



Pulmonary, Gastrointestinal and Urogenital Pharmacology

Characterization of bencycloquidium bromide, a novel muscarinic M_3 receptor antagonist in guinea pig airwaysJun-Xia Jiang^{a,1}, Rui Cao^{a,1}, Wan-Ding Deng^b, Fang Jin^b, Xin-Wei Dong^a, Yu Zhu^a, Xiao-Ping Chen^c, Yi-Cheng Xie^a, Meng-Jing Bao^a, Fen-Fen Li^a, Qiang-Min Xie^{a,*}^a Zhejiang Respiratory Drugs Research Laboratory of State Food and Drug Administration of China, Medical College of Zhejiang University, Hangzhou 310058, China^b Shanghai Institute of Pharmaceutical Industry, Shanghai 200040, China^c Jiashilianbo Medicine Science & Technique Co., Beijing 100080, China

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ABSTRACT

In this study we have investigated the antagonist affinity, efficacy and duration of action of bencycloquidium bromide (BCQB), a selective muscarinic M_3 receptor antagonist, as a possible clinical bronchodilator for the treatment of chronic obstructive pulmonary disease (COPD) and asthma. In competition studies, BCQB showed high affinity toward the M_3 receptor in Chinese hamster ovary (CHO) cells (M_3 $pK_i = 8.21$, M_2 $pK_i = 7.21$, and M_1 $pK_i = 7.86$); $pA_2 = 8.85$, 8.71 and 8.57 in methacholine-induced contraction of trachea, ileum and urinary bladder, 8.19 in methacholine-induced bradycardia of right atrium in vitro, respectively. In function studies, duration of inhibition of carbachol-induced tonic contraction, BCQB and ipratropium had a very similar onset and offset of action, but onset faster and offset slower than that of tiotropium. After treatment with intratracheally instilled or the inhalation route, BCQB protects against methacholine or antigen-induced bronchoconstriction in a dose-dependent manner in the normal and sensitized guinea pigs in vivo. BCQB and ipratropium-induced inhibitory activity was short lasting, as it declined quickly when compared to tiotropium. These results suggest that BCQB bind muscarinic M_3 receptors with high affinity. On this basis we speculate that a putative BCQB-based therapy for COPD might require more than once-a-day administration to be as effective as the currently employed once-daily therapy with tiotropium. Nevertheless, Inhalable M_3 -selective compounds may spare M_2 -cardiac receptors and reduce the risks of cardiovascular events associated with the long-term treatment of these agents.

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1. Introduction

The prevention and relief of symptoms by regular use of bronchodilators is central to the pharmacological management of chronic obstructive pulmonary disease (COPD). Anticholinergic agents, such as ipratropium bromide and tiotropium bromide, administered by the inhalation route, have demonstrated efficacy as bronchodilators in COPD and asthma (Gross, 2006). Though the role of anticholinergic agents in the treatment of asthma is limited, older patients and those with intrinsic asthma are most likely to respond favourably to anticholinergic agents, clinically useful responses have been reported in some asthmatic children and adults (Rodrigo and Castro-Rodriguez, 2005; Kanazawa, 2006). Apparently, the presence of the Arg16Gly polymorphism in the β_2 -AR may also show a good response to anticholinergic agent (Park et al., 2009).

Parasympathetic nerves provide the dominant autonomic innervation of airways. Muscarinic (M_1 , M_2 , and M_3 receptor subtypes are expressed in airways and have relevant physiological roles. M_1 receptor subtypes are found in parasympathetic ganglia where they facilitate neurotransmission. M_2 receptors are localized prejunctionally on the postganglionic cholinergic nerve terminals and provide a functional negative feedback on acetylcholine release. M_3 receptors are localized on airway smooth muscle, submucosal glands, and blood vessels and mediate bronchoconstriction, mucus secretion, and vasodilatation (Minette and Barnes, 1990). In asthma and COPD, bronchoconstriction and mucus secretion is increased and the airways are hyperresponsive to contractile agents. These changes are due to increased parasympathetic nerve activity (Gross et al., 1989). The number and function of postjunctional M_3 muscarinic receptors in the airways are unchanged in asthma and COPD. Rather, it is the supply of acetylcholine to the postjunctional effector cells (smooth muscle and submucosal gland) that is increased. The increase of acetylcholine release occurs because prejunctional inhibitory M_2 muscarinic receptors on the parasympathetic nerves are dysfunctional (Coulson and Fryer, 2003).

Bencycloquidium bromide (BCQB), chemically known as 3-[(2-cyclopentyl-2-hydroxy-2-phenyl) ethoxy]-1-methyl-1-azabicyclo

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[2,2,2] octane bromide, is a quaternary ammonium compound with anticholinergic properties (Zhou et al., 2009; Jiang et al., 2010). A pharmacokinetic study in rats, after intranasal administration of 3.0 mg/kg BCQB, demonstrated a long elimination half-life (11.7 ± 7.1 h) (Xu et al., 2007). However, a pharmacokinetic study in humans using nasal spray of BCQB 45 μ g (Zhou et al., 2009) and in dogs via intratracheal injection of BCQB 100, 200 and 400 μ g (Jiang et al., 2010) did appear to have a shorter elimination half-life compared to rats. In this study we have investigated the potency and duration of action of BCQB in inhibiting cholinergic airway contractile responses evoked by muscarinic receptor agonists in guinea-pig, *in vitro* and *in vivo*. We have also investigated the potency of action of BCQB in inhibiting antigen-induced bronchoconstriction in the sensitized guinea pigs. Parallel studies have investigated the antagonist affinity of the drug from muscarinic receptors in order to evaluate its possible clinical role as a bronchodilator for the treatment of reversible airways obstruction in patients with COPD and in acute exacerbations of asthma. In the experiments, the commonly used muscarinic receptor antagonist ipratropium bromide and tiotropium bromide were included as reference drugs.

2. Materials and methods

2.1. Animals

All animal procedures and experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Research Council, 1996). Hartley guinea-pigs (200–250 g, purchased from Laboratory Animal Center, Zhejiang University, Ltd Certificate No. SCXK 2005-0021) were studied in all experiments. Guinea pigs were housed in groups of five at 20–23 °C under a 12/12 h light/dark cycle with 45–65% humidity. Standard laboratory food and water were provided *ad libitum*.

2.2. Binding affinities of different muscarinic antagonists

2.2.1. Cell culture

Chinese hamster ovary (CHO) cells transfected with the cDNAs encoding the human M_1 , M_2 to M_3 muscarinic acetylcholine receptors were maintained in α -Minimal Essential Medium supplemented with 100 IU/ml of penicillin, 100 μ g/ml of streptomycin, 2.5 μ g/ml of amphotericin B, and 10% fetal calf serum. Cultures were maintained at 37 °C in 5% CO_2 /humidified air at 37 °C, fed every 2–3 days, and passaged when confluent. All cells were harvested for use by the addition of 0.9% saline containing HEPES (10 mM)/EDTA (0.02%). Cells were homogenized at 4 °C in assay buffer (20 mM of HEPES and 1 mM $MgCl_2$ at pH 7.4). The homogenate was centrifuged at 20,000 g for 10 min and the pellet resuspended in assay buffer. This procedure was repeated two more times. Membranes of each cell line were prepared and used fresh daily.

2.2.2. Equilibrium binding experiments

The binding of ^3H -quinuclidinyl benzilate ($[^3H]$ -QNB) (48 Ci/mmol, 0.05–3.00 nmol/L, Amersham Life Science) was performed in 1 ml volumes of the assay buffer containing approximately 100 μ g of membranes at 37 °C for 60 min. Nonspecific binding was defined in the presence of 10^{-5} mol/L atropine. After incubation, each sample was filtered (and washed) under vacuum through filters using a cell harvester. Filter retained radioactivity was extracted for at least 8 h in 4 ml of scintillation fluid. In displacement studies, the interaction of BCQB (10^{-11} – 10^{-3} mol/L), (Jiashilianbo Medicine Science & Technique Co., Beijing, China), tiotropium (10^{-11} – 10^{-3} mol/L), (Boehringer Ingelheim), ipratropium (10^{-11} – 10^{-3} mol/L), (Boehringer Ingelheim) and the M_3 -preferring antagonist 4-diphenylacetoxy-N-methylpiperidine methiodide [$[4-DAMP$ 10^{-11} – 10^{-3} mol/L), Sigma, St. Louis, MO)] with M_1 – M_3 muscarinic receptors was determined by

displacement of 0.8 nmol $[^3H]$ -QNB ($n=5-6$). The concentration of displacers producing 50% displacement of specific binding (IC_{50} ; half-maximal inhibitory concentration) was obtained by computer assisted curve fitting (nonlinear regression; sigmoid concentration–response curve, variable slope with bottom and top set at 0% and 100%, respectively, using GraphPad-PRISM (Graph-Pad Inc, San Diego, CA) and corrected for the competing mass of $[^3H]$ -QNB using K_d s of 0.1 to 2.0 nmol/L at M_1 , M_2 and M_3 receptors, respectively (Cembala et al., 1998), according to Cheng and Prusoff (1973) to yield K_i : $K_i = IC_{50}/[1 + ([^3H]\text{-QNB used}/K_d)]$.

2.3. Isolated guinea pig organ preparation and experiment protocol

2.3.1. Guinea-pig trachea

The experiments were performed essentially as described previously (Xie et al., 1999; Zhu et al., 2010). Briefly, the whole trachea was excised from guinea-pigs and rapidly placed in oxygenated Krebs–Henseleit solution of the following composition (mmol/L): NaCl 118, KCl 5.9, $MgSO_4$ 1.2, $CaCl_2$ 2.5, NaH_2PO_4 1.2, $NaHCO_3$ 25.5 and glucose 5.6. After removal of the excess connective tissues and fat, the trachea was cut into 6–8 approximately equal small rings of about 2 mm in length. Each ring was subsequently cut open through the cartilage, and 3–4 of such open rings were tied together to form a chain. Tracheal chain muscle preparations thus obtained were separately suspended in 5 ml organ bath filled with oxygenated (O_2 95% and CO_2 5%) normal Krebs–Henseleit solution and maintained at 37 °C. Tracheal preparations were connected to an isometric force transducer under a resting tone of 1 g. Data were recorded by means of a MedLab system (Nanjing Biotech Instruments, China). After an equilibration period of 60 min, carbachol (Sigma, St. Louis, MO) was administered to preparations at a concentration of 0.3 μ mol/L that in preliminary experiments had been shown to produce 80–90% of maximal contraction. Isoprenaline 0.1 μ mol/L (Sigma, St. Louis, MO)-induced relaxation was used as the maximal inhibitory reference effect. The test compounds were assayed, as described below.

2.3.1.1. Protocol—estimate of antagonist potency and offset of the inhibitory effect produced by test compounds. The experiments were performed essentially as described by previously (Villetti et al., 2006), with modifications. Briefly, all compounds were tested for their ability to reverse an M_3 receptor-mediated contraction. Thus, a cumulative concentration–response curve to test antagonists was constructed on guinea-pig preparation precontracted with carbachol, until a complete inhibition of smooth muscle tone was achieved. This procedure approximately was 3.0 h. During these periods of time the contractile response to carbachol remained stable (loss of tension $\leq 10\%$) in trachea tissues. In the experiments aiming at assessing the offset of the inhibitory effects produced by three compounds, the minimal concentration of each antagonist known to produce a maximal inhibitory effect was administered to carbachol-precontracted preparations. As soon as carbachol-induced tonic contraction was reversed by the antagonist, the organ bath solution was renewed and preparations were thoroughly washed with fresh Krebs–Henseleit solution. BCQB, tiotropium and ipratropium were washed out only after a complete and sustained relaxation of the smooth muscle was achieved. Carbachol was administered again (at 60 min interval, between washout and next administration) during the next 5 h. The percentage of response to carbachol obtained 5 h after the washout of the test antagonist was taken as a measure of the offset (reversibility) of the compound under investigation.

2.3.2. Guinea pigs urinary bladder

Male Hartley guinea pigs urinary bladders were rapidly removed and placed in 37 °C Krebs–Henseleit solution. The dome of the bladder was isolated from the trigon region and the mucosa was removed. This tissue was then cut into 3-mm-wide by 10-mm-long strips. One end was

secured to the bottom of a water-jacketed tissue bath and the other to an isometric force transducer, and isometric tension was recorded using a MedLab system. Tissues were contracted (1 g) and after 60 min of equilibration were contracted with an additional 0.3 $\mu\text{mol/L}$ methacholine and again allowed to equilibrate. BCQB was administered directly into the tissue baths as sequential concentrations.

2.3.3. Guinea pigs ileum

Segments (approximately 1.5 cm) of ileum from male guinea pigs were set up in organ baths and bathed in 5 ml of Tyrode solution of the following composition (mmol/L): NaCl 115, KCl 5.4, MgCl_2 1, CaCl_2 1.8, NaH_2PO_4 1, NaHCO_3 5 and glucose 10 and maintained at 37 °C and gassed with O_2/CO_2 (O_2 95% and CO_2 5%). Indomethacin (1.0 $\mu\text{mol/L}$) was present in Tyrode buffer at all times. The tissues were connected to force-displacement transducers, and isometric tension was recorded using a MedLab system. Resting tension was equivalent to that generated by a load of 0.5 g. The tissues were allowed to equilibrate for at least 60 min before the initiation of experiments. Tissues were contracted with an additional 0.3 $\mu\text{mol/L}$ methacholine and again allowed to equilibrate. Compounds were administered directly into the tissue baths as sequential concentrations.

2.3.4. Guinea pig right atria

Right atria from male guinea pigs were set up in organ baths and bathed in 5 ml of Krebs–Henseleit solution as described previously (Tanito et al., 2001). Briefly, the lower end of the right atrium was fixed on a hook, and the upper end was connected to force-displacement transducers, and change of isometric tension was recorded using a MedLab system. Resting tension was equivalent to that generated by a load of 0.5 g. The preparation was equilibrated for at least 60 min before the commencement of the experiments. To estimate of BCQB on the spontaneously beating response in guinea pig atria, the concentration–response curve for effect of BCQB and ipratropium were determined in a cumulative manner by increasing concentration of BCQB were added to the atria preparation bath solution in 5 min intervals. A concentration–response curve for methacholine was obtained before and after addition of BCQB. For the right atria assay, the beating rate was measured and the responses were expressed as a percentage of basal beating rate.

2.3.4.1. Protocol effects of BCQB on the contractile response of methacholine in guinea pig trachea, ileum, urinary bladder strip and right atrium. The concentration–response curves for the contractile effect of methacholine were determined in the absence and presence of BCQB at difference concentrations. The concentration–response curves obtained during the second and third applications of methacholine slightly shifted rightward compared with the first response curve, although the maximal responses of the preparation to methacholine did not change. Therefore, the EC_{50} value of methacholine in the presence of BCQB was corrected based on the difference between the EC_{50} values obtained during the first and second or third application of methacholine in the time-matched control preparation. Affinity measurement for BCQB was estimated by Schild plot analysis (Arunlakshana and Schild, 1959).

2.4. Methacholine-induced bronchoconstriction in anaesthetized guinea-pig

2.4.1. In vivo potency of action

Experiment was performed essentially as previously described (Xie et al., 2003). Briefly, guinea pigs were anesthetized with an intraperitoneal injection of 1.25 g/kg urethane (Sinopharm Chemical Reagent Co, Shanghai) and divided into six groups, BCQB (4.11, 8.22 and 16.44 $\mu\text{g/kg}$), ipratropium (8.61 $\mu\text{g/kg}$) and tiotropium (4.72 $\mu\text{g/kg}$) dissolved in saline were administered locally: 100 μl of solution was instilled intratracheally, directly through the tracheal cannula.

The model group was given intratracheally the same volume of saline.

The lung function of anesthetized and tracheostomized the guinea pigs was assessed as described (Xie et al., 2003). Briefly, each animal was anaesthetized, the trachea and esophagus were cannulated and placed supine inside a Plexiglas whole-body plethysmograph. The flow rate was monitored with a Fleisch tube connected to the airway in a pressure transducer. Changes in lung volume were measured by detecting pressure changes in the plethysmographic chamber through a port in the connecting tube with a pressure transducer. The pleural pressure was measured by a needle with a multiholed tip inserted into the pleural cavity through a port in the connecting tube with a differential pressure transducer. Transpulmonary pressure was calculated as the difference between pleural and esophagus pressure. The signal of all pressure transducers were continuously computed (MedLab, Nanjing Biotech Instruments, China) by fitting flow, volume, and pressure to an equation of motion. The values of airway resistance (R_L , $\text{cmH}_2\text{O/ml/s}$), and lung dynamic compliance (C_{dyn} , $\text{ml/cmH}_2\text{O}$) were calculated after detection. Cumulative doses of methacholine 0, 3.125, 6.25 and 12.5 mmol was inhaled for 30 s in a jet nebulizer (BARI Co. Ltd, Germany) for a period of 5 min per dose with lung function measurements taken between each concentration. Cumulative dose–response curves were thus established provocation dose (PD) of methacholine which cumulatively produced a 100% increase in airway resistance (PD_{100}) or a 25% fall in lung dynamic compliance (PD_{25}) was determined for each animal.

2.4.2. In vivo duration of action

Following stabilization of breathing, animals were inhaled with methacholine every 3 min, until three stable and reproducible basal responses were obtained. BCQB (8.22 and 16.44 $\mu\text{g/kg}$), ipratropium (8.61 $\mu\text{g/kg}$) and tiotropium (4.72 $\mu\text{g/kg}$) were administered locally: 100 μl of solution was instilled intratracheally, directly through the tracheal cannula. Methacholine challenge was then repeated at 15, 30, 45, 60, 90, 120, and 180 min from the intratracheal administration of BCQB, ipratropium and tiotropium. The effect of the three compounds was expressed as percentage of inhibition of methacholine-evoked bronchoconstriction, as compared to basal response.

2.5. Antigen-induced airway constriction in sensitized guinea pigs

2.5.1. Sensitizing procedures

Guinea pigs were sensitized by an intraperitoneal injection (i.p.) and intramuscular (i.m.) in hind legs of ovalbumin (Sigma, St. Louis, MO) 10 mg mixed with 100 mg of aluminum hydroxide in saline 1.0 ml per animal on day 0. After day 14, a repeated i.p. ovalbumin 5 mg in saline 0.5 ml per animal was administered. These animals were used 25–35 days later for an aerosol challenge with ovalbumin.

2.5.2. Treatment procedures

Guinea pigs were divided into six groups. To investigate the treatment effects of BCQB, tiotropium and ipratropium on antigen-induced airway constriction, treatment groups inhaled a single dose BCQB 30, 60, and 120 $\mu\text{g/ml}$ (inhaled volume about 100 μl per animal), tiotropium 30 $\mu\text{g/ml}$ and ipratropium 60 $\mu\text{g/ml}$ by a jet nebulizer for 30 s before antigen challenge. The control group was treated with an equal volume of saline.

2.5.3. Measurement of R_L and C_{dyn}

The sensitized guinea pigs were anesthetized with an intraperitoneal injection of 1.25 g/kg urethane and placed in a whole body plethysmograph for the measurement of R_L and C_{dyn} . After the treatment, all animals were challenged with an antigen via the airway cannula, with an aerosolized ovalbumin (1% in saline) or control (equal volume saline) in a jet nebulizer for 15 s. After antigen challenge R_L and C_{dyn} were monitored for 30 min and maximal

changes from baseline for each parameter were recorded by a Medlab system. The effect of BCQB, tiotropium and ipratropium were determined by comparing the ovalbumin-induced changes in R_L and C_{dyn} after drug treatment with the mean of antigen responses alone in the same guinea pig on previous and successive control periods.

2.6. Statistical analysis

Results are expressed as mean $\pm$ S.E.M. and the significance of differences was determined using the two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test (SNK) as post hoc. Differences were considered significant if $P < 0.05$. Statistical analyses were conducted using Sigma Stat 3.2 Version 2.0. Affinity measurement for the inhibition constant (pK_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of BCQB was estimated by Schild plot analysis (Cheng and Prusoff, 1973). In the airway function studies, potency values for the test antagonists were expressed as the concentration required to inhibit responses by 50% (IC_{50} and ID_{50}), and agonists were expressed as the concentration required to increase responses by 50% (EC_{50}), by 100% (PD_{100}) and 25% (PD_{25}) calculated by weighted probit analysis of Bliss method. The time of onset ($t_{1/2}$ [onset]) was defined as the time from starting the administration of the test antagonist to the attainment of 50% inhibition of carbachol responses. The time for offset of action ($t_{1/2}$ [offset]) was defined as the time from washout of the test antagonist to attainment of 50% recovery of carbachol responses.

3. Results

3.1. Binding affinities of BCQB against the three human muscarinic receptor subtypes

Displacement curves were analyzed according to a model of orthosteric competition between [^3H]-QNB and the unlabeled competitors, so that IC_{50} values were transformed into K_i values according to the Cheng–Prusoff equation. To prove that this is the correct model for interpreting the data, the shifts in IC_{50} values in relation to the increase in amount of radioligand used in a given experiment were monitored for different ligands: the IC_{50} values shifted linearly in correlation with the increase of radioactive probe ([^3H]-QNB), as assumed in the model for competitive interaction. The different muscarinic antagonists were tested, and the pK_i values obtained under these conditions are summarized in Table 1. The receptor subtypes selective of BCQB and 4-DAMP are $M_3 > M_1 > M_2$; tiotropium and ipratropium are not selective on muscarinic M_1 – M_3 subtypes. The results showed BCQB and 4-DAMP mainly block muscarinic M_3 receptor subtypes, while tiotropium and ipratropium antagonize all three muscarinic receptor subtypes.

3.2. Effects of BCQB on the contractile response of methacholine in guinea pig trachea, ileum, urinary bladder strip and right atrium

Methacholine contracted the guinea pig tracheal, ileum and urinary bladder smooth muscle, and induced bradycardia in a

concentration-dependent manner. BCQB shifted the concentration–response curve for airway contractile effect of methacholine to the right in a parallel manner without affecting the maximum force of contraction produced by methacholine, pA_2 value was 8.85, slope of the Schild plot was 0.8758 (Fig. 1A). BCQB shifted the concentration–response curve for bradycardia effect of methacholine, pA_2 value was 8.19, slope of the Schild plot was 0.8914 (Fig. 1B). BCQB shifted the concentration–response curve for the ileum contractile effect of methacholine, pA_2 value was 8.71, slope of the Schild plot was 0.6158 (Fig. 1C). BCQB also shifted the concentration–response curve for the urinary bladder contractile effect of methacholine, pA_2 value was 8.57, slope of the Schild plot was 0.6683 (Fig. 1D).

3.3. Potency and duration of inhibition by BCQB of carbachol-induced tonic contraction in the guinea pig isolated trachea

Carbachol (0.3 mmol/L) produced prompt and sustained tonic contractions of the tracheal smooth muscle, which were highly reproducible over a period of several hours. In carbachol (0.3 mM)-precontracted preparations, BCQB, tiotropium and ipratropium produced a slowly developing relaxation of smooth muscle tone, showing a potency ($-\log IC_{50}$) in the subnanomolar range, BCQB ($-\log IC_{50}$) 8.69 ± 0.08 , ipratropium 9.36 ± 0.07 and tiotropium 9.25 ± 0.03 . The maximal inhibitory effect of tiotropium was obtained at 10 nmol/L, but for BCQB and ipratropium at 20 nmol/L (Fig. 2A). Duration of inhibition of carbachol-induced tonic contraction, BCQB and ipratropium had a very similar onset of action. The $t_{1/2}$ (onset) for BCQB (20 nmol/L) was 6.8 ± 1.1 min compared to that for ipratropium (20 nmol/L) 6.6 ± 0.8 min. After washout, the inhibitory effect of ipratropium (20 nmol/L) on cholinergic responses was significantly pronounced $t_{1/2}$ (offset) = 185.2 ± 3.7 min compared to BCQB (20 nmol/L) $t_{1/2}$ (offset) = 180.4 ± 4.5 min. In contrast, the $t_{1/2}$ (onset) and $t_{1/2}$ (offset) for tiotropium (10 nmol/L) was 29.8 ± 5.8 min and > 300 min, respectively. Five hours after washout of tiotropium, tracheal preparations had only recovered approximately 10% of its initial responsiveness to carbachol. BCQB and ipratropium (20 nmol/L)-induced inhibition showed to be a more rapidly reversible (Fig. 2B). Five hours after washout of BCQB and ipratropium tracheal preparations had recovered over 40% of their initial responsiveness to carbachol (Fig. 2B).

3.4. Action potency of BCQB on methacholine-induced airway constriction in anaesthetized guinea-pig

The effect potency of intratracheally instilled BCQB, ipratropium and tiotropium were assessed against methacholine-induced bronchospasm in anaesthetized guinea-pigs. Inhalation of methacholine induced bronchospasm in anaesthetized guinea-pigs in a concentration-dependent manner (Fig. 3). Concentration of methacholine-induced increase in airway resistance (methacholine PD_{100}) and decrease in lung dynamic compliance (methacholine PD_{25}) were $4.623(3.983–5.415)$ nM and $2.692(2.118–3.421)$ nM. After treatment with BCQB 4.11, 8.22 and 16.44 μ g/kg, concentration of methacholine PD_{100} for airway resistance were $6.290(5.185–7.620)$, $10.88(9.896–11.961)$ and > 12.5 mmol/L, methacholine PD_{25} for lung dynamic compliance were $4.038(3.239–5.035)$, $7.561(6.382–8.958)$ and $11.670(7.928–17.20)$ mmol/L. After treatment with ipratropium 8.61 μ g/kg, methacholine PD_{100} and PD_{25} were > 12.5 and 3.238 mmol/L. After treatment with tiotropium 4.72 μ g/kg, methacholine PD_{100} and PD_{25} were > 12.5 and > 12.5 mmol/L (Fig. 3).

3.5. Action duration of BCQB on methacholine-induced airway constriction in anaesthetized guinea-pig

The effect duration of intratracheally instilled BCQB, ipratropium and tiotropium were assessed against methacholine-induced bronchospasm in anaesthetized guinea-pigs. Both the maximum inhibition

Table 1

Binding affinities of different muscarinic antagonists against the three human muscarinic receptor subtypes. The equilibrium dissociation constants of the different muscarinic antagonists were determined in heterologous competition experiments against [^3H]-QNB. The pK_i values shown are the average of at least three independent experiments performed in triplicate, and the mean $\pm$ S.E.M.

Compounds	M1	M2	M3
Bencycloquidium	7.86 ± 0.04	7.21 ± 0.04	8.21 ± 0.03
Tiotropium	9.82 ± 0.02	9.45 ± 0.08	9.85 ± 0.06
Ipratropium	9.45 ± 0.05	9.52 ± 0.04	9.42 ± 0.04
4-DAMP	8.35 ± 0.05	7.92 ± 0.02	9.03 ± 0.03

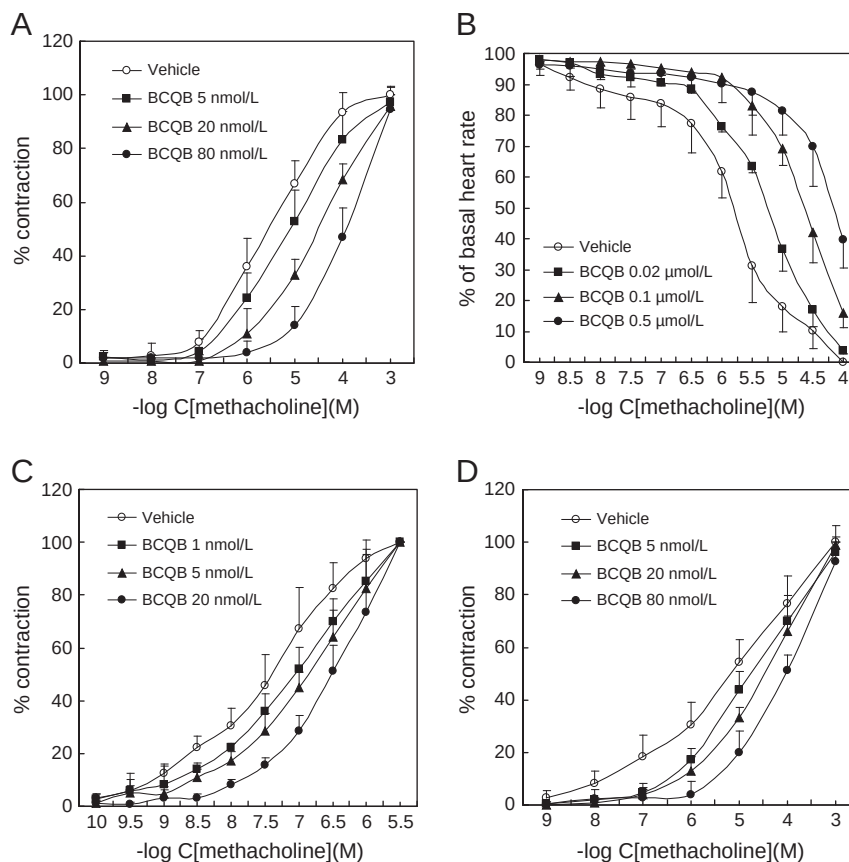


Fig. 1. Cumulative concentration–response curves for methacholine-induced contractions in the guinea-pig trachea, ileum, urinary bladder strip and right atrium in the absence and presence of different concentrations of BCQB. A: trachea, B: right atrium, C: ileum, D: urinary bladder; BCQB preincubation time was 10 min. Each value is the mean $\pm$ S.E.M. of six to eight animals.

(peak effect) of methacholine-induced bronchoconstriction and the inhibition still present 3 h and 24 h after the administration of each antagonist were evaluated. All three compounds exerted a dose dependent prevention of methacholine-induced bronchospasm, and produced a peak effect averaging over 80% inhibition (Fig. 4). Tiotropium was 4–10 fold more potent than ipratropium and BCQB. ID_{50} value of tiotropium, ipratropium and BCQB was $0.24(0.19–0.28)$, $0.98(0.85–1.31)$ and $2.36(1.91–3.70)$ $\mu\text{g}/\text{kg}$, respectively. Tiotropium (4.72 $\mu\text{g}/\text{kg}$)-induced inhibition lasted almost unchanged over a period of 3 h and up to 24 h from antagonist administration. BCQB (40 nmol/L)-induced inhibition remained quite stable up to 3 h, whereas BCQB (8.22 $\mu\text{g}/\text{kg}$) and ipratropium (8.61 $\mu\text{g}/\text{kg}$)-induced inhibition did not remain complete stable up to 3 h (Fig. 4). BCQB and ipratropium-induced inhibitory activity was short lasting, as it declined quickly when compared to tiotropium. At 3 h from antagonist administration, inhibitory rate of tiotropium (4.72 $\mu\text{g}/\text{kg}$), ipratropium (8.61 $\mu\text{g}/\text{kg}$) and BCQB (8.22 and 16.44 $\mu\text{g}/\text{kg}$) was 91.4% , 39.7% , 42.4% and 68.4% , respectively. At 24 h from antagonist administration, inhibitory rate was 43.9% , -10.3% , -8.4% and 10.3% , respectively.

3.6. Effects of BCQB on antigen-induced airway constriction in sensitized guinea pigs

Antigen induced changes of R_L and C_{dyn} in sensitized guinea pigs. Comparison with baseline value before aerosol challenge of antigen, bronchial challenge of ovalbumin-sensitized guinea pigs induced 179% increase of R_L and 58% reduction of C_{dyn} with maximal response at 5 min. Mean value increase of R_L and mean value reduction of C_{dyn} from

1 to 30 min after antigen challenge were up to $150\% \pm 19\%$ and $44\% \pm 14\%$, respectively in vehicle group (Fig. 5). BCQB 30, 60 and 120 $\mu\text{g}/\text{ml}$ via inhalation induced dose-related inhibition of the bronchoconstrictive responses to aerosolized ovalbumin. Maximal increase of R_L and reduction response of C_{dyn} at 5 min was 143% , 132% and 93% , and 48% , 40% and 33% . Mean increase of R_L and reduction response of C_{dyn} from 1 to 30 min were $128\% \pm 10\%$, $109\% \pm 20\%$, $73\% \pm 16\%$ and $35\% \pm 9\%$, $29\% \pm 8\%$, $21\% \pm 8\%$; ID_{50} (95%CL) for R_{aw} and ID_{50} (95%CL) for C_{dyn} were $119.2(89.5–158.6)$ and $111.0(79.8–154.57)$ $\mu\text{g}/\text{ml}$, respectively (Fig. 5). Tiotropium 30 $\mu\text{g}/\text{ml}$ via inhalation induced a marked inhibition of the bronchoconstrictive responses to aerosolized ovalbumin. Maximal increase of R_L and reduction response of C_{dyn} at 5 min was 63% and 26% . Mean increase of R_L and reduction response of C_{dyn} from 1 to 30 min were $47\% \pm 13\%$ and $18\% \pm 5\%$, respectively (Fig. 5). Ipratropium 60 $\mu\text{g}/\text{ml}$ via inhalation also induced a significant inhibition of the bronchoconstrictive responses to aerosolized ovalbumin. Maximal increase of R_L and reduction response of C_{dyn} at 5 min was 99% and 34% . Mean increase of R_L and reduction response of C_{dyn} from 1 to 30 min were $81\% \pm 15\%$ and $24\% \pm 7\%$, respectively (Fig. 5).

4. Discussion

The present results show that the new muscarinic receptor antagonist BCQB showed subtype selectivity for muscarinic receptors from cloned human receptors. In radioligand binding experiments, BCQB was found to compete with [^3H]-QNB with pK_i values of 7.86 , 7.21 and 8.21 for the M_1 , M_2 , and M_3 receptors, respectively. Another known selective M_3 antagonist 4-DAMP pK_i values 8.35 , 7.92 and 9.03 for the M_1 , M_2 , and M_3 receptors, respectively. 4-DAMP pK_i values

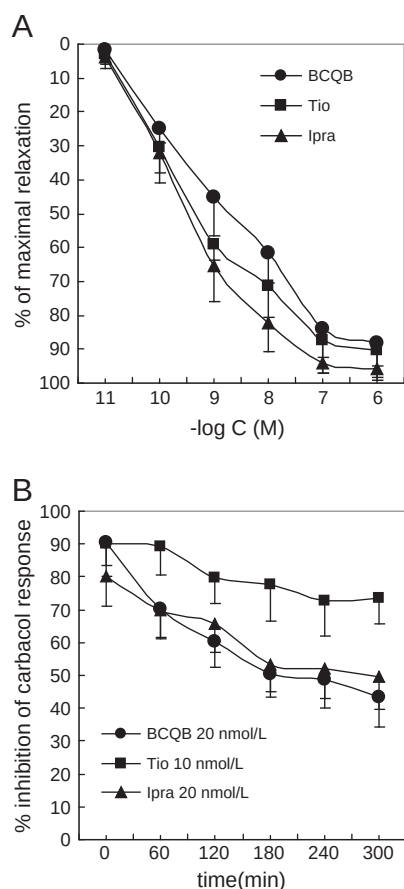


Fig. 2. (A) Concentration-dependent inhibition by bencycloquidium (BCQB), tiotropium (Tio) and ipratropium (Ipra) of carbachol (3 $\mu\text{mol/L}$)-induced contraction in the guinea pig isolated trachea. (B) Decay of the inhibitory effects produced by BCQB 20 nmol/L, Tio 10 nmol/L and Ipra 20 nmol/L against contractile responses to carbachol (3 $\mu\text{mol/L}$) in the guinea pig isolated trachea. After washout of the antagonist (time = 0), carbachol was administered at 60 min intervals during the next 5 h. Each value is the mean $\pm$ S.E.M. of six to eight animals.

were similar to those previously reported by other investigators (Cembala et al., 1998; Watson et al., 1999). The pK_i values of tiotropium and ipratropium (Table 1) were similar to those recently reported by other investigators (Casarosa et al., 2009; Dowling and Charlton, 2006). The results demonstrate that BCQB and 4-DAMP display high selectivity for the M_3 over the M_2 receptor in human binding assays. Conversely, both tiotropium and ipratropium have no selectivity for muscarinic receptors.

Muscarinic M_3 receptor subtype is mainly expressed in airway smooth muscles and shows the effect of the constrictive response, so M_3 receptor antagonist will be widely used to treat COPD and asthma (Belmonte, 2005; Barnes, 2004). Similarly, in many smooth muscles including guinea-pig ileum and urinary bladder the M_3 receptor is the most abundant subtype. Pharmacological functional studies indicated that the contractile response is mediated by M_3 fraction (Watson and Eglon, 1994; Clague et al., 1985; Tran et al., 2006; Griffin et al., 2009). Indeed, guinea-pig ileum and urinary bladder have been employed for many years as a convenient bioassay for M_3 receptor function (Zholos and Bolton, 1997; Wallis et al., 1993). Bladder contractility is mainly controlled by the cholinergic input from the parasympathetic nervous system. The pharmacological profile of muscarinic antagonist obtained on detrusor muscle of several animal species suggested a predominant role for the M_3 subtype. This hypothesis is also confirmed by an experiment performed on mutant male mice lacking the M_3 receptor that showed severely distended urinary bladder

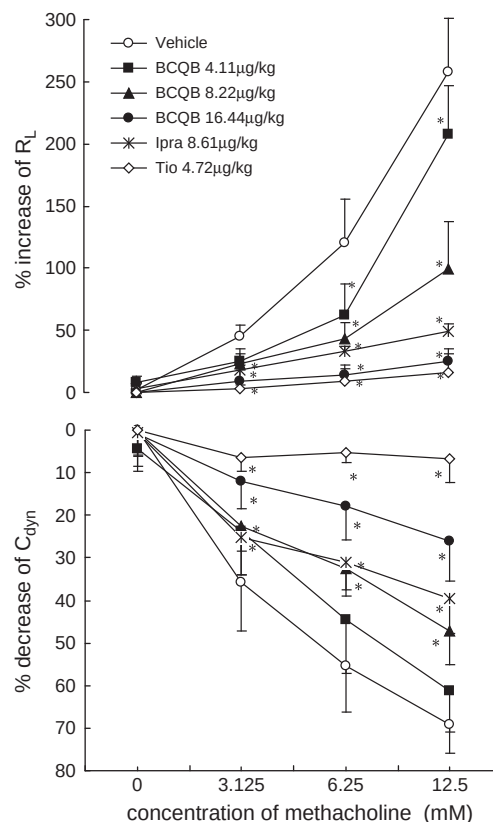


Fig. 3. Dose-dependent response of R_L and C_{dyn} to methacholine. Guinea pigs were anesthetized with an intraperitoneal injection of 1.25 g/kg urethane. In each animal, the trachea and esophagus were cannulated to measure R_L and C_{dyn} . BCQB, ipratropium and tiotropium dissolved in saline were administered locally: 100 μl of solution was instilled intratracheally, directly through the tracheal cannula. The model group was given intratracheally the same volume of saline. 5 min after intratracheal administration of BCQB, ipratropium and tiotropium, cumulative doses of methacholine 0, 3.125, 6.25 and 12.5 mmol/L were inhaled for 30 s in a jet nebulizer for a period of 5 min per dose with lung function measurements taken between each concentration. R_L and C_{dyn} against aerosol application of methacholine were determined as described in Materials and methods. Data are expressed as mean $\pm$ S.E.M. of eight to ten animals. $P < 0.05$, test compounds treated guinea pigs versus vehicle-treated guinea pigs.

(Hanna-Mitchell et al., 2007; Wang et al., 1995; Ehler et al., 2005). In addition, the most convincing results were obtained for M_2 receptors in heart preparations. The reason is that constitutive activity can be observed only in tissues containing high concentrations of relatively

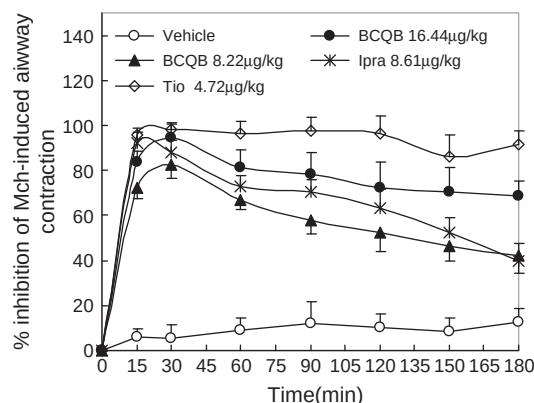


Fig. 4. Inhibition afforded by intratracheally administered test compounds of methacholine (12.5 mM, inhalation)-induced bronchoconstriction in anesthetized guinea-pigs. Methacholine was inhaled before (baseline value) and up to 180 min after administration of the test compounds. Data are expressed as mean $\pm$ S.E.M. of eight to ten animals.

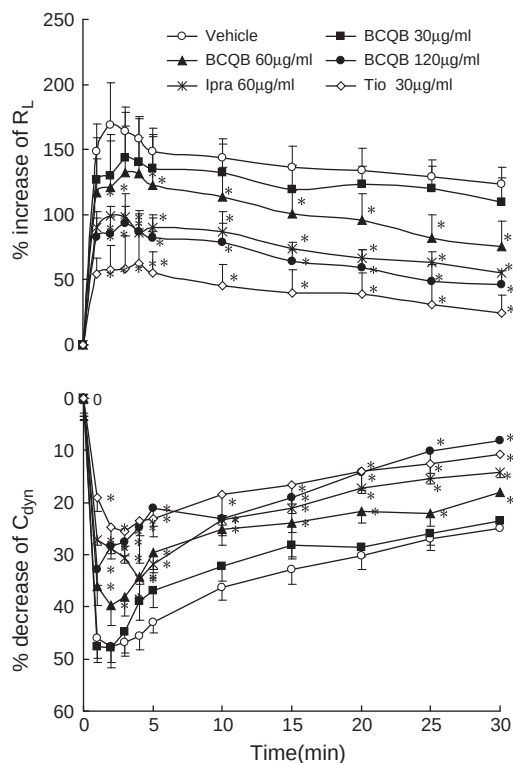


Fig. 5. Effects of BCQB, tiotropium and ipratropium on antigen-induced airway constriction in the sensitized guinea pigs. A single dose of BCQB 30, 60 and 120 µl/ml, tiotropium 30 µl/ml and ipratropium 60 µl/ml inhaled by a jet nebulizer for 30 s before antigen challenge. The control group was treated with an equal volume of saline. R_L and C_{dyn} against aerosol application of antigen were determined as described in **Materials and methods**. Data are expressed as mean $\pm$ S.E.M. of eight to ten animals. $P < 0.05$, test compounds treated guinea pigs versus vehicle-treated guinea pigs.

pure receptor subtypes such as is the case for M_2 and heart tissue (Dhein et al., 2001). In the present studies, using guinea pig isolated tissue assay, BCQB has approximately 100-fold in vitro selectivity for trachea, 250-fold for urinary bladder and 800-fold for ileum over right atrium. These findings suggest that BCQB may be a highly selective M_3 antagonist in vitro, and that cholinergic contraction responses in airway smooth muscle are mainly mediated through the M_3 , but not M_2 receptors. Although nonselective muscarinic antagonists have proven clinical efficacy in the treatment of obstructive airway diseases, gastrointestinal disorders and urinary dysfunction, a limitation in the systemic use of such compounds is failure to obtain desired therapeutic responses without concomitant side effects such as tachycardia, which is mediated by the cardiac M_2 receptor (Feinberg, 1993; Hendrix and Robinson, 1997). Thus, muscarinic M_3 receptor antagonists with high selectivity for the M_3 over the M_2 receptor such as BCQB are anticipated to have minimal cardiac effects, and may be of value in anticholinergic therapy.

Inhaled administration of anticholinergic drugs may result in unwanted adverse events. Therapy with ipratropium and tiotropium commonly leads to adverse events such as dry mouth, constipation, and urinary retention (Oba et al., 2008). There has also been concern that anticholinergic drugs might increase the risk for cardiovascular adverse events. A recent meta-analysis on the cardiovascular risks of anticholinergic drugs reported that this class of drugs is associated with cardiovascular adverse effects in COPD patients, including tiotropium and ipratropium (Singh et al., 2008). However, a 4-year study on tiotropium therapy (UPLIFT) demonstrated that tiotropium reduced cardiac morbidity in COPD patients (Tashkin et al., 2008). Treatment options for COPD that minimize systemic exposure may result in improved safety. Therefore, a newer anticholinergic drug

should have more favorable safety profiles than ipratropium and tiotropium. Preclinical studies showed that inhaled BCQB at 4 mg/dog or intratracheal injection at 400 µg/dog did not produce a significant change of heart rate, with no effect on QT intervals (Jiang et al., 2010). In the Phase I studies, BCQB appears to be safe and well tolerated with repeated doses up to 800 µg by aerosol administration for three times-a-day and consecutive five days, and did not produce any cardiovascular adverse event in healthy participants (unpublished data).

In the present studies, we addressed the question of the preclinical estimate of duration of action of BCQB in producing smooth muscle relaxation in the airways. The ability of BCQB to sustain an inhibition of smooth muscle contraction triggered by carbachol in the airways was evaluated after the antagonist was washed out from the tissue. It is conceivable to speculate that the inhibitory effect of BCQB measured in vitro experiments may be due to a slow dissociation of the compound from muscarinic receptors. In isolated guinea-pig trachea, the $t_{1/2}$ (onset) and $t_{1/2}$ (offset) of BCQB were similar to that obtained with ipratropium, but $t_{1/2}$ (onset) of BCQB was faster and $t_{1/2}$ (offset) was slower compared to tiotropium. The onset of action of tiotropium in our result was similar to that reported by Takahashi et al. ($t_{1/2}$ [onset] of 34–43 min) (Takahashi et al., 1994). However, there is a marked difference in the dissociation profile of BCQB with tiotropium in guinea-pig airways. We have also estimated the duration of the antimuscarinic action of BCQB in vivo, as the ability of this compound to prevent methacholine-induced bronchoconstriction in the guinea pig. In this model, we presume that the inhibitory effect of BCQB results from both the rate of dissociation from muscarinic receptors and from additional pharmacokinetic factors including the retention of BCQB in the lung, its metabolic stability and other factors. In our experiment, the enduring inhibitory effects of tiotropium lasted practically unchanged in vivo. In this bioassay tiotropium-induced inhibition lasted up to 24 h after intratracheal administration of the drug. The results are in agreement with previous reported data (Villetti et al., 2006; Takahashi et al., 1994). BCQB-induced effects lasted markedly short as compared to tiotropium, and similar to that of ipratropium. Duration of action off 50% of BCQB in both in vitro and in vivo bioassays was approximately 3 h. This result is in correlative with pharmacokinetic study of BCQB in humans using nasal spray 45 µg and in dogs treated with 200 µg via intratracheal injection, a single dose administration, both $t_{1/2}$ were 2–3 h (Zhou et al., 2009; Jiang et al., 2010). However, a pharmacokinetic study in rats, after intranasal administration of 3.0 mg/kg BCQB, demonstrated a longer elimination half-life (11.7 ± 7.1 h) (Xu et al., 2007). The results also suggest that elimination half-life of BCQB may be associated with dose and route of administration.

The methacholine provocation test by the increase in dose step by step method, a procedure used to measure airway responsiveness in humans, has been used to assess the activity of several bronchodilators (Fish and Kelly, 1979; Juniper et al., 1981). By modifying the clinical methodology, the measurement methods of airway responsiveness in guinea pigs had been established to evaluate the bronchodilators (Featherstone et al., 1988; Opazo Saez et al., 1996). In this guinea pig methacholine provocation test, intratracheal administered BCQB at 4.11 to 16.44 µg/kg induces a potent bronchodilation. Then our study evaluated and compared the bronchodilating activity of BCQB, tiotropium and ipratropium, given by inhalation in anaesthetized guinea-pigs and intratracheal administration in actively sensitized guinea-pigs. Inhalation of BCQB (from 30 to 120 µg/ml) caused a dose-dependent inhibition of the antigen-induced bronchoconstriction. Tiotropium 30 µg/ml and Ipratropium 60 µg/ml via inhalation also induced a marked inhibition of the bronchoconstrictive responses to aerosolized ovalbumin. These results are consistent with earlier findings that tiotropium can control the changes of airway induced by antigen challenge in guinea-pigs (Rossoni et al., 2007). Anticholinergic bronchodilators, like tiotropium, BCQB also can produce relaxation of antigen-induced constriction of airway smooth muscle through

antagonism of acetylcholine at the muscarinic M_3 -receptor. On the other hand, the assumption that activation of parasympathetic (cholinergic) nerve pathway is an important mechanism for producing airway obstruction, justify the use of anticholinergic compounds in asthma treatment (Westby et al., 2004). Furthermore, although it is widely held view that anticholinergic agents are less effective than β_2 -agonists in the symptomatic treatment of chronic asthma (Cazzola et al., 1998), the use of these compounds in combination with β_2 -agonists in particular subgroups of patients has been considered (Bolule et al., 1999; Gross and Morton, 1984; Berti et al., 1981).

In conclusion, by following original procedures with classical in vitro and in vivo bioassays, we have obtained reproducible data on the duration of antibronchospastic activity of BCQB, The $t_{1/2}$ (onset) up to 6.8 min and $t_{1/2}$ (offset) up to 180 min from its administration. BCQB, a selective antimuscarinic compound with bronchodilator activity potentially useful in asthmatic and COPD patients, have been demonstrated that it has a potency of action in our guinea-pig bioassay, but is shorter acting at equipotent doses, than tiotropium. On this basis we speculate that a putative BCQB-based therapy for COPD might require more than once-a-day administration to be as effective as the currently employed once-daily therapy with tiotropium. Nonetheless, the proposed benefit of using a selective M_3 antagonist reducing the side effect profile such while maintaining therapeutic efficacy. Inhalable M_3 -selective compounds may spare M_2 -cardiac receptors and reduce the risks of cardiovascular events associated with the long-term treatment of these agents.

Conflict of interest

There are no conflicts of interest.

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