Alternative mechanisms for tiotropium

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ABSTRACT

Tiotropium is commonly used in the treatment of chronic obstructive pulmonary disease. Although largely considered to be a long-acting bronchodilator, its demonstrated efficacy in reducing the frequency of exacerbations and preliminary evidence from early studies indicating that it might slow the rate of decline in lung function suggested mechanisms of action in addition to simple bronchodilation. This hypothesis was examined in the recently published UPLIFT study and, although spirometric and other clinical benefits of tiotropium treatment extended to four years, the rate of decline in lung function did not appear to be reduced by the addition of tiotropium in this study. This article summarizes data from a variety of investigations that provide insights into possible mechanisms to account for the effects of tiotropium. The report summarizes the discussion on basic and clinical research in this field.

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1. Introduction

The long-acting anticholinergic tiotropium is indicated for maintenance treatment for chronic obstructive pulmonary disease (COPD). Clinical trials have consistently shown tiotropium to improve lung function [1–3], which has been attributed to bronchodilation – considered the orthodox mechanism of action for anticholinergics in COPD. More surprising has been the efficacy of tiotropium in reducing the frequency of exacerbations, which are considered transient inflammatory events. Furthermore, post-hoc analysis of 1-year maintenance treatment data with tiotropium suggested an ability to slow the accelerated decline in forced expiratory volume in 1 second (FEV_1) [4] that is associated with COPD. This observation prompted a review of the pharmacologic effects of tiotropium in human biology and, specifically, whether mechanisms other than bronchodilation might account for some of the benefits of treatment with this drug. It also provided the basis for a large 4-year clinical study – the Understanding Potential Long-term Impacts on Function with Tiotropium (UPLIFT®) trial [5,6] – involving approximately 6000 patients, the results of which have now been published, and are summarized below.

This article is a summary of a Boehringer Ingelheim/Pfizer-sponsored round-table discussion held in Chicago, Illinois, USA on 26–27 October 2007 that considered current knowledge on the non-bronchodilator pharmacological mechanisms of tiotropium,

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their clinical relevance and whether tiotropium is unique in these effects. Inhibition of exacerbations and, in particular, amelioration of progressive loss of lung function are major unmet needs in COPD and the possibility that tiotropium could address these, at least in part, and that novel mechanisms may be responsible, provided the motivation for this review. As clinical evidence for a non-bronchodilator mechanism is sparse, this article first considers the theoretical possibilities suggested from laboratory findings and current clinical experience with this drug.

2. Function of cholinergic innervation in the lungs – the orthodox mechanism

The innervation of human airways is predominantly by the cholinergic parasympathetic system. This has been well described in previous publications (Fig. 1) [7]. Unlike that of dogs, for example, the sympathetic innervation of the pulmonary system in humans is sparse and this system plays little direct role in regulating airway function. However, since there are sympathetic endings on ganglion cell bodies, the sympathetic system may have a role in modulating cholinergic traffic. Evidence also suggests that paracrine effectors such as tachykinins may enhance cholinergic traffic through the ganglion [8].

Of the two classes of receptor for acetylcholine, nicotinic and muscarinic, nicotinic receptors are confined to the peribronchial ganglion, whereas, muscarinic receptors are more widely distributed in the lung. Five subtypes of muscarinic receptors subtypes are expressed in humans. Three (M_1, M_2 and M_3) are expressed in the lungs. The muscarinic receptors are typical G-protein-coupled receptors and the function of each has been elucidated over the last two decades. The system provides for exquisite fine-tuning of airway physiology (Fig. 2; Table 1) [9,10]. M_2 receptors, in addition to their location on the post-ganglionic nerves where they function as autoreceptors, are also abundant on smooth muscle cells where they are tightly coupled to inhibitory G-proteins. Their activation in the latter location appears to inhibit β_2-agonist induced smooth muscle relaxation. However, this finding is not conclusive due to limited availability of specific agonists and antagonists with which to investigate muscarinic subclasses. In murine heart and bladder, similar autoreceptors are found but appear to be of M_4 subtype [11]. Muscarinic M_3-receptors may be up-regulated in COPD airways compared with normal airways of smokers and non-smokers, whereas there appears to be no change in the expression of M_1 or M_2 receptors (Caramori G, et al. Manuscript in preparation). The
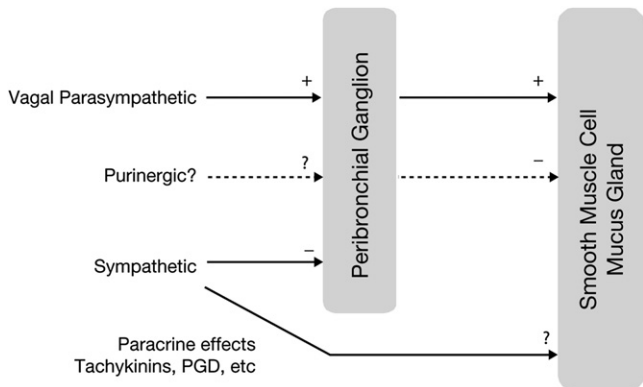
Fig. 1. The innervation of human airways. The cholinergic parasympathetic system is the predominant innervation of human airways. Sympathetic innervation of the pulmonary system in humans is sparse but may have a role in modulating cholinergic traffic. The role of purinergic nerves and paracrine effectors is largely unknown. PGD, prostaglandin D. Reproduced in modified form with permission from Nadel et al., 1986 [7].
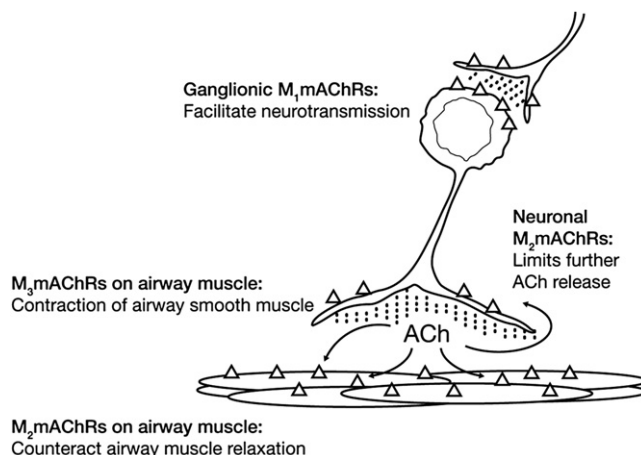
Fig. 2. Muscarinic receptors in human lungs. Three muscarinic receptors subtypes (M_1, M_2 and M_3) are expressed in human lungs. The function of each receptor subtype has been elucidated over the last two decades and suggests a system for fine-tuning airway physiology. Reproduced with permission from Belmonte, 2005 [9].

precise specific cellular location of this increase in receptors is not certain.

The role of muscarinic receptor abnormalities in COPD, if any, is unknown. Dysfunction of airway muscarinic receptors has been linked to airway hyper-responsiveness (AHR) in asthma, particularly neuronal M_2 receptors, which are susceptible to products released from eosinophils and to viral infection [8]. To date no genetic polymorphisms of muscarinic receptors that are associated with AHR have been identified.

The mechanism of action of bronchodilators in COPD is believed to be due to reduction in the basal level of bronchomotor tone in the small airways. Muscarinic antagonists cause a small degree of dilation of airways in normal volunteers, and small changes in airway calibre are associated with more sizeable effect on airways resistance in the narrowed airways of patients with COPD. Parasympathetic activity [7] is likely to be responsible for most bronchomotor tone in human airways, although basal leukotriene release may also play a role (Fig. 1). It is not possible, however, to definitively attribute bronchodilator tone to parasympathetic activity, since most antimuscarinics antagonize both M_2 and M_3 receptors on smooth muscle cells. It is possible, therefore, that functional antagonism of M_2 receptors could remove an inhibitory effect on relaxation of smooth muscle to allow relaxation induced by another agonist rather than direct inhibition of bronchoconstriction, mediated via the M_3 receptor. Regardless, bronchoconstriction due to parasympathetic activity that originates in the central nervous system (CNS) is the probable mechanism for basal bronchomotor tone. A current study is investigating whether bronchial responses to anticholinergics remain intact following lung transplantation. If they do not, this will strongly suggest that bronchomotor tone originates in the CNS rather than from local cholinergic sources.

Bronchomotor tone appears to be increased in both COPD and asthma, although evidence for this is indirect [12]. Anticholinergic agents are relatively more effective in COPD than in asthma. In part, this may be due to the co-presence in asthma of additional factors that are not amenable to anticholinergic therapy [13].

3. Non-neuronal acetylcholine in the airways – a potential unorthodox mechanism

Cholinergic communication and regulation was probably established long before the development of neurons. Acetylcholine as

Table 1
Main function of muscarinic receptors associated with the innervation of human lungs.

Muscarinic subtype	Location	Function
M_1	Peribronchial ganglia	Believed to facilitate cholinergic traffic through ganglia
M_2	Post-ganglionic nerves	Inhibit the further release of acetylcholine
	Smooth muscle	Believed to inhibit smooth muscle relaxation
M_3	Smooth muscle and mucus glands	Promote the action of these cells

a mediator is present in uni- and multicellular organisms, such as bacteria, algae, protozoa, sponges, primitive plants and fungi [14,15]. Phylogenetically, the lungs and airways would have been external to the body, similar to the present-day gills of fish or skin of amphibians. Of interest is that the surface secretion of mucin in many marine animals is controlled by acetylcholine-mediated mechanisms. Thus, acetylcholine may be considered a non-neuronal mediator in human lungs, in much the same way as nitric oxide is a ubiquitous mediator in this and other tissues in the human body.

Further circumstantial support for acetylcholine having a role beyond the parasympathetic nervous system is that most human cells (including epithelial, endothelial, immune and mesenchymal cells) synthesize and release acetylcholine. Acetylcholine modulates phenotypic cell functions by auto- and paracrine loops via nicotinic and muscarinic receptors [16–18]. Thus, the regulation of individual cells outside and independent of the nervous system operates in a way comparable with neurotransmission at neuroeffector junctions.

Importantly, non-neuronal acetylcholine can affect several known signal transduction pathways of individual cells (Table 2) [14,19–21]. Acetylcholine is believed to maintain and optimize cell function, such as cell cycle, proliferation, differentiation, formation of a physical barrier, chemokinesis, migration and ion and water movements. Hence, blockade of nicotinic and muscarinic receptors in vitro can cause cellular dysfunction and cell death. A further possibility is that acetylcholine receptors may not be responsible for all muscarinic effects upon cells. Recent evidence from research on receptors employing fluorescence and bioluminescence resonance energy transfer (FRET/BRET) and immunoprecipitation points to the formation of heterodimers in some systems that may result in co-stimulation through alternative receptor pathways. These findings raise the possibility that muscarinic receptors may be stimulated by mediators other than acetylcholine.

Antagonist-induced phenotypic effects depend on pharmacodynamic (subtype receptor specificity) and pharmacokinetic (dose,

Table 2
Signal transduction pathways of acetylcholine. Reproduced with permission from Wessler et al., 1999 [14].

Classical ion channels (Na^+, K^+, Ca^{2+}, Cl^-)
Large-conductance, voltage- and calcium-activated potassium (BK_{Ca}) channels
Non-selective cation channels
Transient receptor potential (TRP) cation channels
G-proteins
Phospholipase C
Effector kinases
Phosphatases
Cyclic nucleotides
Small GTPase
Non-receptor tyrosine kinase and transporter

distribution, half time) properties. Moreover, the endogenous use of non-neuronal acetylcholine by individual cells or interacting networks of cells is likely to vary with tissue type and pathology. Additionally, blockade of muscarinic receptors could cause a shift in the action of non-neuronal acetylcholine to nicotinic receptors, when both receptor populations are localized close together, as is the case, for example, on epithelial and immune cells. Non-neuronal production of acetylcholine could be particularly relevant for anticholinergics in small airway obstruction, as there are few cholinergic neurons in the lung periphery. In theory, basal levels of non-neuronal acetylcholine production in airway smooth muscle could also contribute to bronchomotor tone, though this has not been investigated. Non-neuronal production of acetylcholine may increase in disease states. A 15-fold increase in acetylcholine has been found in atopic dermatitis [22]. However, in COPD there is no evidence that the synthetic pathways for acetylcholine are increased, though this does not rule out the possibility of subtle changes in the activity of synthetic enzymes and transporter proteins, either at baseline or during exacerbations of COPD.

4. Potential and demonstrated effects of tiotropium on inflammatory cells

4.1. Epithelial cells

The entire respiratory tract is lined by epithelial cells, which are continually exposed to the external environment. In addition to acting as a physicochemical barrier, the epithelium plays a crucial role in initiating and augmenting pulmonary host defence mechanisms [23,24]. The epithelium is the first site of absorption of inhaled anticholinergics, suggesting that epithelial cells play a role in the pharmacological action of anticholinergic medications. The epithelium can also serve both as a source and a target of cholinergic signalling.

The key components of acetylcholine transport and metabolism are present in epithelial cells [20]. Furthermore, prominent ChAT-like immunoreactivity is expressed at the apical part of ciliated cells within the basal body and rootlet, where cilia are embedded [25], suggesting synthesis of acetylcholine on the airway surface. Both muscarinic and nicotinic receptors (M_1, M_3; α1,3,5,7,9, β1,2,4, δ, ε) are expressed on epithelial cells [8,26,27].

Stimulation of epithelial cells with muscarinic agonists (M_1 receptor) in vitro is associated with increased proliferation and may promote cell survival [27]. In vivo, this could represent a beneficial effect of acetylcholine in repair and would only be problematic if the process continued unchecked for a prolonged period. In vitro, acetylcholine stimulates apical chloride secretion and basolateral potassium secretion and inhibits sodium re-absorption, thereby contributing to airway tract fluid, mucus production and mucociliary clearance [27,28]. Thus in theory, sustained blockade of epithelial M_1 receptors by inhaled cholinergic antagonists may be detrimental. In this context it might be significant that tiotropium dissociates more slowly from M_3 ($t_{1/2}$ 34.7 h) and M_1 receptors (14.6 h) than from M_2 receptors (3.6 h); in addition, tiotropium binds with a 3-fold higher affinity at M_3 (K_D 0.014 nM) than at M_1 (0.041 nM) receptors [29]. These properties may facilitate a continuous blockade of M_3 receptors, but a somewhat reduced degree of antagonism at the remaining receptor subtypes.

Potential anti-inflammatory effects of anticholinergic agents have also been postulated in vitro [27,30]. Acetylcholine induces the release of proinflammatory mediators like granulocyte macrophage-colony stimulating factor (GM-CSF), leukotriene B_4 (LTB_4) and prostaglandin E_2 from bronchial epithelial cells [27,31,32]. A recent unpublished investigation on the carbachol-stimulated release of an unknown neutrophil chemokinesis factor (independent of IL-8) from bronchial

epithelial cells showed a dependency on the expression of the M_3 receptor (Pfeifer M et al, data on file). As functional M_3 receptors are not present on cultures of bronchial brushings from all subjects, and since the M_3 receptor does not show evidence of internalization following stimulation, this suggests some heterogeneity in the sensitivity to anticholinergic agents. This requires further elucidation and may warrant specific investigation for other potential inflammatory mechanisms of acetylcholine.

A hypothetical mechanism of stimulation of epithelial cells by acetylcholine could be through the activation of the epidermal growth factor receptor (EGFR), which is present on the epithelial cell surface. Activation of the EGFR, which forms part of the innate immune system to inhaled irritants, stimulates secretion of mucins, antibacterial peptides, as well as inflammatory mediators [33]. Muscarinic receptors, as G-protein-coupled receptors [34–36], could stimulate the pathway that leads to EGFR activation (Fig. 3) [37]. Hence, acetylcholine could share the effects of EGFR. However, this potential relationship has not been investigated.

Further hypotheses discussed at the round table involved the folding of the bronchial wall that occurs during bronchoconstriction. This could potentially bring acetylcholine-releasing nerve endings of the smooth muscle and goblet cells into closer proximity to the basal cell walls of epithelial cells, thereby providing potential for neuronal acetylcholine to behave as non-neuronal acetylcholine. Similarly, the folding and deformation of the epithelium could cause the apical surface of epithelial cells to come into contact with each other and cause irritation and activation of EGFR. In this way, bronchoconstriction could potentiate the stimulation of epithelial cells by acetylcholine.

4.2. Inflammatory cell recruitment

Chronic inflammation in COPD is characterized by accumulation of neutrophils, macrophages and lymphocytes, most prominently $CD8^+$ lymphocytes in pulmonary tissue [38]. When studying inflammation in COPD, distinguishing patients with chronic bronchitis from those with emphysema could be important, because the inflammation may differ between the two conditions; however, both conditions can co-exist in the same individual. It is also important to consider the changes in inflammatory cells that occur during exacerbations.

ChAT-like immunoreactivity is found in many inflammatory cells, including alveolar macrophages, mast cells, mononuclear cells, and granulocytes [27,39]. Again, these cells express both muscarinic and nicotinic receptors (M_1–M_5, $\alpha 2,3,5$–$7,9,10$, $\beta 2,4$) [27,39,40].
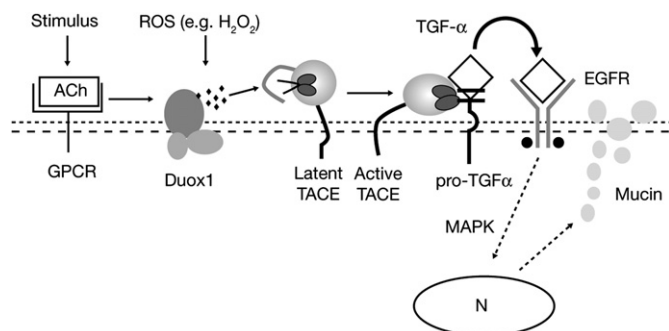
Fig. 3. A hypothetical mechanism of stimulation of epithelial cells by acetylcholine (ACh) through the activation of the epidermal growth factor receptor (EGFR). GPCR, G-protein-coupled receptors; ROS, reactive oxygen species; Duox1, dual oxidase 1; TACE, tumor necrosis factor lambda converting enzyme; TGF-α, transforming growth factor-alpha; MAPK, mitogen-activated protein kinase; N, nucleus. Reproduced in modified form with permission from [37]. Copyright (2005) National Academy of Sciences, USA.

Like epithelial cells, inflammatory cells release chemotactic mediators after incubation with acetylcholine *in vitro*. For example, one recent study has shown human alveolar macrophages and MonoMac6 (monocyte cell line) cells to release chemotactic activity for granulocytes that could be attributed chiefly to LTB_4, which was similarly shown with A549 (epithelial cell line) cells [41]. The secretion of LTB_4 was suppressed by more than 70% by the addition of tiotropium at the time of acetylcholine stimulation (Fig. 4). In this study tiotropium alone did not influence the *in vitro* migration of granulocytes from healthy donors [41].

Mast cells are also an important source of chemotactic mediators stimulating the recruitment of inflammatory cells. Histamine has been shown to stimulate the generation of the proinflammatory cytokine, IL-6 [42]. Elevated histamine and tryptase levels have been found in the bronchoalveolar lavage fluid of smokers [43], indicating enhanced mast-cell activity. Although a protective effect of terfenadine on adenosine-induced bronchoconstriction has been shown in COPD, histamine receptor blockers are not effective in COPD [44]. In human airways, mucosal mast cells are strongly controlled by inhibitory M_1 receptors [45]. Blockade of these inhibitory M_1 receptors by cholinergic antagonists could, in theory, be associated with diverse effects. How tiotropium, with its selective action on M_3, M_1 and M_2 receptors as described above, may affect this pathway has not yet been identified.

This body of information provides evidence that acetylcholine may induce inflammatory mediators from most inflammatory cells via a muscarinic-mediated mechanism that may be inhibited by tiotropium. Whether this occurs during basal conditions or during acute exacerbations in patients with COPD, however, is yet to be evaluated. Of note is that tiotropium has been shown to reduce inflammation in a guinea-pig model of allergen-induced bronchoconstriction [46].

5. Potential effects of tiotropium on airway remodelling

The effect of tiotropium on airway remodelling may be relevant to its hypothesized role in reducing the decline in FEV_1 [4]. A number of studies have investigated the potential role that acetylcholine could play in remodelling the airways. 'Remodelling' of airways is generally considered to be detrimental to structure and function of the lung. However, this may not be true, since proliferation and fibrosis are normal parts of wound healing. Fibrosis of a collapsing airway may have a stabilizing effect and reduce the tendency to dynamic hyperinflation during exercise. Like inflammation, remodelling is probably only a problem when it is excessive and progressive.

5.1. Airway smooth muscle

Airway smooth muscle is a key determinant of airflow limitation and AHR in obstructive airways disease [47]. AHR results from an altered local inflammatory environment that promotes contraction in combination with structural and phenotypic changes of the muscle. These include increased muscle mass, increased contractile capacity, indicative of a more contractile smooth muscle phenotype, and the increased presence of proinflammatory markers, suggestive of an active immunomodulatory/secretory function for airway smooth muscle in COPD [48–51].

Regulatory roles for muscarinic receptors have been reported for each of these structural and phenotypic smooth muscle abnormalities using *in vitro* and/or *in vivo* experimental models. Muscarinic receptors synergistically augment growth factor-induced airway smooth muscle cell proliferation, an effect that appears to be regulated exclusively by muscarinic M_3 receptors [52]. Intracellular signalling targets that are cooperatively regulated by muscarinic agonists and growth factor receptors in airway smooth muscle
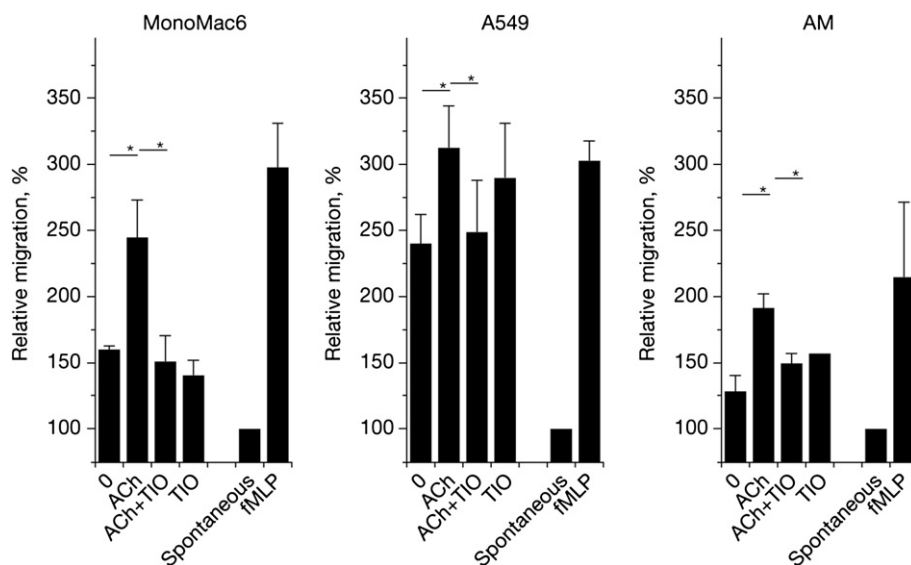
Fig. 4. Inhibition of chemotactic activity by tiotropium. MonoMac6 cells, A549 cells, and alveolar macrophages (AM) were stimulated without (0) or with acetylcholine (ACh; 100 mmol/l) and tiotropium (TIO, 20 nM) for 24, 48 or 8 h. The conditioned supernatants were used to stimulate granulocyte migration. Tiotropium alone did not significantly influence the migration of human neutrophilic granulocytes. In control experiments, granulocytes were incubated with fMLP. The spontaneous migration of granulocytes that were incubated with native culture medium was set to 100%. The results of four experiments are presented as mean ± SEM. Asterisks indicate significant ($p < 0.05$) differences between the indicated samples. Reproduced with permission from [41].

include p70 S6 kinase and GSK-3β, both of which are involved in protein translation and cell-cycle progression [53–55]. The functional importance of this mechanism is yet to be established in COPD or in animal models of COPD.

Although not a model of COPD, treatment with tiotropium reduced bronchiolar smooth muscle hyperplasia by approximately 75% in repeatedly allergen-challenged guinea pigs [56]. This suggests that endogenous acetylcholine may perform a regulatory role in the remodelling of airway smooth muscle bundles in allergic asthma. In the same model, tiotropium partially prevented the increased expression of the contractile protein sm-myosin heavy chain (MHC) and reduced smooth muscle contractility ex vivo [56]. These data suggest that acetylcholine regulates smooth muscle contractile phenotype, a contention supported by an *in vitro* study [57,58].

It has recently been demonstrated that muscarinic receptors also regulate the expression of proinflammatory markers in airway smooth muscle (in particular IL-6, IL-8 and COX-2), an effect strongly amplified by mechanical stimulation of the muscle [59]. As in inflammatory cells (discussed above), muscarinic receptor stimulation of airway smooth muscle could contribute to the induction and perpetuation of local airway inflammation.

Together, these data suggest that prolonged inhibition of muscarinic receptor function by tiotropium has significant therapeutic potential in preventing abnormalities in the airway smooth muscle that are observed in COPD, including increased muscle mass, contractility and expression of proinflammatory markers. However, although ChAT-like immunoreactivity is found in smooth muscle fibres, these data do not confirm that these cells are a source of non-neuronal acetylcholine [27].

5.2. Fibroblasts

Human fibroblasts predominantly express M_2 receptors with relatively fewer M_3 receptors. *In vitro*, muscarinic agonists stimulate the incorporation of ^{3}H-thymidine (as a measure of cell proliferation) in human lung fibroblast cell lines and primary cells [60]. This effect is mediated by the M_2 receptor, which then involves a pertussis-sensitive G-protein [60] and p42/p44 mitogen-activated protein kinase [61] in the transduction pathway. Similarly,

muscarinic agonists enhance ^{3}H-proline incorporation into cellular proteins (as a measure of collagen synthesis) to a degree of circa 50–70% of that expected by transforming growth factor-β_1 (TGF-β_1). This effect also involves the same transduction pathway [62,63].

Tiotropium inhibited the muscarinic agonist-stimulated proliferation of fibroblasts described above [60]. Recently, this has been confirmed in a second study with acetycholine-stimulated proliferation of primary human fibroblasts isolated from biopsies of patients with lung fibrosis, myofibroblasts derived from these cells, and a human lung fibroblast cell line [64]. Tiotropium inhibited the acetylcholine-induced proliferation in both fibroblasts and myofibroblasts in a concentration-dependent manner.

These findings support the possibility that cholinergic mechanisms play a role in profibrotic airway remodelling processes. Whether inhibition of these processes by prolonged blockade of the M_2 receptor contributes to the long-term beneficial effects of tiotropium is uncertain and must be questioned since blockade of the M_2 receptor by tiotropium is relatively short-lived.

6. The effect of tiotropium on mucus production

Mucus secretion is physiological and contributes to maintaining a hydrated mucosal surface as a first line of defence in the lungs. The mucus barrier assists with the trapping and clearance of inhaled particles and microbes, and has microbicidal properties. In disease, mucus hypersecretion may contribute to airflow obstruction and increase the risk of pulmonary infections, and is associated with troublesome symptoms of cough and expectoration. In such circumstances, reduced mucus secretion might be beneficial and improve clearance of the airways. Tiotropium is now the most commonly used inhaled bronchodilator for the treatment of COPD [65]. Despite the widespread acceptance of this medication, an often-expressed concern among clinicians is that it might lead to drying of the bronchial mucosa with increased viscosity, making it more difficult for patients to clear secretions. This fear is based in part on the known effect of anticholinergics upon the salivary glands, and the development of a dry mouth in a small percentage of patients using tiotropium. Although impairment of mucociliary clearance has been observed after administration of some of the older anticholinergic

drugs [66,67], in healthy animals pre-treatment with atropine blocks induced but not basal secretion of mucus [68].

No published data describe the effects of tiotropium on the volume and rheology of mucus. However, a number of clinical studies using other anticholinergics, primarily ipratropium, in subjects with COPD provide some insight (Table 3) [67,69–77]. Ipratropium has no direct effect on mucociliary clearance in COPD, but cough clearance may be decreased. Using radiolabelled, monodispersed aerosols and gamma camera analysis, Bennett and colleagues have reported that ipratropium decreases cough clearance in subjects with stable, moderate-to-severe COPD [74]. Pavia et al. reported a decrease in 6-h expectorated sputum volume in subjects with reversible airway obstruction [71]. A similar reduction in the volume of airway mucus in patients with chronic bronchitis has been reported with the long-term administration of oxitropium bromide [76]. The effect of ipratropium on cough clearance may be due to either changes in rheology (thus detrimental) or to a decreased volume of airway secretions (considered to be of clinical benefit) – which of these it is has yet to be determined.

There are also no published studies evaluating the effect of tiotropium on mucus secretion, sputum properties or sputum clearability. However, Hasani et al. conducted a 3-week randomized clinical study of tiotropium in 34 subjects with stable COPD who were between 40 years and 75 years of age [77]. Although there were no significant changes in mucociliary clearance, cough clearance was mildly impaired after tiotropium (Fig. 5). Because aerosol deposition was significantly increased with tiotropium, the authors speculated that the transit pathway for mucus clearance was lengthened and thus mucociliary clearance might increase slightly after tiotropium.

Mucus hypersecretion is a risk factor for an accelerated loss of lung function in COPD [78] and this is related to increased risk and severity of infectious exacerbations [79]. Thus, while there is concern that anticholinergic medications may impair sputum clearance, especially by cough, there are also data suggesting that these medications reduce the volume of secretions without altering viscoelasticity and, thus, may be of value in patients with mucus hypersecretion.

7. The effect of tiotropium on cough

Acute cough (defined as cough of up to 3 weeks' duration) is most often caused by a viral upper respiratory tract infection (URI) and is usually transient and self-limited. However, some patients will develop a prolonged postviral cough after resolution of all other symptoms or a URI that is non-responsive to treatment [80]. In spite of very limited evidence of their effectiveness in clinical trials, prescription and non-prescription anti-tussive therapies
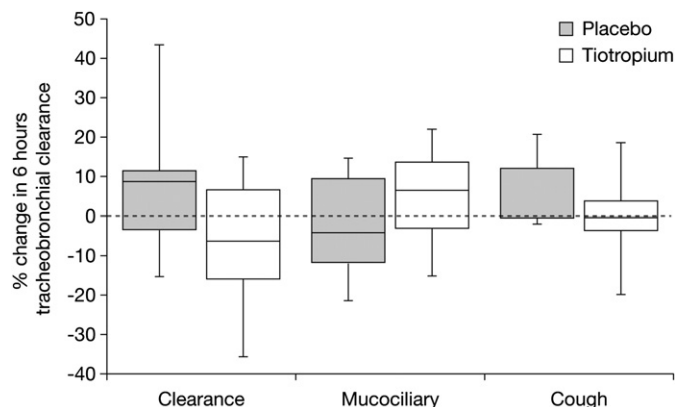

Fig. 5. The change in tracheobronchial clearance at 6 h after radioaerosol inhalation and in productive coughing (represented by radioactivity of expectorated sputum) together with their difference (mucociliary). Reproduced with permission from Hasani et al., 2004 [77].

continue to be widely used [81] and there is an urgent need for new agents for the symptom of cough.

Cough is entirely a vagal nerve phenomenon [82], though, surprisingly, only a few small studies have investigated the effects of anticholinergics on cough and cough reflex sensitivity (Table 4) [83–87]. Methodological differences make interpretation of these studies difficult. One small, cross-over study evaluated the effect of inhaled ipratropium bromide compared with placebo in postviral cough. A dose of 320 μg/d of ipratropium was found to be effective in suppressing subjectively described cough [83]. In another small, placebo-controlled, cross-over study, the ability of ipratropium to diminish citric acid-induced cough in asthmatics was demonstrated, but no effect was observed in healthy volunteers [84]. Oxitropium bromide has been shown to inhibit cough induced by ultrasonically nebulized distilled water in asthmatic and non-asthmatic volunteers [85], but not in subjects with acute URI [86].

A recent placebo-controlled pilot study has demonstrated that tiotropium reduces capsaicin-induced cough sensitivity in volunteers with URI, but not in otherwise healthy volunteers [87]. However, the effect of tiotropium upon symptoms was not recorded and further studies are required to examine the clinical relevance of these findings.

The mechanism by which tiotropium inhibits cough reflex sensitivity is unclear. It is unlikely to be a direct airway effect on the cough reflex, as there are no muscarinic receptors on airway sensory afferent nerves. An alternative mechanism is through its bronchodilator effect in counteracting transient increases in bronchial cholinergic tone caused by viral infection. However, numerous prior studies have demonstrated the inability of induced bronchodilation

Table 3
Clinical studies on the effects of anticholinergics on mucus [67,69–77].

Drug	1st author	Year	N	Diagnosis	Results
Atropine	Groth	1991	14	COPD	Decrease in MCC relative to placebo
Ipratropium	Francis	1977	12	Healthy subjects	No change in MCC vs. placebo
Ipratropium	Ruffin	1978	6	COPD	No change in MCC vs. placebo
Ipratropium	Pavia	1979	12	COPD/asthma	No change in MCC vs. placebo Decrease in 6-h sputum weight
Ipratropium	Matthys	1985	14	COPD	No change in MCC vs. placebo
Ipratropium	Taylor	1986	12	Smokers and ex-smokers	No change in MCTR or in sputum weight in 7 expectorators
Ipratropium	Bennett	1993	15	COPD	Significant decrease in CC vs. placebo
Ipratropium	Guleria	2003	10	COPD	No change in MCC vs. placebo
Oxitropium	Tamaoki	1994	17	Chronic bronchitis	Significant 31% decrease in sputum volume Increased sputum % solids and elasticity
Tiotropium	Hasani	2004	34	COPD	MCC preserved but CC slightly decreased

MCC, mucociliary clearance; CC, cough clearance; MCTR, mucociliary transport rate.

Table 4
Selected clinical studies on the effects of anticholinergics on cough [83–87].

Drug	1st author	Year	N	Diagnosis	Results
Ipratropium	Holmes	1992	14	PVC	Suppressed subjectively described cough
Ipratropium	Pounsford	1985	8	Asthma	Increase in citric acid-induced cough threshold
			7	Normal	No change in citric acid-induced cough
Oxitropium	Lowry	1988	10	Asthma	Decreased frequency of ultrasonically nebulized distilled water-induced cough
			16	Normal	
Oxitropium	Lowry	1994	56	Acute URI	No change in ultrasonically nebulized distilled water-induced cough
Tiotropium	Dicpinigaitis	2007	11	Acute URI	Reduced capsaicin-induced cough sensitivity

PVC, postviral cough; URI, viral upper respiratory tract infection.

or bronchoconstriction to alter cough-receptor sensitivity (albeit mainly in healthy volunteers) [88–90]. Other potential mechanisms are an effect on mucus glands, inflammatory mediators, inflammatory cells, epithelial permeability, vascular blood flow and clearance of substances applied to the airway lumen, all of which could induce an alteration in cough-receptor sensitivity.

8. Clinical perspective of effects of tiotropium on non-neuronal acetylcholine and non-mechanical properties of tiotropium

Overall, no clinical evidence currently exists to demonstrate that tiotropium or any other muscarinic antagonist has either an anti-inflammatory effect or an effect on airway remodelling in asthma or COPD. Similarly, evidence for a clinical effect of tiotropium on mucus clearance and cough is sparse.

There is evidence that patients with lower FEV_1 values have a greater degree of inflammation, suggesting that inflammation may increase as disease progresses [49]. The role of endogenous acetylcholine in inflammation has been assessed by studying the effects of cholinergic agonists on inflammatory cell chemotaxis and activation and there is good evidence for the expression of M_1, M_2 and M_3 receptors on various inflammatory cells in the airways, as discussed above. However, it is much less certain that tiotropium and other muscarinic antagonists have anti-inflammatory effects. In the only clinical trial to investigate the effect of tiotropium on inflammation, tiotropium failed to reduce the concentrations of IL-6 and myeloperoxidase (as evidence of the presence of neutrophils) in induced sputum of COPD patients after 12 months of therapy [91]. In contrast, the concentration of IL-8 increased compared with placebo. Yet, in the same study, there was a 50% reduction in exacerbations, suggesting that the clinical benefit of tiotropium is not exerted through an effect on airway inflammation, as assessed by the sputum measures made. However, concentrations of LTB_4 or TGF-β, which may be more relevant to cholinergic effects, were not measured in this study. Additionally, in this study the measurements of inflammatory cells and mediators were made on sputum and airway secretions (from both induced and spontaneous sputum) and may not reflect changes in bronchial mucosa. It is also possible that the increased IL-8 concentrations with tiotropium may be related to xerostomia ("dry mouth"), particularly since reduced sputum production was more frequently reported with tiotropium than with placebo.

The failure to demonstrate changes in inflammatory cells with tiotropium in COPD can be viewed in relation to the effect of corticosteroids. This class of anti-inflammatory drugs has also failed to show significant changes in inflammatory cells in some studies assessing induced sputum, but has recognizable clinical benefits. It is possible, therefore, that any anti-inflammatory effects of tiotropium may not be observable with the methods used.

Cholinergic agonists activate pulmonary fibroblasts and may thereby induce fibrosis [61]. Since it is not yet possible to quantify fibrosis in small airways *in vivo*, it is not possible to evaluate the potential effects of tiotropium on airway fibrosis and any resultant structural changes in airways or lung tissue that may result. However, peribronchiolar fibrosis may be a key mechanism in progressive loss of FEV_1 in COPD [49]. Any inhibitory effect of tiotropium on the development of fibrosis would presumably only be detectable after many years as a slowed decline in lung function.

In addition to the observed effects of cholinergic agonists on fibroblasts, it is possible that acetylcholine (from neuronal or non-neuronal sources) closes small airways and that the strain and compression of airway epithelial cells may release growth factors, such as TGF-β, that result in fibrosis [92]. If this is a genuine mechanism in humans, it would predict that any long-acting bronchodilator (including both anticholinergics and long-acting β$_2$-agonists) would reduce airway fibrosis in COPD and asthma.

Evidence that anticholinergics may also be involved in airway smooth muscle proliferation, smooth muscle hypertrophy, increased contractility and inflammatory mediator synthesis [56,59] predicts that, in addition to bronchodilation, tiotropium may affect airway smooth muscle contractility and the thickness of airway smooth muscle. This potentially could be tested by measuring AHR in asthma or COPD induced by bronchoconstrictors other than inhaled cholinergic agonists, such as histamine or leukotriene D_4. Assessment of whether any treatment reduces airway smooth muscle proliferation is very difficult as this cannot be assessed in biopsies. High-resolution imaging techniques may make it possible to measure airway thickness, but any treatment would be expected to change this very slowly. In addition, airway smooth muscle in COPD is not increased to the same extent as in asthma [93,94].

More research is needed on the mechanical consequences of closure in small airways in COPD, particularly with respect to air trapping, which could stimulate inflammatory processes and remodelling in the lung due to stretching of lung tissue. If a link is found, this may provide some justification for earlier intervention with bronchodilators such as tiotropium. Although all bronchodilators reverse air trapping to some extent, the prolonged reversal of cholinergic tone appears to be most effective in this respect [95]. A comparison with a β-agonist with a similar duration of action to tiotropium, would provide more information on whether tiotropium is genuinely better than β-agonists at reducing air trapping.

To date, only two studies have examined the effect of regular use of anticholinergic drugs on the progression of COPD; the Lung Health Study and the UPLIFT study. In the former, ipratropium bromide (36 μg given three times daily over 3 years) had no effect on the decline in FEV_1 in smokers with mild airflow limitation [96]. In the UPLIFT study, tiotropium added to usual treatment (which included long-acting β$_2$-agonist and/or inhaled corticosteroids in almost two thirds of patients) resulted in sustained clinical benefit in most secondary endpoints [6]. Relevant to this review, improvements in pre-bronchodilator FEV_1 and forced vital capacity levels with tiotropium did not return to baseline values within the 4-year treatment period. However, the rates of decline in pre- and post-bronchodilator FEV_1, the co-primary endpoints, were not different in patients receiving tiotropium compared with those receiving control treatment. A possible reason for the difference between these results and the earlier study is the effects of concurrent treatment. For instance,

Table 5
Summary of non-bronchodilator effects of tiotropium.

System/parameter	Potential effect of tiotropium
Epithelial cells	• Antagonism of the effects of acetylcholine. Reduction of: apical chloride secretion (and facilitation of sodium re-absorption); airway fluid and mucus production; mucociliary clearance. • Potential reduction of epithelial cell survival. • Antagonism of inflammatory effects of acetylcholine: reduction in release of GM-CSF, LTB_4 and PGE_2 from bronchial epithelium. • Inhibition of M_3-receptor-dependent-, IL-8-independent release of a chemokinetic factor for neutrophils. • Inhibition of the effect of acetylcholine on EGFR, thereby reducing secretion of mucins, antibacterial peptides and inflammatory mediators in response to inhaled irritants. • Bronchodilation of airways may prevent mucosal folding thereby both reducing epithelial cell-cell contact which provokes EGFR stimulation, and increasing the cholinergic nerve ending-epithelial distance (reducing the possibility of non-neuronal activation of epithelium by acetylcholine).
Inflammatory cells	• Suppression of LTB_4-induced stimulation of neutrophil chemotactic activity by monocytes and alveolar macrophages. • Inhibition of mast-cell function though M_1-receptor blockade in COPD may reduce IL-6 recruitment of inflammatory cells and release of histamine and tryptase. • In humans, in one study, tiotropium failed to reduce the concentrations of IL-6 and myeloperoxidase (as evidence of the presence of neutrophils) in induced sputum of COPD patients after 12 months of therapy.
Airway remodelling	Smooth muscle: • Reduction in bronchiolar smooth muscle hyperplasia. • Antagonism of acetylcholine-induced regulation of smooth muscle contractile protein phenotype. • Inhibition of acetylcholine-induced expression of proinflammatory markers in airway smooth muscle (e.g. IL-6, IL-8 and COX-2). Fibroblasts: • Inhibition of acetylcholine-induced fibroblast and myofibroblast proliferation and collagen synthesis.
Mucus production	• Effects are uncertain. Tiotropium may reduce the volume of secretions without altering viscoelasticity. Reduced cough clearance and mucus volume has been demonstrated with anticholinergics, but mucociliary clearance is not impaired.
Cough	• Reduction of capsaicin-induced cough sensitivity in volunteers with upper respiratory infections. Its role in COPD and COPD exacerbations is unknown.

GM-CSF, granulocyte macrophage-colony stimulating factor; LTB_4, leukotriene B_4; PGE_2, prostanglandin E_2; IL, interleukin; EGFR, epidermal growth factor receptor; COX, cyclooxygenase.

a post-hoc analysis of the 13.5% of patients in the UPLIFT study who were maintenance-treatment-naïve (ie, were not receiving treatments such as long-acting β_2-agonists and/or inhaled corticosteroids at baseline) showed a statistically significantly lower rate of decline in pre-bronchodilator FEV_1 in the tiotropium group vs control group (difference of 9 ml/year [$p = 0.026$] and 134 ml over 4 years [$p < 0.001$]) [97]. This provides further support for the need to continue exploring mechanisms of disease progression in COPD [5].

9. Summary

Reviewing laboratory studies alone, tiotropium could function in the lung by both mechanical (bronchodilator) and non-mechanical (anti-inflammatory and anti-proliferative) mechanisms (Table 5). These mechanisms could co-exist and could interact.

Bronchodilation could reduce physical stress to the lung and, thereby, reduce the possible resulting damage to epithelium and other cells (e.g. via shearing forces, deformation). In this way, the mechanical effects of tiotropium could also reduce inflammation occurring in the lungs of patients with COPD. These effects would probably not be unique to anticholinergics, but could be generalized to all long-acting bronchodilators.

In vitro studies suggest that a more direct anti-inflammatory effect of tiotropium is theoretically possible. This is a potential explanation for the effect of tiotropium on exacerbations of COPD. Further investigations into both inflammatory and mechanical mechanisms are warranted. This is particularly important as, through such actions, tiotropium may have potential to improve the clinical course of COPD in addition to providing symptomatic benefit.

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