Bronchodilator Activity of (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 bromide (CHF5407), a Potent, Long-Acting, and Selective Muscarinic M3 Receptor Antagonist


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ABSTRACT

The novel quaternary ammonium salt (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 bromide (CHF5407) showed subnanomolar affinities for human muscarinic M1 (hM1), M2 (hM2), and M3 (hM3) receptors and dissociated very slowly from hM3 receptors (t1/2 = 166 min) with a large part of the receptorial complex (54%) remaining undissociated at 32 h from radioligand washout. In contrast, [3H]trotropium dissociated quickly from hM2 receptors (t1/2 = 31 min), whereas [3H]trotropium 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 24 h) 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 to anesthetized guinea pigs, potently inhibited acetylcholine (Ach)-induced bronchoconstriction with an ED50 value of 0.15 nmol/kg. The effect was sustained over a period of 24 h, with a residual 57% inhibition 48 h after antagonist administration at 1 nmol/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 anesthetized guinea pigs were not significantly changed by CHF5407, up to 100 nmol/kg i.v. and up to 1000 nmol/kg i.t. In conclusion, CHF5407 shows a prolonged anticholinergic activity both in vitro and in vivo, caused by a very slow dissociation from M3 receptors. In contrast, CHF5407 is markedly short-acting at M2 receptors, a behavior 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 and Rousell, 1998; Barnes, 2004b). In addition, ample evidence has been accumulating for a role for ACh released from pulmonary cells that can activate various muscarinic receptor subtypes expressed by non-neuronal cells (Racké et al., 2006). This is the case for pulmonary fibroblasts, in which muscarinic-mediated activation (mainly of the M2 subtype) leads to increases in proliferation (Matthiesen et al., 2006) and collagen synthesis (Haag et al., 2008). Currently, 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 (Krishna et al., 2004; Barnes and Stockley, 2005). Anticholinergic drugs have been found to be 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 such as 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 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, which allows COPD patients to follow a 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 versus 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 the pulmonary level, because 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 antimuscarinic therapy less effective, because 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, because it apparently 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-agonist 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. Numerous compounds, namely LAS-34273 (aclidinium), GSK233705 (darotropium), and NVA-237 (glycopyrrolate), are now under development with the hope of achieving quicker onset of action and improved safety profile in comparison with tiotropium (Hanania and Donohue, 2007).

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

### Materials and Methods

#### Radioligand Binding Studies

**Cell Lines.** Chinese hamster ovary (CHO-K1) clone cells expressing human M1, M2, or M3 receptors (Swiss-Prot P11129, P08172, and 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 of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of Fungizone (Gibco, San Giuliano Milanese, Italy).

**Membrane Preparation.** Cells were harvested in Ca2+/Mg2+-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 tissue homogenizer (Dispergerate; PBI International, Milan, Italy) (setting 5 for 15 s). The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000g 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, 12.5 mM MgCl2, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose), and aliquots were stored at −80°C. The day of the experiment, frozen membranes were resuspended in buffer C (50 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 1 mM EDTA). Membrane synthesis was initiated by the addition of 40 μl of buffer A containing 1 μCi of [35S]GTPγS (0.05 mCi/ml) to each sample in the presence of 1 μM of specific agonists or ACh.

**Materials.** Acetylcholine chloride (AChCl) (Gibco, San Giuliano Milanese, Italy) was added to the buffer in the presence of 100 μM EGTA and homogenized using the tissue homogenizer (Dispergerate; PBI International, Milan, Italy). The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000g 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, 12.5 mM MgCl2, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose), and aliquots were stored at −80°C. The day of the experiment, frozen membranes were resuspended in buffer C (50 mM Tris-HCl, pH 7.4, 2.5 mM MgCl2, 1 mM EDTA). Membrane synthesis was initiated by the addition of 40 μl of buffer A containing 1 μCi of [35S]GTPγS (0.05 mCi/ml) to each sample in the presence of 1 μM of specific agonists or ACh.

### Abbreviations

- CHF5407
- COPD: chronic obstructive pulmonary disease
- β1: human M1
- β2: human M3
- Ach: acetylcholine
- CV: cardiovascular
- dP/dt max: maximum rate of LVP rise
- EFS: electrical field stimulation
- CHO: Chinese hamster ovary
- NMS: N-methyl scopolamine
- G418: (2R,3S,4R,5R)-3-amino-6-[(1S)-5-amino-6-[(1R,2S,3S,4R,6S)-4,6-diamino-3-[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol
- GPA: guinea pig paced left atrium
- GPA: guinea pig paced left atrium
- hM1: human M1
- hM2: human M2
- hM3: human M3
- MAP: mean arterial pressure
- Penh: enhanced pause
- LVP: left ventricular pressure
- HR: heart rate
- LVP: left ventricular pressure
- PEF: peak expiratory flow
- EF: electrical field stimulation
- CHO: Chinese hamster ovary
- NMS: N-methyl scopolamine
- G418: (2R,3S,4R,5R)-3-amino-6-[(1S)-5-amino-6-[(1R,2S,3S,4R,6S)-4,6-diamino-3-[(2R,3R,4R,5R)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol
- GPA: guinea pig paced left atrium
protein concentration was determined by using the Quant-iT Protein Assay Kit (Molecular Probes, San Giuliano Milanese, Italy). Final protein concentrations were 1 to 2, 10 to 15, and 15 to 25 μg/ml for hM1, hM2, and hM3 receptor assays, respectively. The nonspecific binding was determined in the presence of 10 μM cold N-methyl scopolamine (NMS).

**Receptor Binding Assays.** The nonspecific muscarinic radioligand [³H]NMS (PerkinElmer, Milan, Italy; SA 81 Ci/nmol) was used to label the hM1, hM2, and hM3 binding sites. The nonspecific binding was determined in the presence of 10 μM cold N-methyl scopolamine. 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 concentrations of 0.1 to 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 (Milan, Italy) Filtermate Harvester. After the addition of the scintillation cocktail Microscint-20 (PerkinElmer), radioactivity on the filters was measured by a microplate scintillation counter (TopCount NXT, PerkinElmer). In addition, the binding affinity of CHF5407 for 66 different neurotransmitter/hormone receptors and ion-channels at radioligand concentrations of 0.1–3 nM was measured, according to established methods, by MDS Pharma Services (Taiwan Ltd. Pharmacology Laboratories, Taipei, Taiwan).

**[³H]Tiotropium and [³H]CHF5407 Binding Studies.** Experiments were performed in polypipetone tubes in triplicate. The nonspecific binding was determined in the presence of 10 μM cold N-methyl scopolamine. Dissociations were initiated after equilibration of membrane in the presence of the radioligands (0.15–0.25 nM) for 3 h, by adding 10 μM cold N-methyl scopolamine 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 and three washes with cold buffer C (4 ml) using a filter box (Millipore SpA, Vimodrone, Italy) and three washes with cold assay buffer C (4 ml) using a filter box (Millipore SpA, Vimodrone, Italy). Filters used for [³H]CHF5407 binding were presoaked for at least 3 h in buffer C containing 0.01% bovine serum albumin. The filters were placed in vials with 3 ml of the scintillation cocktail (PerkinElmer) and counted with a Packard scintillation counter (TopCount NXT, PerkinElmer). In addition, the binding affinity of CHF5407 for 66 different neurotransmitter/hormone receptors and ion-channels at radioligand concentrations of 0.1–3 nM was determined as described for [³H]NMS. Samples were incubated at room temperature for 3 h. Membrane protein concentration was determined by using the Quant-iT Protein Assay Kit (Molecular Probes).

**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 a 20-ml organ bath filled with oxygenated (95% O_2 and 5% CO_2) Krebs-Henseleit solution and maintained at 37°C. Tracheal preparations were connected to isometric force transducers under a resting tone of 10 mN. Changes in basal tone were recorded as described above. After that, carbachol (1 μM) was administered to preparations up to 24 h 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 to 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 produced 80 to 90% of maximal contraction. At steady tonic contraction, preparations were relaxed by isoprenaline (0.3 μM). After a thorough washout and a 60-min stabilization period, the test compounds were analyzed, 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 that point preparations were relaxed by a maximally effective concentration of tiotropium or CHF5407 (1.0 nM each). After a full relaxation had developed, preparations were washed out thoroughly, and the washout was repeated every 30 min for the next 4 h. Subsequently, preparations were dismounted and transferred into ice-cold oxygenated Krebs-Henseleit solution, in which they were kept overnight. The next day, each ring was placed in the same bath of the day before and, after stabilization, was challenged again with carbachol (1 μM) at 24 h 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 that aim, cumulative concentration-response curves to test antagonists were constructed on preparations precontracted by carbachol (trachea and bronchus) or EFS (trachea) or on atrial preparations inhibited by carbachol, until a complete reversal of EFS or carbachol-induced effects was achieved. The antagonist concentration producing a 50% inhibitory effect (IC50) 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 intervals, between washout and the next administration) during the next 4.5 h in the guinea pig trachea and left atrium preparations and at 60-min intervals during the next 6 h in the human bronchus. EFS-stimulated tracheal preparations were followed for 4 h after antagonist washout. A t50 (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 percentage response to carbachol or to EFS obtained 4 to 6 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 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 ratio 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):

\[
pK_B = \log \left[ \frac{\text{dose ratio} - 1}{1 - \text{dose ratio}} \right] - \log [\text{antagonist concentration}]
\]

(1)

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 Anesthetized Guinea Pigs**

The experiments were performed as described previously (Villetti et al., 2006). In brief, guinea pigs (450–550 g) were anesthetized with pentobarbital sodium (90–100 mg/kg i.p.). Pentobarbital was subsequently infused at the rate of 5 to 10 mg/kg/h into the carotid artery to maintain a stable level of anesthesia 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 submaximal 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 intravenous bolus of 20 µg/kg ACh, was quantified according to the method of Konzett and Rossauer (1940). CHF5407 (0.1, 0.3, and 1 nmol/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) to facilitate the distribution of the solution into the airways (Lundberg and Saria, 1983). ACh challenge was then repeated at 5, 15, and 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 percentage inhibition of ACh-evoked bronchoconstriction, compared with basal response. In animals undergoing ACh challenge 24 to 48 h after antagonist administration, anesthesia was induced with isoflurane (4% in O2, 2 l/min), and the trachea was exposed by a laryngoscope. Test compounds (or vehicle) were then instilled locally as described previously. Guinea pigs were allowed to recover from anesthesia and fed normally. The day of testing, animals were anesthetized with pentobarbital sodium and surgically prepared as above reported. ACh (20 µg/kg) was administered at 3-min intervals until three stable bronchospasms were recorded. ACh challenges did not ever exceed 10. Time-matched, vehicle-treated animals were used as controls.

**Bronchoconstriction in Conscious Guinea Pigs**

Airway sensitivity to ACh was measured with a single-chamber whole-body plethysmograph (Buxco, Sharon, CT), according to the method of Hamelmann et al. (1997) and Chong et al. (1998). Guinea pigs (body weight 500–600 g) were individually placed in plethysmograph chambers and acclimated 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 (pretreatment control). ACh dosage (selected on the basis of a previously determined dose-response curve) was set to produce a submaximal bronchoconstriction, i.e., 3 to 5 s of apnea in 50% of guinea pigs (data not shown). After a 60-min washout period, 5-min aerosol administration of vehicle (H2O), CHF5407 (5, 12.5, 25, 50, 100, and 250 µM), ipratropium (200–400 µM), and tiotropium (50–100-250 µM) was applied, and ACh challenge was repeated after 2, 5, 24, 48, and 72 h. In the case of 100% inhibition of ACh-evoked bronchoconstriction and at 24, 48, and 72 h after antagonist administration the animals were challenged with 1-min aerosol of histamine (0.75–1.0 mg/ml) to verify bronchial reactivity to a contracting agent. The pressure waveforms generated by respiration of the guinea pigs were recorded with an air flow transducer (model TRD5700; Buxco) and processed by using MAX II hardware and BioSystem XA software for Windows (Buxco). Enhanced pause (Penh), defined as pause × peak inspiratory flow/peak inspiratory flow, was used as readout. Penh was recorded for 5 min after each challenge. The average value of this recording was used as an index of bronchoconstriction. The effects of the test compounds were expressed as percentage of inhibition of the basal ACh-induced increase in Penh. Time-matched, vehicle-treated animals were used as controls.

**Measurement of Cardiovascular Parameters in Anesthetized Guinea Pigs**

Guinea pigs (390–410 g) were anesthetized 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, Palo Alto, CA) for the measurement of arterial blood pressure. A 2F Millar MikroTip catheter, with one high-fidelity pressure sensor (model SRR-249; Millar Instruments Inc., Houston, TX), was inserted via the right carotid artery into the left ventricle to measure left ventricular pressure transducer 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):
pressure (LVP). ECG was recorded by inserting needle electrodes subcutaneously according to standard limb leads after Einthoven I–II. Mean arterial pressure (MAP) and LVP values were calculated by using a PowerLab/400 device (ADInstruments Ltd., Chalgrove, Oxfordshire, UK). In addition, the maximal rate of LVP rise (dP/dt\text{max}) and heart rate (HR) were calculated from LVP. ECG was recorded with a Cardioline apparatus (model 6-1; Remco Italia, Milan, Italy). The signals were continuously transmitted into ECG module (ADInstruments Ltd.), and the number of extrasystoles was counted. Signals obtained from each module of the system were analyzed by Chart 5 software (ADInstruments Ltd.). Data were recorded for 30 s after 5 or 10 min from intravenous or intratracheal administration of test compounds, respectively.

In the first set of experiments, cumulative doses (1–10,000 nmol/kg) of CHF5407, tiotropium, methoctramine, or vehicle were injected intravenously via the right jugular vein (0.5 ml/kg) every 5 min. In the 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 Italia 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 approved by local ethical committees.

**Chemicals**

All materials used for cell culture were from Invitrogen with the exception of chemicals. Purities were confirmed by thin-layer chromatography. Tiopronin bromide and CHF5407 bromide or chloride (CHF5407), 

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<th>Chemicals</th>
<th>Synthesis</th>
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|             | synthesized at the 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% dimethyl sulfoxide. Further dilutions were prepared with saline. In control in vitro or in vivo experiments, administration of 1% dimethyl sulfoxide in saline was ineffective per se. [H]tiotropium and [H]CHF5407 were custom-synthesized by GE HealthCare (Little Chalfont, Buckinghamshire, UK) (84 and 62 µCi/nmol, respectively).

**Statistical Analysis**

All values are expressed as mean ± S.E.M. of the given number (n) of experiments. Statistical analysis was performed by using Student’s t test for paired or unpaired data or by one-way analysis of variance followed by Dunnett’s t test when applicable. P < 0.05 was considered a level of statistical significance. Kinetic and competition binding curves were analyzed by nonlinear regression analysis using the Solver tool of Excel program (Microsoft, Milan, Italy).

Dissociation binding results were analyzed by using the classic first-order equation and a model considering an irreversible binding component:

\[
\text{Ir:} \quad B_i = [B_0 \times (e^{-k \times t}) + \text{Ir}] 
\]

where \(B, B_0, k, \text{ and } t\) 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 (Ir) on the goodness of fit was determined by 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 by using a four-parameter logistic equation, and results were transformed into \(K_i\) values by using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Saturation experiments were analyzed by using the nonlinear fitting program Ligand (Munson and Rodbard, 1980). ED\(_{50}\) (i.e., the dose of antagonist reducing by 50% Ach-induced bronchoconstriction in anesthetized guinea pigs) was calculated from dose-response curves by using a least-squares linear regression analysis in Prism version 4.02 (GraphPad Software Inc., San Diego, CA). Data from studies in anesthetized and conscious guinea pigs were analyzed by Student’s t test for paired or unpaired data or by one-way analysis of variance 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, glycopyrrolate, and ipratropium produced a concentration-dependent full displacement of [H]N-methyl scopolamine from hM1, hM2, and hM3 receptors stably expressed in CHO-K1 cells with comparable subnemomolar affinities (\(K_i\) values; see Table 1). None of the antagonist compounds considered was selective toward any of the three muscarinic receptor subtypes. In saturation experiments, [H]CHF5407 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, [H]CHF5407 bound to hM1, hM2, and hM3 with high subnemomolar affinities (\(K_i\) values, 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 following: adenosine A1, A2A, A3, adrenergic α1A, α1B, α1D, α2A; β1, β2, bradykinin B1, B2; calcium channel L type; calcium channel N type; dopamine D1, D2A, D3, D4; endothelin ET-A, ET-B; epidermal growth factor; estrogen ERα; GABA(A), GABA(B1A); glucocorticoid; glutamate kainite; glutamate NMDA; histamine H1, H2; imidazole I1, interleukin-1; leukotrienes; cysteinyl CysLT1; melatonin MT1; neuropeptide Y Y1, Y2; nicotinic acetylcholine; nicotinic acetylcholine, bungarotoxin-sensitive; opiate δ, κ, and µ; potassium channel HERG; potassium channel [KATP]; phorbol ester.

**Table 1**

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

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CHF5407</th>
<th>Tiotropium</th>
<th>Ipratropium</th>
<th>Glycopyrrolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>hM1</td>
<td>0.19 ± 0.05</td>
<td>0.27 ± 0.014</td>
<td>0.21 ± 0.02</td>
<td>0.27 ± 0.014</td>
</tr>
<tr>
<td>hM2</td>
<td>0.21 ± 0.06</td>
<td>0.63 ± 0.05</td>
<td>0.59 ± 0.08</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td>hM3</td>
<td>0.96 ± 0.07</td>
<td>1.14 ± 0.08</td>
<td>0.99 ± 0.06</td>
<td>1.14 ± 0.08</td>
</tr>
</tbody>
</table>

Values are mean ± S.E.M. of four to eight observations.
TABLE 2

Kinetic binding parameters obtained in association and dissociation experiments performed with [3H]CHF5407 and [3H]tiotropium at human M3, M2, and M1 receptors.

All values are mean ± S.E.M. from two independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Radioligand</th>
<th>Association</th>
<th>Dissociation</th>
<th>Irr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$k_{1\text{obs}}$</td>
<td>$k_1$</td>
<td>$t_{1/2}$</td>
</tr>
<tr>
<td>hM3</td>
<td>[3H]CHF5407</td>
<td>0.0116 ± 0.0011</td>
<td>NC</td>
<td>59.8 ± 10</td>
</tr>
<tr>
<td>hM2</td>
<td>[3H]tiotropium</td>
<td>0.0170 ± 0.0014</td>
<td>NC</td>
<td>40.8 ± 6.5</td>
</tr>
<tr>
<td>hM1</td>
<td>[3H]CHF5407</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>

$k_{1\text{obs}}$, association rate constant observed ($k + 1 \times [L] + k - 1$), where $[L]$ is the radioligand concentration; $k_1$, association rate constant; $k_{-1}$, dissociation rate constant; $t_{1/2}$, time required for half association; $t_{1/2}$, time required for half dissociation from the reversible binding component; Irr, irreversible binding component expressed as percentage of control binding; %Bc, percentage of control binding (samples without 10 μM cold N-methyl scopolamine addition); NC, not calculated.

Association and Dissociation Kinetics of [3H]CHF5407 Versus [3H]tiotropium at hM1, hM2, and hM3 Receptors.

In association kinetic experiments, both [3H]CHF5407 and [3H]tiotropium bound to hM3 or hM1 with much slower association rates compared with hM2 receptor (Table 2).

Dissociation kinetics of [3H]CHF5407 and [3H]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 [3H]CHF5407 or [3H]tiotropium was effectively dissociated from the hM3 receptor (45.9 and 34.8%, respectively; Fig. 2; Table 2). As for the reversible binding components, the half-life ($t_{1/2}$) of the [3H]CHF5407–hM3 receptor complex was almost identical to $t_{1/2}$ of the [3H]tiotropium–hM3 receptor complex: 166 versus 163 min, respectively (Table 2). In contrast, the binding of [3H]CHF5407 at the hM2 receptor was totally reversible, with the dissociation curve of this radioligand best described by a simple first-order exponential function, with a $k_{-1}$ equal to 0.0224 min$^{-1}$ and a corresponding half-life of 31.8 min (Table 2). On the other hand, the dissociation curve of [3H]tiotropium from the hM2 receptor was best-fitted by a mathematical model considering an irreversible component, corresponding to 14.5% of total binding (Table 2; Fig. 2). The estimated $t_{1/2}$ of the hM2/[3H]tiotropium complex was much longer than that of the [3H]CHF5407/hM2 complex: 297.3 versus 31.8 min, respectively (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 that 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 other antimuscarinic compounds (Fig. 3a; Table 3). Likewise, 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, because 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 aimed at evaluating the offset of action, CHF5407 (10 nM) was administered to carbachol (0.3 μM)-precontracted or 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 to 4.5 h, despite the removal of the antagonist from the physiological solution (Table 3; Figs. 3b and 4). The offset of tiotropium (10 nM)-induced inhibitory effects was superimposable to that shown by CHF5407 (Figs. 3b and 4), whereas ipratropium (Fig. 4) or glycopyrrolate produced more quickly reversible effects (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 (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.

![Fig. 3](image-url) a, concentration-dependent inhibition by CHF5407 and tiotropium (Tio) of carbachol (0.3 μM)-induced tonic contraction in 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 over the next 4.5 h. Each value is the mean ± S.E.M. of four to six observations.

**TABLE 3** In vitro potency and duration of action of CHF5407 as compared with 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. All data are mean ± S.E.M. of at least four observations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Guinea Pig Trachea (Carbachol-Contracted)</th>
<th>Guinea Pig Trachea (EFS-Stimulated)</th>
<th>Human Bronchus</th>
<th>Guinea Pig Left Atrium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pIC50 (μg/mL)</td>
<td>t1/2 (%)</td>
<td>% Recovery</td>
<td>pIC50 (μg/mL)</td>
</tr>
<tr>
<td>CHF5407</td>
<td>9.0 ± 0.01</td>
<td>10.5 ± 0.5</td>
<td>50 ± 0.5</td>
<td>9.0 ± 0.01</td>
</tr>
<tr>
<td>Tiotropium</td>
<td>9.1 ± 0.04</td>
<td>10.3 ± 0.3</td>
<td>50 ± 0.3</td>
<td>9.1 ± 0.04</td>
</tr>
<tr>
<td>Ipratropium</td>
<td>8.6 ± 0.02</td>
<td>10.5 ± 0.5</td>
<td>50 ± 0.5</td>
<td>8.6 ± 0.02</td>
</tr>
<tr>
<td>Glycopyrrolate</td>
<td>9.0 ± 0.07</td>
<td>10.5 ± 0.5</td>
<td>50 ± 0.5</td>
<td>9.0 ± 0.07</td>
</tr>
</tbody>
</table>

pIC50 represents the log molar concentration of the test antagonist producing a 50% reversal of carbachol or electrical-induced effect. t1/2 is the time taken for response to carbachol or electrical field stimulation to return to 50% of offset after a washout of the test antagonist. % Recovery indicates percentage response to carbachol or electrical field stimulation obtained 4 or 6 h after the washout of the test antagonist. Data for glycopyrrolate and part of data for ipratropium are from Villetti et al., 2006. NT, not tested.

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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 (Table 3; Fig. 5b). As observed in the guinea pig trachea, ipratropium or glycopyrrolate produced more quickly reversible effects compared with CHF5407 (Villetti et al., 2006) (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 Materials and Methods for details). In control preparations, the responsiveness of bronchial smooth muscle to carbachol (1 μM) was almost halved at 24 h 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 (Fig. 6).

**Antagonist Potency and Duration of Action of CHF5407 at M2 Receptors in the Guinea Pig Paced Left Atrium.** Carbachol (100 nM to 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 antagonized carbachol-induced inhibition of twitch response, with a potency (pK<sub>B</sub> = 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 to 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 (Fig. 7) and tiotropium produced concentration-dependent excitatory effects caused by reversal of the agonist-induced inhibitory activity (Fig. 8a). CHF5407 and tiotropium produced similar excitatory effects, both in terms of pEC<sub>50</sub> or E<sub>max</sub> (Table 3; Fig. 8a).

In experiments aimed 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 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 (t<sub>1/2</sub> = 1.0 h), whereas the recovery was much slower.
(t½\textsubscript{1/2}; = 3.3 h) in tiotropium (10 nM)-pretreated preparations (Table 3; Fig. 8b). CHF5407 and tiotropium (2-h 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\textsubscript{B} = 8.7 ± 0.08; n = 12), because it produced a parallel shift of the curves to carbachol, without depressing E\textsubscript{max} (Fig. 9). Schid plot analysis of CHF5407 antagonism provided an unitary slope, as expected for a competitive antagonism (Fig. 9). In contrast, tiotropium produced an insurmountable noncompetitive antagonism, as expected from a slowly dissociating M2 receptor antagonist (Fig. 9).

**Antibronchoconstrictor Effect in Anesthetized Guinea Pigs.** CHF5407 intratracheal treatment exerted a dose-dependent and potent inhibition of ACh-induced bronchospasm, with an ED\textsubscript{50} value of 0.15 nmol/kg (Fig. 10). At 1 nmol/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 to 0.3 nmol/kg (maximum effect at ~180 min from CHF5407 administration). At any dose, CHF5407-induced inhibition lasted almost unchanged over a period of 24 h (Fig. 11). At 48 h from CHF5407 administration, a slight, but significant, decrease of the inhibitory effect was observed only at the dose of 1 nmol/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 to 250 μM solutions. The dose response of the CHF5407 effect seemed 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 had a reduced or no effect (Fig. 12). However, after nebulisation of the 25 μM solution endowed with a submaximal antibronchospastic 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 (Fig. 12).

**Cardiovascular Effects of CHF5407 in Anesthetized Guinea Pigs.** The effects on MAP, HR, LVP, and dP/dt\textsubscript{max} of cumulative doses of CHF5407, tiotropium, and methoctramine administered intravenously or intratracheally are reported in Figs. 13 and 14.

When test compounds were administered intratracheally, minor changes in MAP, HR, LVP, and dP/dt\textsubscript{max} were ob-
tained compared with that observed after intravenous administration (Figs. 13 and 14). Up to the dose of 100 nmol/kg i.t., the three compounds did not significantly modify all the parameters considered. At dosages of 1000 and 10,000 nmol/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 intrathecal treatment, the activity of CHF5407 was comparable with 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 to 11. 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 is as potent as tiotropium in preventing M3 receptor-mediated bronchoconstriction both in vitro and in vivo. It is noteworthy that CHF5407 is as long-acting as tiotropium, because its effects remain measurable up to 24 to 48 h from antagonist administration. It is worth noting that other antimuscarinic drugs that are currently used 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 with CHF5407 (present data; 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 fact that 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 \( t_{1/2} = 166 \) min) of CHF5407 from M3 receptors, as measured in radioligand binding experiments. Moreover, we observed that radiolabeled 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 to 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, which is in keeping with previously published data. In particular, Casarosa et al. (2009) described a fully reversible dissociation of \(^3\text{H}\)tiotropium from
M3 but with a slower kinetic than from M1 or M2. However, in contrast with results from 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 data and that of Casarosa et al. (2009) might be caused by the different protocols used, particularly for the following procedures: 1) dissociation started after 3 h (our procedure) or 2 h of incubation with test compounds; and 2) dissociation started by diluting samples with buffer containing unlabeled NMS (10 μM; our procedure) versus 10 μM atropine.

Bronchoconstriction induced by intravenous injection of Ach in anesthetized guinea pigs is a widely used model for assessing the efficacy of bronchodilators (e.g., Mohammed et al., 2000; Rossoni et al., 2007). Because the duration of action of bronchodilators cannot be followed for more than 6 h with this model we modified the experimental procedure and established a simple and reliable protocol for evaluating the duration of the antimuscarinic activity in the airways up to 24 to 48 h from antagonist administration (Villetti et al., 2006). Under those conditions, CHF5407 was at least 2- to 4-fold more potent than tiotropium, ipratropium, or glycopyrrolate (present data; Villetti et al., 2006). We also observed that whereas 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). It is noteworthy that CHF5407 inhibition of Ach-induced bronchoconstriction at 24 h from antagonist administration was comparable with that produced by tiotropium at doses endowed with a comparable peak effect (present data; Villetti et al., 2006).

Present data show that the antibronchoconstrictor effect of CHF5407 was also long-lasting in conscious, unrestrained guinea pigs. In these 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 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 anesthetized animals in either 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 versus M2 receptors on the basis of both radioligand binding and functional experiments that suggested that tiotropium apparently dissociated quickly from M2 receptors (Disse et al., 1993; Haddad et al., 1994; Takahashi 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 32 h from antagonist washout), as observed for tiotropium–M3 receptor binding was practically irreversible (14% of total receptors bound at 32 h from antagonist washout), as observed for tiotropium–M3 receptor binding whose irreversible fraction (65% of total receptors bound at 32 h 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 (\(t_{1/2} = 297\))

![Fig. 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-evoked bronchoconstriction was measured up to 72 h after the administration. Data are expressed as mean ± S.E.M. of data obtained from 5 to 12 animals per group. ns, not, significantly different from time-matched, vehicle-treated animals. All other points are significantly different from time-matched, vehicle-treated animals (\(p < 0.05\)).](Image)
versus 163 min, respectively). In sharp contrast to tiotropium, CHF5407 dissociated quickly ($t_{1/2}$ = 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- to 5-fold less potent than tiotropium ($pIC_{50}$ 8.2 versus 8.7, respectively), a result that is in good agreement with binding affinities shown by the two antagonists at M2 receptor (Table 1). In addition, CHF5407
duration of action in the GPA was significantly shorter than that of tiotropium. We also demonstrated diverging antagonist behavior of CHF5407 (competitive) versus tiotropium (not competitive) in the GPA, two drug profiles fitting with reversible versus irreversible M2 receptor antagonists, respectively. On this basis, we conclude that CHF5407, much more than tiotropium, possesses a kinetic selectivity for M3 versus 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 of which is mediated mainly by the cardiac M2 receptor (Hendrix and Robinson, 1997). A modest, although significant, difference between CHF5407 (less effective) versus tiotropium (more effective) in changing cardiovascular parameters became evident only at very high doses (1000–10,000 nmol/kg). 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 large clinical trial with tiotropium has confirmed the benefits of tiotropium in COPD management and provided reassurance regarding its CV profile (UPLIFT study; Tashkin, 2010). However, the same CV safety has not been established for other inhaled antimuscarinic compounds, such as ipratropium. On the basis of the UPLIFT trial (Tashkin, 2010), it may be speculated that tiotropium takes advantage from its short-acting activity at M2 receptors, which might reduce CV risks associated to systemic absorption of the drug. If this latter interpretation is correct, then CHF5407 should be 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 (Sarria et al., 2002). Nevertheless, we found that CHF5407 was as effective as tiotropium in preventing ACh-induced bronchoconstriction in conscious guinea pigs until 48 h from drug administration. This result suggests that the short duration of action at M2 receptors, assuming this characteristic actually happens, mimics antimuscarinic compounds, apparently does not penalize CHF5407 in comparison with tiotropium.

In conclusion, CHF5407 possesses a very promising pharmacological profile in vitro and in vivo as an anticholinesterase. Only tiotropium, among several currently used antimuscarinic drugs, matches CHF5407 for the duration of the antimuscarinic activity that is caused for both compounds by a very slow dissociation from the M3 receptor. It is noteworthy that CHF5407 is very short-acting at M2 receptors, a behavior not shared by tiotropium.

Acknowledgments

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References

Skroden MS, Gross NJ, Morita T, King PW, Armstrong W, Wells D, Galavan E, and

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