μM. Dissociations were initiated after equilibration of membrane in presence of the radioligands (0.15-0.25 nM) for 3 hours, by adding cold N-methyl scopolamine (10 μM) to avoid tracer rebinding. Samples (final volume 2 ml) were incubated at room temperature for different times. The reaction was terminated by rapid vacuum filtration through Whatman GF/B unifilter filters (Biomap SNC, Agrate Brianza, Italy) and three washes with cold assay buffer C (4 ml) using a filter box (Millipore Spa, Vimodrone, Italy). Filters used for [3H]-CHF-54070 binding were pre-soaked for at least 3 hours in buffer C containing 0.01% BSA. The filters were placed in vials with 3 ml of the scintillation cocktail Filtercount (PerkinElmer, Milan, Italy) and counted with a PerkinElmer 2500 TR beta counter (Milan, Italy). Saturation binding experiments were performed in duplicate in 96 well plates at radioligands concentration of 0.1-3 nM as described for [3H]-NMS. Samples were incubated at room temperature for 3 hours. Membrane protein concentration was determined using the ‘Quant-iT Protein Assay Kit’ (Molecular Probes, San Giuliano Milanese, Italy).

Guinea-pig isolated trachea
Zig-zag tracheal segments were obtained from male albino Dunkin-Hartley guinea pigs (450-550 g, Charles River, Laboratories Italia, Calco, Italy), as described previously (Emmerson and Mackay, 1979). Each preparation was placed in 20-ml organ bath filled with oxygenated (O2 95% and CO2 5%) normal Krebs-Henseleit solution and maintained at 37º C. Tracheal preparations were connected to isometric force
transducers under a resting tone of 10 mN. After an equilibration period of 60 min, tracheal preparations were contracted by administration of carbachol (0.3 μM: a concentration that in preliminary experiments had shown to produce 80-90% of maximal contraction) or by application of electrical field stimulation (EFS, trains of pulses of 4Hz, 0.5 msec pulse duration at 700 mA, for 10 sec every 4 min). This latter protocol was adopted to study potency and duration of action of CHF5407 under more physiological conditions than exogenous muscarinic receptor activation. All EFS experiments were performed in the presence of indomethacin (10 μM) and propranolol (1 μM). After that constant and reproducible contractions elicited by carbachol or EFS application were obtained, the effect of the test compounds was assessed, as described below. Isoprenaline (0.3 μM)-induced relaxation was used as the maximal inhibitory reference effect in carbachol-pretreated tissues. Data were recorded by means of a PowerLab system (www.AD instruments.com).

Guinea-pig isolated left atrium

All the experiments were performed according to Eglen and coworkers (Eglen et al., 1988), with modifications. Briefly, the left atrium was quickly excised from male albino Dunkin-Hartley guinea pigs (450-550 g, Charles River, Laboratories Italia, Calco, Italy), and placed in 20-ml organ bath filled with oxygenated (O₂ 95% and CO₂ 5% ) normal Krebs-Henseleit solution, maintained at 32° C. Atrial preparations were connected to isometric force transducers under a resting tone of 10 mN. Inotropic contractile responses were evoked by application of EFS (pulses of 5 msec duration, 1Hz, 8 V). Carbachol was used to produce an M2-receptor mediated inhibition of EFS-induced contraction, and test compounds were assayed for their ability to prevent
carbachol effect, as described below. Ethical approval of the experimental protocols with animals was obtained from the local Ethics Committee.

Human isolated bronchus

Specimens of human lung were obtained from 12 patients (6 males) age 46–66 years, undergoing surgery for lung cancer. Ethical approval for the experiments performed on human tissue was obtained from the Ethics Committee of the University of Florence. No patient received radio- or chemo-therapy before the operation. In all patients pre-anaesthetic medication included intramuscular atropine (1 mg) and diazepam (10 mg). Anaesthesia was induced by sodium thiopental (500 mg i.v.) and maintained with $N_2O/O_2$ (1/2) and halothane (0.6-1 %). The patients received pancuronium bromide (6 mg i.v.) during induction of anaesthesia. Immediately after the surgical removal of the lung tissue, bronchial rings of about 1-5 mm (internal diameter) were excised from the parenchyma, deprived of bronchial blood vessels and carefully rubbed with a cotton-tip applicator in order to remove the epithelium. All specimens appeared macroscopically normal without signs of tumor or inflammation. Several preparations (group A) were used to measure potency and reversibility of test compounds, up to 6h from their washout. The rest of preparations (group B) was used to assess reversibility of test compounds up to 24h from their washout. Bronchial rings of group A were rapidly placed in ice-cold gassed (96 % $O_2$ and 4 % $CO_2$) Krebs-Henseleit solution, overnight. The next day, 15-20 h after excision, the rings were placed in 5-ml organ baths filled with oxygenated Krebs-Henseleit solution at 37° C, under a resting tension of 20 mN. Changes in basal tone were recorded isometrically. The experiments commenced after a
120-min equilibration period. Carbachol was administered twice to preparations at a concentration (1 µM), that in preliminary experiments had shown to produce 80-90% of maximal contraction. At steady tonic contraction, preparations were relaxed by isoprenaline (0.3 µM). After a thorough washout and a 60 min stabilisation period, the test compounds were analysed, as reported below. Group B bronchial rings were mounted in the organ baths immediately after their preparation, and stabilized as described above. After that, carbachol (1 µM) was administered to preparations, and kept in contact until a steady tonic contraction had developed. At this point preparations were relaxed by a maximally-effective concentration of tiotropium or CHF5407 (1.0 nM each). After that a full relaxation had developed, preparations were washed out thoroughly, and the washout was repeated every 30 min for the next 4h. Subsequently, preparations were dismounted and transferred into ice-cold oxygenated Krebs-Henseleit solution, in which were kept overnight. The next day, each ring was placed in the same bath of the day before and, after stabilisation, was challenged again with carbachol (1 µM) at 24h from antagonist washout. Control matched preparations receiving the vehicle of the test antagonists were used to assess the reproducibility of the response to carbachol in the absence of M3 receptor blockade. All experiments were carried out in accordance with the Declaration of Helsinki.

**Estimate of antagonist potency and offset of the effects produced by test compounds in isolated tissues.**

All compounds were tested for their ability to revert a M3 receptor-mediated contraction (trachea and bronchus) or a M2 receptor-mediated twitch inhibition (left atrium). To this aim, cumulative concentration-response curves to test antagonists were
constructed on preparations precontracted by carbachol (trachea and bronchus) or by
EFS (trachea), or on atrial preparations inhibited by carbachol, until a complete reversal
of EFS or carbachol-induced effects was achieved. The antagonist concentration (IC_{50})
producing a 50% inhibitory effect was taken as a measure of antagonist potency in each
bioassay. To assess the offset of the effects produced by test compounds, the minimal
concentration of each test antagonist producing a (almost) maximal effect was used. As
soon as EFS- or carbachol-induced contractions or carbachol-induced twitch inhibition
were reversed by the antagonist, the organ bath solutions were renewed and
preparations were thoroughly washed with fresh Krebs-Henseleit solution. Carbachol
was administered again (at 45 min interval, between washout and next administration)
during the next 4.5 h in the guinea-pig trachea and left atrium preparations, and at 60
min interval during the next 6.0 h in the human bronchus. EFS-stimulated tracheal
preparations were followed for 4.0 h after antagonist washout. A t1/2 (offset) value – i.e
the time taken for response to carbachol or to EFS to return to 50% recovery after
washout of the test antagonist - was estimated whenever possible. In addition, the %
response to carbachol or to EFS obtained 4.0 – 6.0 h after the washout of the test
antagonist was taken as a measure of the offset (reversibility) of the compound under
investigation. Control matched preparations receiving the vehicle of the test antagonists
were used to assess the reproducibility of the response to carbachol or to EFS in the
absence of M3 or M2 receptor blockade. In the guinea-pig left atrium only, antagonist
potency of CHF5407 was estimated as the ability of the compound to rightward shift
concentration-response curves to carbachol. pK_B (negative logarithm of the antagonist
dissociation constant, K_B) was estimated as the mean of the individual values obtained
with the equation (Kenakin, 1997):
(1) $pK_B = \log \left[ \frac{\text{dose ratio} - 1}{\text{antagonist concentration}} \right]$ 

Competitive antagonism was checked by the Schild plot method: a plot with linear regression line and slope not significantly different from unity was considered as proof of simple reversible competition (Kenakin, 1997).

**Bronchoconstriction in anaesthetized guinea-pigs**

The experiments were performed as described previously (Villetti et al., 2006). Briefly, guinea pigs (450 - 550 g) were anaesthetized with pentobarbital sodium (90-100 mg/kg, i.p.). Pentobarbital was subsequently infused at the rate of 5-10 mg/kg/h into the carotid artery, in order to maintain a stable level of anaesthesia throughout the duration of the experiment. Animals were mechanically ventilated with room air (rate: 70 strokes/min; tidal volume: 10 ml/kg) by a rodent ventilator after pancuronium bromide treatment (2 mg/kg i.v.). A full dose-response curve to ACh was obtained before testing the antagonists, to identify a sub-maximal dose of the agonist. The dose of 20 µg/kg was selected because this dose evoked a bronchospasm that was ~70% of the effect achieved by the complete occlusion of the tracheal cannula (maximal bronchospasm theoretically achievable). Bronchoconstriction, induced by i.v. bolus of ACh, 20 µg/kg, was quantified according to the method of Konzett and Roessler (1940). CHF5407 (0.1 - 0.3 - 1 nmoles/kg) was instilled intratracheally in a volume of 100 µl after stabilization of the bronchoconstrictor response to ACh (baseline reading). Test compound instillation was aided by two consecutive air insufflations (3 ml each), in order to facilitate the distribution of the solution into the airways (Lundberg and Saria, 1983). ACh challenge was then repeated at 5, 15, 30 min and then every 30 min up to 180 min from test compound administration. At the end of the experiment, the animals
were challenged with histamine (5 mg/kg i.v.) to verify bronchial reactivity to a different spasmogen. The effect of test compounds was expressed as % inhibition of ACh-evoked bronchoconstriction, as compared to basal response. In animals undergoing Ach challenge 24-48 h after antagonist administration, anaesthesia was induced with isoflurane (4% in O2, 2 l/min) and the trachea exposed by a laryngoscope. Test compounds (or vehicle) were then instilled locally as previously described. Guinea pigs were allowed to recover from anaesthesia and fed normally. The day of testing, animals were anaesthetized with pentobarbital sodium and surgically prepared as above reported. ACh (20. µg/kg) was administered at 3 min intervals, until 3 stable bronchospasms were recorded. ACh challenges did not ever exceed the number of 10. Time-matched, vehicle-treated, animals were used as controls.

**Bronchocostriction in conscious guinea pigs**

Airway sensitivity to Ach was measured using a single-chamber whole-body plethysmograph (Buxco, Sharon, CT, USA), according to the method of Hamelmann and colleagues (1997) and Chong and colleagues (1998). Guinea pigs (body weight 500-600 g) were individually placed in plethysmograph chambers and acclimatated for 5 min. The bias flow (air removed from chamber) was set at 2.4 l/min. Guinea pigs were then challenged for 1 min with saline using the Buxco Aerosol Delivery System (Micropump Aeroneb Laboratory Nebulizer; nebulized air flow 2 l/min) to obtain baseline airway readings. Animals were then exposed to Ach aerosol (2.5 mg/ml, 1 min) to determine their sensitivity to the spasmogen (pre-treatment control). Ach dosage (selected on the basis of a previously determined dose-response curve) was set to produce sub-maximal bronchocostriction, i.e. 3-5 sec of apnea in 50% of guinea-pigs
(data not shown). After 60-min washout period, 5-min aerosol administration of vehicle (H2O), CHF5407 (5-12.5-25-50-100-250 µM), ipratropium (200-400 µM) and tiotropium (50-100-250 µM) were applied and Ach challenge was repeated after 2, 5 24, 48 and 72 hours. In case of 100% inhibition of Ach-evoked bronchocostriction and at 24, 48 and 72 hours after antagonist administration the animals were challenged with 1-min aerosol of histamine (0.75 - 1.0 mg/ml) in order to verify bronchial reactivity to a contracting agent. The pressure waveforms generated by respiration of the guinea pigs were recorded by Air Flow Transducer (mod. TRD5700 Buxco) and processed using MAX II hardware and BioSystem XA software for Windows (Buxco). Enhanced pause (Penh), defined as pause x peak expiratory flow /peak inspiratory flow was used as read-out. Penh was recorded for 5 min after each challenge. The average value of this recording was used as index of bronchoconstriction. The effects of the test compounds were expressed as percent inhibition of the basal Ach-induced increase in Penh. Time-matched, vehicle-treated animals were used as controls.

Measurement of cardiovascular parameters in anaesthetized guinea pigs
Guinea pigs (390-410) were anaesthetized with 1.5 g/kg i.p. urethane solution, tracheotomized and intubated, to allow spontaneous breathing. Rectal temperature was monitored and maintained at 38 ± 0.2 °C by means of infrared lamps. A polyethylene catheter (PE-50) was inserted into the left femoral artery and connected to a pressure transducer (model 1280C; Hewlett Packard, Waltham, MA, USA) for the measurement of arterial blood pressure. A 2F Millar Mikro-Tip® catheter, with one high-fidelity pressure sensor (model SPR-249; Millar Instruments Inc., Houston, Texas, USA), was inserted via the right carotid artery into the left to measure the left ventricular pressure
ECG was recorded by inserting needle electrodes subcutaneously according to standard limb leads after Einthoven I–II. Mean arterial pressure (MAP) and left ventricular pressure (LVP) values were calculated by using a PowerLab/400 device (ADInstruments, Hastings, UK.) In addition, the maximal rate of LVP rise (dP/dtmax) and heart rate (HR) were calculated from LVP. ECG was recorded with a Cardioline apparatus (model Delta-1; Remco Italia, Milan, Italy), the signals were continuously transmitted into ECG module (ADInstruments), and the number of extrasystoles counted. Signals obtained from each module of the system were analyzed by Chart 5™ software (ADInstruments). Data were recorded for 30 s after 5 min or 10 min from intravenous or intratracheal administration of test compounds, respectively.

In a first set of experiments, cumulative doses (1-10000 nmoles/kg) of CHF5407, tiotropium, methoctramine or vehicle were injected intravenously via the right jugular vein (0.5 ml/kg) every 5 min. In a second set of experiments, cumulative doses of the same compounds were administered intratracheally through a tracheal cannula (50 µl for each dose; 250 µl total volume) every 10 min.

Animals
Male albino Dunkin-Hartley guinea pigs were obtained from Charles River Laboratories Italy (Calco, Lecco, Italy) and housed in plastic cages (Tecniplast Gazzada, Varese, Italy) in air-conditioned rooms at 22° C in a 12-h light/dark cycle. Food and water were available at libitum. All animals were acclimatized for at least 5 days before any experimental work began. All the experiments were carried out in accordance with National and European legislation and were approved by local ethical committees.
Chemicals

All the materials used for cell culture were from Invitrogen SRL (San Giuliano Milanese Italy) with the exception of G 418 that was purchased from VWR, Milan, Italy. Tiotropium bromide and CHF5407 bromide or chloride, (3R)-3-[[[3-fluorophenyl][3,4,5-trifluorophenyl)methyl]amino]carbonyl]oxy]-1-[2-oxo-2-(2-thienyl)ethyl]-1-azoniabicyclo[2.2.2]octane) (Fig.1), were synthesized at Chiesi Chemical Department. All other drug substances were from Sigma Aldrich (Milan, Italy). Stock solutions (10 mM) of CHF5407 and tiotropium were prepared in 100% DMSO. Further dilutions were prepared with saline. In control in vitro or in vivo experiments, administration of 1 % DMSO in saline was ineffective per se. [3H]-tiotropium and [3H]-CHF-5407 were customer synthesized by GE Healthcare (84 and 62 μCi/nmole, respectively).

Statistical analysis

All values in the text, table and figures are expressed as mean ± S.E.M. of the given number (n) of experiments. Statistical analysis was performed using Student’s t-test for paired or unpaired data, or by one-way analysis of variance (ANOVA) followed by Dunnett’s t test when applicable. P < 0.05 was considered a level of statistical significance. Kinetic and competition binding curves were analysed by nonlinear regression analysis using the ‘Solver’ tool of Excel program (Microsoft, Milan, Italy). Dissociation binding results were analysed using the classical first order equation and a model considering an irreversible binding component:

\[
B_t = [Bo*e^{-k1*t}] + Ir
\]
where \((B_t), (B_0), (k_{-1})\) and \((t)\) represent residual binding at the different times, binding at time 0, dissociation constant and time expressed in minutes, respectively. The impact of the introduction of the new parameter \((I_r)\) on the goodness of fit was determined using the F test, by comparing the residual sum of square errors obtained by data fitting with the two models, as described by (Munson and Rodbard, 1980). IC\(_{50}\) values were determined using a four parameters logistic equation and results transformed into Ki values using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Saturation experiments were analysed using the non-linear fitting program ‘Ligand’ (Munson and Rodbard, 1980). ED\(_{50}\) (i.e. the dose of antagonist reducing by 50% Ach-induced bronchoconstriction in anaesthetized guinea pigs) was calculated from dose-response curves using a least-squares linear regression analysis in Prism version 4.02 (GraphPad Software Inc., San Diego, USA). Data from studies in anaesthetized and conscious guinea pigs were analyzed using Student’s t-test for paired or unpaired data, or by one-way analysis of variance (ANOVA) followed by a one-sided Dunnett’s multiple comparison test, versus the time-matched vehicle control.
Results

Affinity and selectivity of CHF5407 for hM1, hM2 and hM3 receptors

In competition experiments, CHF5407, tiotropium and ipratropium produced a concentration-dependent full displacement of $[^3]$H-N-methyl scopolamine from hM1, hM2 and hM3 receptors stably expressed in CHO-K1 cells, with comparable, subnanomolar affinities (Ki values, see Table 1). None of the antagonist compounds considered was selective toward anyone of the three muscarinic receptor subtypes. In saturation experiments, $[^3]$H-CHF-5407 binding at the three muscarinic receptor subtypes was saturable and best fitted by a single site model. In agreement with data calculated in competition experiments, $[^3]$H-CHF-5407 bound to hM1, hM2 and hM3 with high, subnanomolar affinities (Kd values, cfr. Table 1). Receptor selectivity of CHF5407 for muscarinic receptors was checked by measuring its affinity for a variety of different receptors / ion channels / transporters, including the followings: Adenosine A1, A2A, A3; Adrenergic $\alpha_1A$, $\alpha_1B$, $\alpha_1D$, $\alpha_2A$, $\beta_1$, $\beta_2$; Bradykinin B1, B2; Calcium Channel L-Type; Calcium Channel N-Type; Dopamine D1, D2s, D3, D4,2; Endothelin E$\tau_A$, E$\tau_B$; Epidermal Growth Factor (EGF); Estrogen ER$\alpha$; GABA$\alpha$, GABA$\beta_1$; Glucocorticoid; Glutamate Kainate, Glutamate NMDA; Histamine H1, H2; Imidazoline I2; Interleukin IL-1; Leukotriene, Cysteiny1 CysLT1; Melatonin MT1; Neuropeptide Y Y1, Y Y2; Nicotinic Acetylcholine, Nicotinic Acetylcholine, Bungarotoxin-Sensitive; Opiate $\delta$, k and $\mu$; Potassium Channel HERG; Potassium Channel [K$\text{ATP}$]; Phorbol ester; PAF; Prostanoid EP4; Purinergic P$_{2X}$, P$_{2Y}$; Rolipram; Serotonin 5-HT$_{1A}$, 5-HT$_{3}$; Sigma $\sigma_1$, $\sigma_2$; Sodium Channel, Site 2; Tachykinin NK-1; Testosterone; Thyroid hormone; Transporter, Dopamine (DAT); Transporter, GABA; Transporter,
Norepinephrine (NET); Transporter, Serotonin (5-HT) (SERT) (data not shown). At the above receptorial sites, CHF5407 displayed >1000-fold lower affinity (i.e. IC$_{50}$ or $K_I$ > 0.25 $\mu$M) than at the target receptor M$_3$ ($K_D = 0.23$ nM; cfr Table 1).

Association and dissociation kinetics of $[^{3}\text{H}]$-CHF-5407 vs. $[^{3}\text{H}]$-tiotropium at hM1, hM2 and hM3 receptors

In association kinetic experiments, both $[^{3}\text{H}]$-CHF-5407 and $[^{3}\text{H}]$-tiotropium bound to hM3 or hM1 with much slower association rates as compared to hM2 receptor (cfr. Table 2).

Dissociation kinetics of $[^{3}\text{H}]$-CHF-5407 and $[^{3}\text{H}]$-tiotropium from hM3 or hM1 were best fitted by a mathematical model considering an irreversible binding component for both radioligands (F test, p<0.001). Indeed, at 32 h from the beginning of the dissociation, only a fraction of $[^{3}\text{H}]$-CHF-5407 or $[^{3}\text{H}]$-tiotropium was effectively dissociated from the hM3 receptor (45.9 % and 37.8 %, respectively: Fig. 2 and Table 2). As for the reversible binding components, the half-life (t$_{1/2}$) of $[^{3}\text{H}]$-CHF-5407 – hM3 receptor complex was almost identical to t$_{1/2}$ of $[^{3}\text{H}]$-tiotropium – hM3 receptor complex: 166 vs.163 min, respectively (cfr. Table 2). In contrast, the binding of $[^{3}\text{H}]$-CHF-5407 at hM2 receptor was totally reversible, with the dissociation curve of this radioligand best described by a simple first order exponential function, with a $\kappa_{off}$ equal to 0.0224 min$^{-1}$ and a corresponding half-life of 31.8 min (cfr. Table 2; Fig. 2). On the other hand, the dissociation curve of $[^{3}\text{H}]$-tiotropium from the hM2 receptor was best fitted by a mathematical model considering an irreversible component, corresponding to 14.5 % of total binding (cfr. Table 2; Fig. 2). The estimated t$_{1/2}$ of the hM2/$[^{3}\text{H}]$-
tiotropium complex was much longer than that of [3H]-CHF-5407/hM2 complex: 297.3 vs. 31.8 min, respectively (cfr. Table 2).

**Antagonist potency and duration of action of CHF5407 at M3 receptors in the guinea-pig trachea**

Carbachol (0.3 µM) elicited reproducible and sustained tonic contractions of the tracheal preparations, over a period of several hours. EFS elicited constant twitch contractions which remained unaltered for hours. In carbachol (0.3 µM)-precontracted preparations, CHF5407 produced a slowly-developing inhibition of smooth muscle tone, with a potency overlapping that shown by tiotropium and by other antimuscarinic compounds (cfr. Fig. 3a; Table 3). Similarly, CHF5407 produced a slowly-developing inhibition of EFS-induced twitch contractions with unaltered potency (Table 3).

CHF5407-induced inhibitory effects of M3-receptor mediated contractions were selective, as a maximally-effective concentration of CHF5407 (10 nM) failed to revert a tracheal smooth muscle tone (averaging ~ 85% of that produced by carbachol 0.3 µM) raised by neurokinin A (1 µM) (n=4, data not shown). CHF5407 (up to 1 µM) failed to produce any contractile response in quiescent tracheal preparations, thus showing no agonist efficacy at M3 receptors (n=4; data not shown).

In experiments aiming at evaluating the offset of action, CHF5407 (10 nM) was administered to carbachol (0.3 µM)-precontracted, or to EFS-stimulated preparations and left in contact with the tissues until attainment of complete inhibition of the contractions. After washout of CHF5407, both the contractile response to carbachol (0.3 µM, given every 45 min) and responsiveness to EFS of the trachea remained depressed over a period of 4.0 - 4.5 h, despite the removal of the antagonist from the physiological solution (Table 3; Fig. 3b, Fig. 4). The offset of tiotropium (10 nM)-induced inhibitory
effects was superimposable to that shown by CHF5407 (Fig. 3b, Fig.4), whereas ipratropium (Fig. 4) or glycopyrrolate produced more quickly reversible effects (cfr. Table 3).

Antagonist potency and duration of action of CHF5407 at M3 receptors in the human bronchus

Carbachol (1 µM) produced reproducible tonic contractions of the human bronchial smooth muscle over a period of several hours. CHF5407 concentration-dependently completely reverted carbachol (1 µM)-induced tonic contraction with a potency similar to that shown by tiotropium (cfr. Fig. 5a; Table 3). CHF5407 (10 nM) failed to inhibit a bronchial smooth muscle tone (averaging ~ 100% of that raised by carbachol 1 µM) produced by neurokinin A (1 µM) (n=4 each, data not shown). CHF5407 (1 nM)-induced inhibitory effect persisted almost unaltered over a period of 6 h, irrespective of washout of the antagonist from the buffer solution. Tiotropium (1 nM) effect was lasting as long as (or less than) CHF5407 over the same time interval (cfr. Table 3; Fig.5b). As observed in the guinea-pig trachea, ipratropium or glycopyrrolate produced more quickly reversible effects compared to CHF5407 (Villetti et al., 2006) (cfr. Table 3). A modified protocol was set up, to check whether CHF5407 inhibitory effect on M3–receptor mediated bronchial smooth muscle contraction could be maintained up to 24 h from antagonist washout (see Methods for details). In control preparations, the responsiveness of bronchial smooth muscle to carbachol (1 µM) was almost halved at 24h from the first administration of the agonist. However, in CHF5407 (1 nM)-pretreated preparations the response elicited by carbachol was significantly less, indicating that an inhibitory effect of the antagonist was still present (Figure 6).
Antagonist potency and duration of action of CHF5407 at M2 receptors in the guinea-pig paced left atrium

Carbachol (100 nM - 3 µM) produced a concentration-related, M2-receptor mediated inhibition of EFS-induced twitch contraction of the left atrium (not shown). Methoctramine (0.3 – 10 µM), an M2-receptor preferring antagonist, competitively antagonised carbachol-induced inhibition of twitch response, with a potency (pK_B = 7.9 ± 0.06, n=16) exactly matching that reported previously in this bioassay (Eglen et al., 1988). A submaximal concentration of carbachol (0.3 µM), producing 80-90% of maximal inhibition was chosen for testing the ability of CHF5407 to revert M2 receptor-mediated effects in the left atrium. In carbachol (0.3 µM)-pretreated electrically-driven preparations, both CHF5407 (Figure 7) and tiotropium produced concentration-dependent excitatory effects due to reversal of the agonist-induced inhibitory activity (Figure 8a). CHF5407 and tiotropium produced similar excitatory effects, both in terms of pEC_50 or E_max (cfr. Table 3; Figure 8a).

In experiments aiming at evaluating the offset of action, CHF5407 (30 nM) was administered to carbachol (0.3 µM)-pretreated preparations and left in contact with the organ until that a complete reversal of agonist-induced inhibition was attained. After washout of CHF5407, the inhibitory response to carbachol (0.3 µM, given every 45 min) was recovered quickly (t1/2 = 1.0 h), whereas the recovery was much slower (t1/2 = 3.3 h) in tiotropium (10 nM)-pretreated preparations (cfr. Table 3; Figure 8b). CHF5407 and tiotropium (2h incubation period, each) were then challenged for their ability to rightward shift a concentration-response curve to carbachol. CHF5407 behaved as a competitive antagonist (pK_B = 8.7 ± 0.08; n=12 ), as it produced a parallel shift of the curves to carbachol, without depressing E_max (Figure 9). Schild plot analysis of CHF5407 antagonism provided an unitary slope, as expected for a
competitive antagonism (Figure 9). In contrast, tiotropium produced an insurmountable non competitive antagonism, as expected from a slowly-dissociating M2 receptor antagonist (Figure 9).

**Antibronchoconstrictor effect in anaesthetized guinea-pigs**

CHF5407 intratracheal treatment exerted a dose-dependent and potent inhibition of ACh-induced bronchospasm, with an ED50 value of 0.15 nmoles/kg (Figure 10). At 1 nmoles/kg the onset of CHF5407 inhibitory action raised quickly with time and reached a maximum at 30 min from antagonist administration, whereas it was slower at 0.1 - 0.3 nmoles/kg (maximum effect at ~180 min from CHF5407 administration). At any dose, CHF5407-induced inhibition lasted almost unchanged over a period of 24 h (Figure 11). At 48 h from CHF5407 administration, a slight but significant decrease of the inhibitory effect was observed only at the dose of 1 nmoles/kg (p < 0.05).

**Antibronchoconstrictor effect in conscious guinea pigs**

Two hours after inhaled administration in conscious, unrestrained animals, CHF5407 produced a dose-dependent inhibition of Penh increase induced by Ach challenge, with a maximal effect observed after nebulisation of 50-250 µM solutions. The dose response of the CHF5407 effect appeared to increase with time. Indeed, the highest concentrations nebulised (50-250 µM) afforded a significant protection lasting up to 48 h from administration, whereas at later stages of the observation period the lower doses were having a reduced or no effect (Figure 12). However, after nebulization of the 25 µM solution endowed with a sub-maximal anti-bronchospastic activity at the 2 h time point (84.0 ± 6.3 % inhibition), the effect persisted significantly up to 24 h (64.0 ±
16.1% inhibition). Tiotropium profile of action was similar to that of CHF5407, whereas ipratropium was short acting in this model (Figure 12).

**Cardiovascular effects of CHF5407 in anaesthetized guinea pigs**

The effect on MAP, HR, LVP and dP/dtmax of cumulative doses of CHF5407, tiotropium and methoctramine, administered intravenously or intratracheally, are reported in Figures 13 and 14.

The lowest dose of CHF5407 studied on cardiovascular (CV) parameters was the dose which produced a maximal inhibition of Ach-induced bronchospasm, namely 1 nmole/kg. Higher doses were 10x the initial dosage, up to 10,000 nmoles/kg. Starting from the doses of 100-300 nmoles/kg, intravenous administration of CHF5407, tiotropium or methoctramine, dose-dependently and significantly decreased both MAP and LVP, and increased HR, without affecting the dP/dtmax. At 300 nmoles/kg, a significant fall in MAP was observed only after methoctramine treatment. The increase in HR and the modest changes in LVP and dP/dtmax observed after CHF5407 and tiotropium treatment were not significantly different from that obtained with methoctramine. More severe changes in MAP, HR, and LVP were attained with the three compounds at doses from 1000 to 10000 nmoles/kg i.v. However, in this case, the differences of CHF5407 from tiotropium were modest but significant (p < 0.05).

When test compounds were administered intratracheally, minor changes in MAP, HR, LVP and dP/dtmax were obtained as compared to that observed after intravenous administration (Figures 13 and 14). Up to the dose of 100 nmoles/kg i.t., the three compounds did not significantly modify all the parameters considered. At the dosage of 1000 and 10000 nmoles/kg more substantial modifications on both MAP and HR were
observed, with methoctramine being more effective than both CHF5407 and tiotropium in changing MAP and HR. After i.t. treatment, the activity of CHF5407 was comparable to that of tiotropium.

The total number of extrasystoles, counted via ECG signal at the end of intravenous administration of cumulative doses of CHF5407, tiotropium and methoctramine, was very low with a variation in 45 min of the extrasystolic beats from 5 ± 3 to 11 ± 5. In the case of intratracheal instillation of the three compounds, no extrasystoles were recorded (data not shown).
Discussion

Our data provide evidence that CHF5407 is a potent and selective muscarinic receptor antagonist, and that it is as potent as tiotropium in preventing M3 receptor-mediated bronchoconstriction both in vitro and in vivo. Importantly, CHF5407 is as long acting as tiotropium, as its effect remains measurable up to 24 - 48h from antagonist administration. It is worth noting that other antimuscarinic drugs that are currently employed as bronchodilators (e.g. ipratropium; (Gross, 1988)) or suggested to be used for this purpose (e.g. glycopyrrolate; (Hansel et al., 2005)) show a significantly shorter duration of action compared to CHF5407 (cfr. present data and (Villetti et al., 2006). In the present study, we observed that CHF5407 blocked M3-mediated responses in either human or guinea-pig isolated airway smooth muscles for several hours, despite the antagonist had been washed out from the organ bath. This result can be easily explained with the partial and very slow kinetic of dissociation (t1/2 = 166 min) of CHF5407 from M3 receptors, as measured in radioligand binding experiments. Moreover, we observed that radiolabelled CHF5407 remains tightly bound to a large fraction of M3 receptors (~54% of total receptors) at 32 h from its removal from the buffer solution: a characteristic that may explain the residual antibronchoconstrictor effect exerted by our compound up to 24 - 48 h from its administration. The rank order of reversibility of tiotropium from muscarinic receptors, that can be estimated from our data, is the following: hM3 < hM1 <hM2, that is in keeping with previously published data. In particular, Casarosa et al. (2009) described a fully reversible dissociation of [3H]-tiotropium from M3 but with a slower kinetic than from M1 or M2. However, in contrast with Casarosa et al. (2009), we found a consistent irreversible component of
tiotropium binding to all muscarinic receptor subtypes; this component was greater for tiotropium-M3 receptor complex than for the other receptor complexes. The discrepancy between our and Casarosa et al. (2009) data might be due to the different protocols used, particularly for the following procedures: (a) dissociation started after 3h (our procedure) or 2h of incubation with test compounds; (b) dissociation started by diluting samples with buffer containing unlabelled NMS (10 µM; our procedure) vs. atropine 10 µM.

Bronchoconstriction induced by intravenous injection of Ach in anaesthetized guinea pigs is a widely used model to assess the efficacy of bronchodilators (e.g. Mohammed et al., 2001; Rossoni et al., 2007). Because the duration of action of bronchodilators can not be followed for more than 6 h with this model we modified the experimental procedure, and established a simple and reliable protocol to evaluate the duration of the antimuscarinic activity in the airways up to 24 - 48 h from antagonist administration (Villetti et al., 2006). Under that conditions, CHF5407 was at least ~ 2-4 folds more potent than tiotropium, ipratropium or glycopyrrolate (cfr. present data and Villetti et al, 2006). We also observed that while the onset of action of CHF5407 was slightly slower than after glycopyrrolate and ipratropium administration, CHF5407 produced a more prolonged bronchoprotective effect in Ach-challenged animals than ipratropium and glycopyrrolate (Villetti et al., 2006). Importantly, CHF5407 inhibition of Ach-induced bronchoconstriction at 24h from antagonist administration was comparable to that produced by tiotropium at doses endowed with a comparable peak effect (cfr.present data and Villetti et al, 2006).

Present data show that the antibronchoconstrictor effect of CHF5407 was long-lasting also in conscious, unrestrained guinea pigs. In these latter animals, we measured Penh
as a representative indicator of airflow through the airways. Indeed, Penh is an empiric parameter reflecting efforts of breathing that was initially considered proportional to airway obstruction (Hamelmann et al., 1997; Chong et al., 1998). Penh measurement may be affected by many factors, so that it was disputed to represent a direct measure of airway resistance (Lundblad et al., 2002). Nevertheless, we observed that the effects of CHF5407 and tiotropium on Penh matched those observed in anaesthetized animals either in terms of relative potency or duration of action.

We also investigated CHF5407 activity at M2 muscarinic receptors, in comparison with tiotropium. Tiotropium was claimed to possess a kinetic selectivity for M3 vs. M2 receptors, on the basis of both radioligand binding and functional experiments which suggested that tiotropium apparently dissociated quickly from M2 receptors (Disse et al., 1993) (Takahashi et al., 1994) (Haddad et al., 1994). Our present data only partially support this claim. Indeed, in our radioligand binding experiments a fraction of tiotropium-M2 receptor binding was practically irreversible (14% of total receptors bound at 32h from antagonist washout), as observed for tiotropium-M3 receptor binding whose irreversible fraction (65% of total receptors bound at 32h from antagonist washout) was, however, more relevant. We also observed that tiotropium dissociated more slowly from the M2 than from the M3 reversible pool of receptors (t1/2 = 297 vs. 163 min, respectively). In sharp contrast to tiotropium, CHF5407 dissociated quickly (t1/2 = 31 min) and completely from human M2 receptor, but was as slow as tiotropium in dissociating from M3 receptors. These results obtained at the molecular level are matched by data arising from our functional experiments in the guinea-pig paced left atrium (GPA). In the guinea-pig heart, activation of muscarinic receptors leads to a reduction of force of contraction and (in nonpaced tissues) to a
reduction of rate of beating (Eglen et al., 1988). Extensive studies with various muscarinic antagonists have defined this response as being mediated by the M2 receptor (Caulfield and Birdsall, 1998). Our present results show that CHF5407 is 4-5 fold less potent than tiotropium (pIC50 8.2 vs. 8.7, respectively): a result that is in good agreement with binding affinities shown by the two antagonists at M2 receptor (cfr. Table 1). In addition, CHF5407 duration of action in the GPA was significantly shorter than that of tiotropium. We also demonstrated diverging antagonist behaviour of CHF5407 (competitive) vs. tiotropium (not competitive) in the GPA: two drug profiles fitting with reversible vs. irreversible M2 receptor antagonists, respectively. On this basis, we conclude that CHF5407 - much more than tiotropium - possesses a kinetic selectivity for M3 vs. M2 receptors. In vivo, both CHF5407 and Tiotropium, at high dosage, affected heart rate and blood pressure similarly to the M2 receptor antagonist methoctramine (Howell and Kovalsky, 1995), i.e. they decreased MAP and increased HR, an effect the latter which is mainly mediated by the cardiac M2 receptor (Hendrix and Robinson, 1997). A modest, although significant, difference between CHF5407 (less effective) vs. tiotropium (more effective) in changing cardiovascular parameters became evident at very high doses (1000 - 10000 nmoles/kg) only. The discrepancy could actually depend on the faster dissociation of CHF5407 from M2 receptors than tiotropium, even if other factors, such as different pharmacokinetics and tissue distribution, could have contributed to the different cardiovascular profile of the two antagonists. A recent large clinical trial with tiotropium (UPLIFT study) has confirmed the benefits of tiotropium in COPD management and provided reassurance regarding its CV profile (Tashkin, 2010). However, the same CV safety has not been established for other inhaled antimuscarinic compounds, like ipratropium. On the basis of the UPLIFT
trial, it may be speculated that tiotropium takes advantage from its short-acting activity at M2 receptors, that might reduce CV risks associated to systemic absorption of the drug. If this latter interpretation is correct, then CHF5407 should result at least as safe as tiotropium, because of its shorter duration of action at M2 receptors. On the other hand, it has been suggested that blockade of M2 receptors may increase the efficacy of muscarinic M3 blockers, because M2 receptor activation may reverse sympathetically-mediated relaxation (e.g. Sarria et al., 2002). Nevertheless, we found that CHF5407 was as effective as tiotropium in preventing ACh-induced bronchoconstriction in conscious guinea-pigs, until 48h from drug administration. This result suggests that the short duration of action at M2 receptors, assuming this characteristic actually handicaps antimuscarinic compounds, apparently does not penalize CHF5407 in comparison to tiotropium.

In conclusion, CHF5407 possesses a very promising pharmacological profile in vitro and in vivo as antibronchoconstrictor agent. Only tiotropium, among several currently used antimuscarinic drugs, matches CHF5407 for the duration of the antimuscarinic activity that is due, for both compounds, to a very slow dissociation from the M3 receptor. Importantly, CHF5407 is very short-acting at M2 receptors, a behaviour not shared by tiotropium.
Acknowledgments

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References


Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.


airway changes induced by different challenger in guinea-pigs. *Pulm Pharmacol Ther* **20**: 250-257


Footnotes

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Preliminary data of this study have been presented in abstract form at the following meetings: ATS International Conference, San Francisco, May 18-23, 2007 and ERS Annual Congress, Stockholm, September 15-19, 2007.
Legends for the Figures

Figure 1
Chemical structure of CHF5407

Figure 2
[^3]H]-CHF5407 vs. [^3]H]-tiotropium dissociation kinetics from hM3 and hM2 muscarinic receptors expressed in CHO-K1 cells. Dissociations were started by adding cold 10 μM N-methyl scopolamine. Data are expressed as percentage of corresponding control (% C; samples without 10 μM cold N-methyl scopolamine). Each point is the average of triplicate determinations from two independent experiments.

Figure 3
Concentration-dependent inhibition by CHF5407 and tiotropium (Tio) of carbachol (0.3 μM)-induced tonic contraction in the guinea-pig isolated trachea. (b) Decay of the inhibitory effects produced by CHF5407 and tiotropium (10 nM each) against contractile responses to carbachol (0.3 μM). After washout of the antagonist (time=0) carbachol was administered at 45 min intervals during the next 4.5 h. Each value is the mean ± S.E.M. of 4-6 observations.

Figure 4
Typical tracings showing the rate of offset of the inhibitory effects produced by CHF5407, ipratropium and tiotropium on EFS-induced twitch contractions in the
guinea-pig isolated trachea. Arrows indicate administration of test compounds or washout. The upper tracing represents a control experiment.

**Figure 5**
Concentration-dependent inhibition by CHF5407 and tiotropium (Tio) of carbachol (1 µM)-induced tonic contraction in the human isolated bronchus. (b) Decay of the inhibitory effects produced by CHF5407 and tiotropium (1nM each) against contractile responses to carbachol (1 µM). After washout of the antagonist (time=0) carbachol was administered at 60 min intervals during the next 6.0 h. Each value is the mean ± S.E.M. of 4-6 observations.

**Figure 6**
Columns show the contractile response to carbachol (1 µM) in the human isolated bronchus, 24h after a first response to carbachol was elicited in the same preparation, in the absence (control) or presence of CHF5407 or tiotropium (1nM each). After washout of the antagonist (time=0), preparations were dismounted and kept overnight into ice-cold oxygenated Krebs-Henseleit solution. The next day, each bronchial preparation was mounted again in the same bath of the day before, and challenged again with carbachol. Each value is the mean ± S.E.M. of 9-13 observations, made in tissues obtained from 6 donors. (**) Significantly different from control response: P < 0.01.
Figure 7

Typical trace showing the concentration-dependent reversal by CHF5407 of the inhibition of EFS-elicited twitch contractions produced by carbachol (0.3 μM) in the guinea-pig paced left atrium. EFS: pulses of 5 msec duration, 1Hz, 8 V.

Figure 8

Concentration-dependent reversal by CHF5407 and tiotropium (Tio) of the M2-receptor mediated inhibitory effect produced by carbachol on electrically-induced twitch contractions in the guinea-pig paced left atrium. (b) Decay of the antagonist effects produced by CHF5407 (30 nM) or tiotropium (10 nM) against inhibitory responses to carbachol (0.3 μM) in the guinea-pig paced left atrium. After washout of the antagonist (time=0) carbachol was administered at 45 min intervals during the next 4.5 h. Each value is the mean ± S.E.M. of 4 observations.

Figure 9

(a) Concentration-dependent inhibition by carbachol of electrically-induced twitch contractions in the guinea-pig paced left atrium in the absence or presence of CHF5407 or tiotropium. (b) Schild plot analysis of agonist dose ratios vs. CHF5407 concentrations. (slope = -1.2; 95% C.L. –0.8 –1.6). Each value is the mean ± S.E.M. of 4 observations.

Figure 10. Inhibition afforded by intratracheally-administered CHF5407 of Ach (20 μg/kg i.v.)-induced bronchoconstriction in anaesthetised guinea-pigs. Ach was injected...
before (baseline value) and up to 180 min after test compound administration. Each value is mean ± S.E.M. of data obtained from 4-7 animals for group, and is obtained by comparing the effect obtained in the presence of CHF5407 to baseline response to Ach.

Figure 11. Comparison of the inhibitory effects produced by intratracheally-administered CHF5407 (doses = nmoles/kg) on Ach (20 µg/kg i.v.)-induced bronchoconstriction in anaesthetised guinea pigs, at 3 vs. 24 h and 48 h from antagonist administration. ** p < 0.01 significantly different from the value obtained at 3h. Each value is mean ± S.E.M. of data obtained from 4-7 animals for group. Time-matched, vehicle-treated, animals were used as controls.

Figure 12. Duration of action of antimuscarinic compounds in conscious guinea pigs. Groups of guinea pigs were treated by inhalation with either vehicle or test compounds and inhibition of Ach-induced bronchoconstriction was measured up to 72 h after the administration. Data are expressed as mean ± S.E.M. of data obtained from 5-12 animals per group. * p < 0.05 and ** p < 0.01 significantly different from time-matched vehicle-treated animals.

Figure 13. Effect of muscarinic antagonists given in cumulative intravenously (i.v) or intratracheally (i.t.) doses to anaesthetized guinea-pigs upon mean arterial pressure (MAP) and heart rate (HR). Data are means ± S.E.M. of 8 (i.v.) or 7 (i.t.) animals for each drug. * p < 0.05 versus tiotropium, # p < 0.05 and § p < 0.01 versus CHF5407.

Figure 14. Effect of muscarinic antagonists given in cumulative intravenously (i.v) or intratracheally (i.t.) doses to anaesthetized guinea-pigs upon left ventricular pressure...
(LVP) and the maximum rate of LVP rise (dP/dt max). Data are means ± S.E.M. of 8 (i.v.) or 7 (i.t.) animals for each drug. *P < 0.05 versus tiotropium, #P < 0.05 and §P < 0.01 versus CHF5407.
Table 1

Receptor affinity of CHF5407 compared to tiotropium, ipratropium and glycopyrrolate for human M1, M2 and M3 muscarinic receptors, calculated in radioligand binding experiments.

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<th>Compound</th>
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<td>1.08±0.07</td>
<td>1.07±0.09</td>
<td>0.87±0.05</td>
</tr>
<tr>
<td>Ipratropium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki</td>
<td>0.24±0.02</td>
<td>0.69±0.08</td>
<td>0.23±0.01</td>
</tr>
<tr>
<td>n(H)</td>
<td>0.90±0.03</td>
<td>0.92±0.04</td>
<td>0.93±0.05</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki</td>
<td>0.17±0.01</td>
<td>0.81±0.08</td>
<td>0.53±0.07</td>
</tr>
<tr>
<td>n(H)</td>
<td>1.07±0.04</td>
<td>0.96±0.03</td>
<td>1.01±0.04</td>
</tr>
</tbody>
</table>
(a) $K_d$ : equilibrium dissociation constant (nM) calculated for the radioligand compounds $[^3\text{H}]$-CHF5407 and $[^3\text{H}]$-tiotropium in saturation experiments. (b) $K_i$ : inhibition dissociation constant (nM) estimated in competition experiments with non-radiolabelled compounds against $[^3\text{H}]$-N-methyl scopolamine as the radioligand. (c) $n(H)$: Hill coefficient, calculated in competition experiments. Values are mean ± S.E.M. of 4-8 observations.
**Table 2**

Kinetic binding parameters obtained in association and dissociation experiments performed with [³H]-CHF-5407 and [³H]-tiotropium at human M3 and M2 receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radioligand</th>
<th>Association</th>
<th>Dissociation</th>
<th>Dissociation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{+1,obb}^{a}$</td>
<td>$k_{+1}^{b}$</td>
<td>$t_{1/2}^{c}$</td>
</tr>
<tr>
<td>hM3</td>
<td>[³H]-CHF-5407</td>
<td>0.0116 ± 0.0011</td>
<td>NC</td>
<td>59.8 ± 10</td>
</tr>
<tr>
<td></td>
<td>[³H]-tiotropium</td>
<td>0.0170 ± 0.0014</td>
<td>NC</td>
<td>40.8 ± 6.5</td>
</tr>
<tr>
<td>hM2</td>
<td>[³H]-CHF-5407</td>
<td>0.096± 0.0010</td>
<td>0.369 ± 0.04</td>
<td>7.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>[³H]-tiotropium</td>
<td>0.048 ± 0.0005</td>
<td>NC</td>
<td>14.5 ± 2</td>
</tr>
<tr>
<td>hM1</td>
<td>[³H]-CHF-5407</td>
<td>0.014± 0.001</td>
<td>NC</td>
<td>49.9 ± 3.5</td>
</tr>
<tr>
<td></td>
<td>[³H]-tiotropium</td>
<td>0.012 ± 0.0005</td>
<td>NC</td>
<td>58.8 ± 2.5</td>
</tr>
</tbody>
</table>
(a) $k_{+1,obs}$ : association rate constant observed ($k+1^*[L]+k-1$) where $[L]$ is the radioligand concentration. (b) $\kappa_+ :$ association rate constant. $\kappa_+$ value was not calculated for radioligands displaying an irreversible component. (c) $t\frac{1}{2} :$ time required for half association. (d) $\kappa_1 :$ dissociation rate constant. (e) $t\frac{1}{2} :$ time required for half dissociation from the reversible binding component. (f) $\text{Irr} :$ irreversible binding component expressed as percentage of control binding. (g) $\% \text{ Bc} :$ percentage of control binding (samples without 10 $\mu$M cold N-methylscopolamine addition). (h) $\text{NC} = \text{not calculated.}$ All values are mean ± S.E.M. from 2 independent experiments performed in triplicate.
Table 3

*In vitro* potency and duration of action of CHF5407 as compared to tiotropium, ipratropium and glycopyrrolate in blocking muscarinic M3-receptor mediated bronchoconstriction in human and guinea-pig airways, or M2-receptor mediated inhibition of electrically-induced twitch contractions in guinea-pig left atrium.

<table>
<thead>
<tr>
<th>Compound</th>
<th>GP TRACHEA (Carbachol)(^a)</th>
<th>GP TRACHEA (EFS)(^b)</th>
<th>H BRONCHUS</th>
<th>GP LEFT ATRIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pIC_{50}) (t_{1/2})(^d)</td>
<td>(%) recovery(^e)</td>
<td>(pIC_{50}) (t_{1/2})</td>
<td>(%) recovery</td>
</tr>
<tr>
<td>CHF5407</td>
<td>9.0±0.04</td>
<td>&gt;4</td>
<td>11±5</td>
<td>9.1±0.25</td>
</tr>
<tr>
<td>tiotropium</td>
<td>9.1±0.001</td>
<td>&gt;4</td>
<td>10±3</td>
<td>9.3±0.09</td>
</tr>
<tr>
<td>ipratropium(^f)</td>
<td>8.6±0.02</td>
<td>0.5±0.1</td>
<td>70±7</td>
<td>9.2±0.24</td>
</tr>
<tr>
<td>glycopyrrolate(^f)</td>
<td>9.0±0.07</td>
<td>4.0±0.5</td>
<td>50±8</td>
<td>NT</td>
</tr>
</tbody>
</table>

\(^a\) Carbachol

\(^b\) EFS

\(^c\) \(pIC_{50}\) = negative log molar concentration that causes a 50% reduction in response

\(^d\) \(t_{1/2}\) = halflife

\(^e\) \% recovery = recovery at specific time point

\(^f\) NT = not tested
(a) Carbachol-contracted guinea-pig trachea. (b) Electrical field stimulated (EFS) guinea-pig trachea. (c) pIC$_{50}$ represents the −log molar concentration of the test antagonist producing a 50% reversal of carbachol or electrical-induced effect. (d) $t_{1/2}$ (offset) is the time taken for response to carbachol or electrical field stimulation to return to 50% recovery after washout of the test antagonist. (e) % response to carbachol or electrical field stimulation obtained 4 or 6 h after the washout of the test antagonist. (f) Data for glycopyrrolate and part of data for ipratropium are from (Villett et al., 2006). (g) Significantly different from $t_{1/2}$ of CHF5407; $P < 0.05$. NT = not tested. All data are mean ± S.E.M. of at least 4 observation.
Figure 4

Time control

2 g

20 min

Ipratropium 10 nM

CHF 5407 3 nM

Tiotropium 1 nM

2 g

4 h

w

w

w
Figure 5

(a) % maximal relaxation vs. log [Antagonist] (M)

(b) % inhibition of carbachol response vs. time (min)

- Vehicle
- CHF 5407
- Tio
Figure 6

Human isolated Bronchus (24h in vitro assay)

% of first response to carbachol

control  |  CHF 5407  |  Tiotropium

**  |  **  |  **
Figure 9

Graph a: Percentage of maximal effect vs. log [Carbachol] (M).

Graph b: Schild plot with log (DR-1) vs. log CHF5407.
Figure 11

% inhibition of bronchospasm

Hours from administration

- 0.1 nmoles/kg
- 0.3 nmoles/kg
- 1 nmoles/kg

**
Figure 13

MAP (change from baseline, mmHg)

HR (change from baseline, bpm)

nmoles/kg i.v.

nmoles/kg i.t.

- Vehicle
- CHF 5407
- Tiotropium
- Methoctramine