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The design of a novel series of muscarinic receptor antagonists leading to AZD8683, a potential inhaled treatment for COPD

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ABSTRACT

A novel series of muscarinic receptor antagonists was developed, with the aim of identifying a compound with high M_3 receptor potency and a reduced risk of dose-limiting side effects with potential for the treatment of COPD.

Initial compound modifications led to a novel cycloheptyl series, which was improved by focusing on a quinuclidine sub-series. A wide range of N-substituents was evaluated to determine the optimal substituent providing a high M_3 receptor potency, high intrinsic clearance and high human plasma protein binding. Compounds achieving in vitro study criteria were selected for in vivo evaluation. Pharmacokinetic half-lives, inhibition of bronchoconstriction and duration of action, as well as systemic side effects, induced by the compounds were assessed in guinea-pig models.

Compounds with a long duration of action and good therapeutic index were identified and AZD8683 was selected for progression to the clinic.

Muscarinic acetylcholine (ACh) receptors perform important roles in the regulation of many physiological functions of the central and peripheral nervous systems and are widely distributed throughout the body.[1–5] They belong to the G-protein-coupled receptor family and the five different muscarinic receptor subtypes currently known are denoted M_1, M_2, M_3, M_4 and M_5.[6,7] In the lung, the M_3 receptor sub-type is present on airway smooth muscles and sub-mucosal glands and when activated by ACh released from parasympathetic nerves, produces bronchoconstriction, mucus secretion and subsequently an increase in airways resistance, as observed in chronic obstructive pulmonary disease (COPD). Antagonism of the M_3 receptor sub-type leads to improved lung function and is an established part of the therapeutic management of COPD.[8,9] The muscarinic receptor antagonists currently used to treat COPD (ipratropium, tiotropium (1.1) and aclidinium bromide[10](1.2)) and newer antagonists, such as glycopyrrolate[11,12] (1.3) in late stage clinical development, are all non-selective across the M_1 to M_5 receptor subtypes (Fig. 1). All these compounds are quaternary ammonium salts and carry a fixed positive charge which imparts very low permeability and makes them poorly absorbed. These compounds are administered directly to the lung by inhaled delivery and this, in combination with their poor permeability, limits their systemic exposure. All these compounds contain an ester function, which can be metabolized upon systemic exposure, further reducing their potential to cause side effects such as tachycardia, via antagonism of cardiac M_2 receptors. Tiotropium

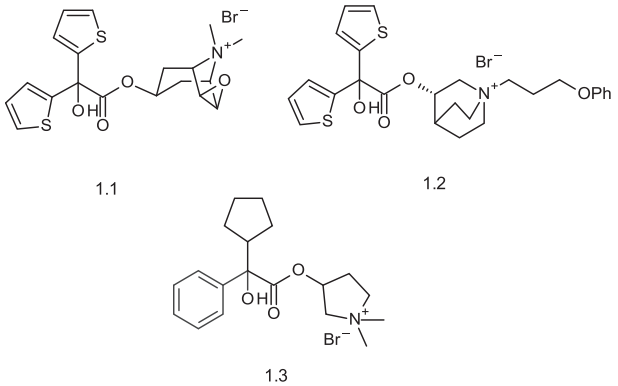

Figure 1. Structure of muscarinic antagonists in clinical use or in development.

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† A.M., K.B., H.C., D.K.D., R.F. and A.M. were former employees of AstraZeneca.

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
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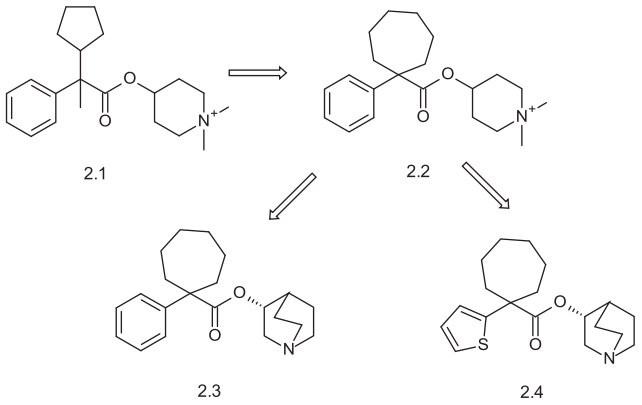


Figure 2. Novel series of muscarinic receptor antagonists.

is currently considered the bronchodilator of choice for the treatment of COPD and is generally well tolerated with dry mouth being the main reported side-effect.[8,9] Tiotropium is a highly potent[13] and long acting muscarinic receptor antagonist and any new muscarinic receptor antagonist developed for COPD should match tiotropium for efficacy and duration of action whilst reducing the dry mouth and other dose-limiting side effects.

We recently reported the discovery of a novel series of muscarinic antagonists which demonstrated a reduced side-effects profile relative to tiotropium in pre-clinical studies.[14] Our approach was to achieve low systemic exposure with the M_3 receptor antagonists by selecting compounds which possessed high intrinsic metabolic clearance coupled with high human plasma protein binding (hPPB) in order to reduce the free concentration of any compound reaching the plasma. In this paper, another series is described with the aim of identifying a chemically differentiated compound which also possesses high M_3 receptor potency and low systemic exposure and hence minimizes the risk of systemic side effects in vivo.

We have previously reported that compounds such as 2.1 (Fig. 2), which bear a methyl group in the position where tiotropium, glycopyrrolate and aclidinium bromide have a hydroxy group, exhibit antagonism of the M_3 receptor[13] coupled with high metabolic clearance and high human plasma protein binding.[15] However, in compounds such as 2.1, constructing the chiral center at the quaternary carbon was synthetically very challenging and a hindrance to rapid analog synthesis. Therefore, in order to maintain the promising biological profiles of these molecules but in a chemically simpler series the cyclopentyl and the methyl groups of 2.1 were combined into a cycloheptyl ring, thus eliminating the chiral center and leading to the derivative 2.2. This type of compound possessed good activity as M_3 receptor antagonists (Table 1). Replacement of the piperidine ring by quinuclidine also led to potent compounds in both the phenyl (2.3) and thiophene (2.4) derivatives. In order to rapidly improve the properties of this novel cycloheptyl series, effort was primarily focused on the quinuclidine sub-series as N-substitution would lead to single isomers, whereas N-alkylation of the piperidine series would give an isomeric mixture at the quaternized center, which would introduce lengthy and difficult separation steps.

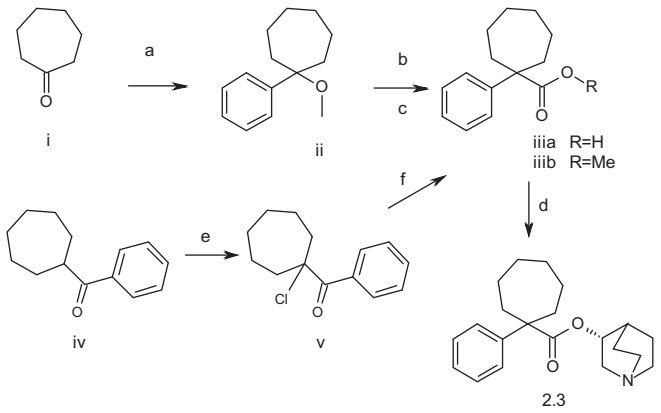
Table 1
In vitro biological data of known muscarinic antagonists compared to the new series

Compound	M_3 pIC_{50}[a]	HM Cl_int[b] (μl/min/mg)	hPPB (%) bound
1.1 Tiotropium bromide	>10.3	<3	48
1.2 Glycopyrrolate	9.9	<3	<30
1.3 Aclidinium bromide	>10.3	>145	No data (not stable in plasma)
2.1	10.5	35	58
2.2	9.6	20	73
2.3	10.2	–	95
2.4	10.1	–	95

[a] Binding estimates measured using recombinant human M_3 receptor ($n \geqslant 2$).
[b] Intrinsic clearance in human liver microsomes.

The synthetic routes used to prepare the types of compounds explored in this work are summarized in Scheme 1.[16] The synthesis started from cycloheptanone (i) which was reacted with phenyl Grignard and the resulting tertiary alcohol was methylated in situ to give (ii). The methoxy group of (ii) could be replaced by a carboxylate under the influence of Na/K (sodium/potassium alloy) followed by quenching the resultant anion with solid CO_2 to give (iiia) and subsequent methylation gives the methyl ester (iiib). This was then reacted with 3-(*R*)-quinuclidinol to afford the compound 2.3. A similar approach could be used for derivatives containing other aromatic rings. An alternative approach started with the ketone (iv) which was chlorinated to give (v). This α-chloroketone rearranged under the influence of silver nitrate followed by heating under basic conditions to afford the acid (iiia) which was then methylated to give ester (iiib). The quinuclidine derivatives, 2.3 and 2.4, (Fig. 2) could be reacted on the tertiary nitrogen with a range of alkylating groups leading to the quaternized derivatives of structural sub-types 2, 3 and 4 shown in Figure 3.

The choice of groups used as N-substituents determines the physical and biological properties of the analogs listed in Table 2. A wide range of groups were used and evaluated to find the optimal substituent to afford a compound which met our desired criteria of high potency at the M_3 receptor together with high intrinsic clearance and high hPPB. We set in vitro criteria of M_3 pIC_{50} >9.5,[13]



Scheme 1. Synthetic routes to the novel series of muscarinic receptor antagonists. Reagents and conditions. (a) PhMgBr, THF, RT, 0.5 h, 66%; then NaH, MeI, THF, reflux, 78 °C h, 55%; (b) Na/K, −10 to 0 °C diethyl ether; solid CO_2 −78 °C to rt, 75%; (c) MeOH, HCl, reflux, 24 h, 80%(d) (3*R*)-quinuclidinol, (2 equiv), NaH (60% disp, 2 equiv), toluene, reflux, 70%; (e) SO_2Cl_2, reflux, 18 h, 95%; (f) $AgNO_3$, water, 75 °C, 4.5 h then K_2CO_3, 90 °C, 1 h, 20%.

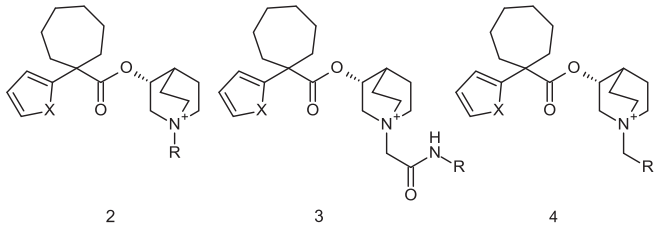


Figure 3. Chemical sub-types made and profiled with data shown in Table 2.

Table 2
In vitro biological data for the novel muscarinic antagonists

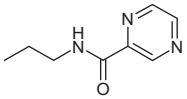
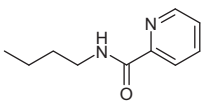
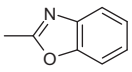
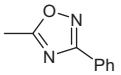
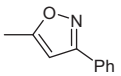
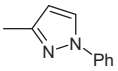
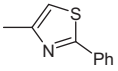
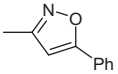
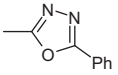
Compound	*R* group	X	M_3 pIC_{50}[a]	GPT pA_2[b]	HM Cl_int[c] (μl/min/mg)	hPPB (%) bound	Log*P*[d]
2.3	–	–	10.2	–	–	95	4.6
2.5	Me–	CH_2	8.6	–	34	73	2.1
2.6	Ph-CH_2–	CH_2	7.4	–	68	–	3.4
2.7	3,4-di-F-Ph-CH_2–	CH_2	7.4	–	55	–	3.4
2.8	3-MeO-Ph-CH_2–	CH_2	8.5	–	–	–	3.3
2.9	Ph-$(CH_2)_2$–	CH_2	10.1	9.5	105	99.3	3.3
2.10	5-Me-Thien-2-yl-$(CH_2)_2$–	CH_2	10.2	9.5	130	99	3.5
2.11	3-Cl-Ph-$(CH_2)_2$–	CH_2	>10.4	9.6	72	99.5	3.9
2.12	4-CN-Ph-$(CH_2)_2$–	CH_2	8.5	–	49	–	2.8
2.13	Ph-O-$(CH_2)_2$–	CH_2	9.8	9.4	106	96	3.4
2.14	Ph-CH_2-O-$(CH_2)_2$–	CH_2	9.9	8.4	120	97	3.7
2.15	Ph-O-$(CH_2)_3$–	CH_2	10.2	9.1	75	91.5	3.4
2.16	4-Pyridyl-$(CH_2)_3$–	CH_2	>10.4	9.9	57	88	2.0
2.17	4-Pyridyl-$(CH_2)_3$–	S	10.3	10.0	55	94	1.6
2.18	2-Me-4-pyridyl-$(CH_2)_3$–	CH_2	>10.5	9.8	135	97	2.4
2.19	6-Me-3-Pyridyl-$(CH_2)_3$–	CH_2	9.8	–	77	95	2.4
2.20	2,6-di-Me-3-pyridyl-$(CH_2)_3$–	CH_2	9.0	–	50	96	2.9
2.21	1-Me-3-Pyrazolyl-$(CH_2)_3$–	CH_2	9.3	–	63	94	1.4
2.22	2-Benzoxazolyl-$(CH_2)_3$–	CH_2	10.3	9.2	82	96	3.3
3.1	H–	CH_2	9.7	10.1	19	82	1.0
3.2	Ph–	CH_2	9.6	–	27	99.4	3.3
3.3	3-F-Ph	CH_2	9.9	8.5	17	99.7	3.8
3.4	-4-Morpholino	CH_2	7.0	–	15	–	1.3
3.5	3-Isoxazolyl	CH_2	>10.2	10.7	150	82	1.2
3.6	5-Me-3-Isoxazolyl	CH_2	9.8	9.6	26	97	1.6
3.7	3-Pyrazolyl	CH_2	10.0	–	37	95	1.4
3.8	1,3,4-Thiazol-2-yl	CH_2	8.1	–	39	94	1.8
3.9	1,2,4-Thiadiazol-5-yl	CH_2	9.4	–	25	–	1.8
3.10	2-Oxazolyl	CH_2	7.9	–	66	85	1.8
3.11	5-^{t}Butyl-3-isoxazolyl	CH_2	8.0	–	–	–	2.8
3.12	2-Pyridyl (R enantiomer)	CH_2	9.8	9.4	55	96	2.7
3.14	2-Pyridyl (S enantiomer)	CH_2	7.5	–	–	–	2.7
3.15	3-Pyridyl	CH_2	9.7	–	40	96	2.5
3.16	4-Pyridyl	CH_2	8.6	–	39	95	2.8
3.17	5-Pyrimidyl	CH_2	9.2	–	32	–	2.0
3.18	4-Pyrimidyl	CH_2	9.8	–	–	89	2.2
3.19	3-Pyridazinyl	CH_2	9.8	10.1	70	90	0.3
3.20	2-Pyrazinyl	CH_2	>9.9	9.9	43	92.5	2.3
3.21	3-F-2-Pyridyl	CH_2	9.6	–	49	94	2.5
3.22	5-Me-2-Pyridyl	CH_2	9.3	–	51	98.1	3.1
3.23	5-F-2-Pyridyl	CH_2	9.2	–	–	97.6	3.0
3.24	4-Me-2-Pyridyl	CH_2	9.1	–	30	98.2	3.1
3.25	6-CF_3-2-Pyridyl	CH_2	8.6	–	–	99.6	3.8
3.26	6-CF_3-3-Pyridyl	CH_2	9.6	–	5	–	3.9
3.27	6-Me-3-Pyridyl	CH_2	9.1	–	–	–	2.9
3.28	2-Me-4-Pyrimidyl	CH_2	9.4	–	–	–	1.0
3.29	6-Me-4-Pyrimidyl	CH_2	9.0	–	26	80	2.7
3.30	6-Me-3-Pyridazinyl	CH_2	9.1	–	16	90	0.8
3.31	6-CF_3-3-Pyridazinyl	CH_2	8.6	–	–	–	1.74
3.32	6-Cl-3-Pyridazinyl	CH_2	9.7	9.7	–	96	1.6
3.33	5-Me-2-Pyrazinyl	CH_2	9.1	–	27	96	2.8
3.34	6-Me-2-Pyrazinyl	CH_2	9.2	–	27	–	2.8
3.35	6-Cl-2-Pyrazinyl	CH_2	10.0	9.3	51	98.5	3.4
3.36		CH_2	8.5	–	74	–	0.8
3.37		CH_2	8.2	–	101	97.4	0.6
4.1		CH_2	10.0	9.8	78	97.7	4.1
4.2		CH_2	9.8	9.4	102	99	4.5
4.3		CH_2	9.6	9.5	63	99	2.8

Table 2 (*continued*)

Compound	*R* group	X	M_3 pIC_{50}[a]	GPT pA_2[b]	HM Cl_int[c] (μl/min/mg)	hPPB (%) bound	Log*P*[d]
4.4		CH_2	9.4	—	103	—	3.2
4.5		CH_2	7.6	—	133	—	4.4
4.6		CH_2	10.1	8.5	79	99.5	3.6
4.7		CH_2	9.5	9.7	97	96.5	2.8

[a] Binding estimates measured using recombinant human M_3 receptors ($n \geqslant 2$).
[b] Antagonist potency at the M_3 receptor measured in guinea pig trachea ($n = 2$).
[c] Intrinsic clearance in human liver microsomes.
[d] Log*P* was calculated using ACD Log*P* software.

Table 3
In vivo pharmacokinetics, efficacy and mechanistic side effect of the novel series of muscarinic antagonists

Compound	IT-PK *T*½ h	BC ED_{80} (μg/kg) 2 or 4 h	BC % inhibition 24 h	BC % inhibition 48 h	Salivation EC_{50} (μg/kg)	TI
Tiotropium	nd	0.3[a]	>80[c]	69[c]	<0.3	⩽3
2.15	10.2	3[a]	31[b]	—	—	—
2.16	19	1	0	—	—	—
2.18	43.7	1	0	—	—	—
2.19	19.2	—	—	—	—	—
2.22	9.3	—	—	—	—	—
3.1	11.4	—	—	—	—	—
3.5	19	1[a]	0	—	>10	—
3.6	26.6	3	56[b]	—	>30	⩾10
3.12	25.6	1[a]	—	84[c]	>30	⩾10
3.19	42.4		—	—	>10	—
3.20	23	1	88[c]	73[c]	>10	⩾10
3.32	56.7	3	89[c]	—	>30	⩾10
4.1	13	—	—	—	>10	—
4.2	17	3	—	0	—	—
4.3	16.5	3	—	0	>30	—
4.6	12.1	—	—	—	—	—
4.7	22	3	—	0	>30	—

[a] Measured ED_{80} dose.
[b] $P < 0.05$.
[c] $P < 0.01$ compared to control group.

guinea pig trachea pA_2 >9,[17] HM Cl_int >50, hPBB >95% bound, and primarily compounds which matched these were considered for progression into a range of in vivo models.

Table 2 summarizes the range of compounds made and some key in vitro data. The parent compound 2.3 was initially N-methylated to afford 2.5 which proved significantly less potent. A range of *N*-benzyl substituted derivatives, 2.6–2.8, were also in general, found to possess lower potency than the parent 2.3. However, derivatives 2.9–2.22 in which an aromatic ring is linked to the quinuclidine by chain lengths of two or more atoms exhibited a range of potencies, with some having pIC_{50} values >10.3 at the M_3 receptor. Some of these compounds also exhibited high human PPB and high HM Cl_int leading to 2.15, 2.16 and 2.18 being taken forward for detailed in vivo evaluation (discussed later).

Another structural sub-type explored is exemplified by compounds 3.1–3.37. In this compound set, the chain linking the aryl group to the quinuclidine contains an amide function. A range of aromatic and heterocyclic rings were evaluated to improve the properties of the final molecules. However, when the aryl was a phenyl ring (e.g., 3.3) the high potency exhibited at the M_3 receptor was not matched by the potency seen in the tissue-based guinea pig trachea assay.[17] When the aromatic ring was a range of more polar heterocycles, similar potency was achieved in both of these assays (3.5–3.35). However, the challenge in the latter structural type was to achieve high hPPB (⩾95%). It became apparent that in this type of compound a Log*P* value of >2.5 was typically required to attain high hPPB. A number of these compounds such as 3.1, 3.2, 3.6, 3.12, 3.19, 3.20 and 3.32 did possess the desired in vitro profile and were selected for evaluation in the in vivo models. Finally, a set of compounds were made in which the terminal aryl was linked to the quinuclidine by an intervening heterocycle, 4.1–4.7, which acted as an amide isostere. Several of these analogs, 4.1, 4.2, 4.3, 4.6 and 4.7 combined the required in vitro properties for progression into the in vivo studies.

The in vivo profiles of compounds which passed our pre-set in vitro criteria are summarized in Table 3. These in vivo guinea pig studies included determining pharmacokinetic half-lives (PK *T*½ (lung)), duration of action (inhibition of bronchoconstriction) and systemic side effects (inhibition of hypersecretion (salivation)). Initially, the PK half-lives (IT-PK *T*½) of the compounds were determined following intratracheal dosing.[18] Compounds with long half-lives were then evaluated in a guinea pig model of bronchoconstriction (BC) to determine their ED_{80} dose and then duration of action at 24 and/or 48 h post-dose.[19] To test duration of action, compounds were administered to the lung at an ED_{80} dose (i.e., dose which produced ~80% inhibition of metha-

choline induced BC at 2 or 4 h).[19] The ED_{80} dose was either measured or predicted from the guinea pig trachea potency data. Compounds which had significant bronchoconstriction beyond 24 h were then evaluated in a guinea pig pilocarpine-induced salivation model to assess the peripheral side effects of the compounds at a 4 h timepoint.[20] Data from the BC and salivation models were used to establish a therapeutic index (TI) for bronchoprotection over salivation effects in the guinea pig. Our objective was to identify compounds with a TI $\geqslant$10 as this would be a significant improvement over tiotropium which has a narrow therapeutic window (TI <3) in these models.

The compounds bearing an alkyl-aryl substituted quinuclidine, 2.13–2.22 exhibited IT-PK half-lives ranging from 9 to 44 h.[18] Several of these compounds were taken into the guinea pig bronchoconstriction (BC) model but demonstrated limited or no efficacy at 24 h post-dose. The compounds from the amide-linked sub-series, 3.1–3.32, also had a range of IT-PK half-lives and a number were taken into the BC model. In general, higher levels of efficacy were observed at 24 h and long duration of action (>48 h) was seen in this class of compound, when the IT-PK *T½* was $\geqslant$23 h. From the third series, 4.1 to 4.7 none of the compounds that were progressed attained IT-PK *T½* >24 h and indeed did not show significant efficacy in the BC model at 48 h. The long duration of action in the BC model for this compound series is thought to be mediated by improved retention of compound in the lung as demonstrated by the IT-PK half-lives >23h. However, the contribution of slow-off rate at the M_3 receptor to the duration action cannot be ruled out and additional receptor kinetic studies would be required to further understand the pharmacology profile of these compounds.

In addition, the systemic side effect profile of some of the more potent compounds with long duration of action were tested using the salivation model.[20] Thus three compounds 3.6, 3.12 and 3.20 demonstrated a therapeutic index (TI) of at least 10-fold when comparing their efficacy in the BC model seen at 4 h and the EC_{50} dose in the salivation model. The improved TI may be attributed to high PPB and metabolic clearance limiting systemic exposure in the salivation model. As the guinea pig bronchoconstriction and salivation models are primarily mediated by M_3 receptors then the inhibitory effects of these compounds are thought to be due to antagonism of the M_3 receptor. In general, compounds from this series are not selective for the M_3 receptor (data not shown) over other muscarinic receptors and so the contribution of other muscarinic receptors to the in vivo findings are unlikely but cannot be excluded at this time.

Compounds 3.12 and 3.20 demonstrated long duration of action and a good TI and so their pharmaceutical properties were further evaluated for development in inhalation delivery devices. The compound chosen to progress to the clinic based on the balance of efficacy, side effect profile and solid state properties (not discussed in this paper) was 3.12.

In summary, we have reported on the design and improvement of a novel series of M_3 receptor antagonists which match a set of criteria including in vitro potency and ADME properties (high PPB and clearance). Key compounds were identified with optimal in vivo properties demonstrating duration of action in the lung and an improved therapeutic index over tiotropium in guinea pigs. Our criteria were selected to improve on the biological profile of existing M_3 receptor antagonists, leading to the selection of compound 3.12, AZD8683, which combined the required biological profile with good pharmaceutical properties and was progressed into the clinic for evaluation as a potential treatment for COPD.

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13. In vitro M_3 radioligand binding assay: The affinity of a compound at the human M_3 receptors was estimated from its ability to compete with specific [^{3}H]NMS binding to membranes from CHO-K1 cells expressing recombinant human M_3 receptors as measured using a SPA assay format. Concentration-effect curves for each compound (0.003−100 nM final concentrations) were constructed using serial dilutions in ½-log unit intervals. For each assay plate, eight replicates were obtained for [^{3}H]NMS binding in the absence of test compound. Non-specific binding of [^{3}H]NMS was determined by replacing test compound with atropine 1 μM. [^{3}H]NMS was used at a concentration of 0.1 nM, which was below the determined dissociation constant (K_d) for [^{3}H]NMS, and about 10% of the radioactivity was specifically bound to the membranes. The assay mixture was incubated at room temperature for at least 16 h before counting to allow [^{3}H]NMS binding with muscarinic receptors to reach equilibrium; binding of [^{3}H]NMS was also reversible. Test compound inhibition was expressed as percent inhibition relative to the specific radioligand binding for the plate.
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16. Ford, R.; Mather, A.; Mete, A.; Bull, R.; Skidmore, E. WO2009138707, 2009, *Chem. Abstr.* 2009, 152, 12539. Synthetic conditions and yields for all new compounds described herein are reported in these publications. New compounds were identified and characterized by their ^{1}H NMR and mass spectra, chiral HPLC and elemental analyses.
17. *In vitro guinea-pig trachea assay:* The antagonist potency of compounds was measured by the ability of compounds to inhibit methacholine-induced airway smooth muscle contraction using a functional, guinea-pig trachea organ bath assay. Dunkin Hartley guinea-pigs were sacrificed by cervical dislocation and the trachea removed. Tracheal rings were prepared and then suspended in 10 mL organ baths containing a modified Krebs solution (containing Indomethacin at 2.8 M final concentration), gassed with 5% CO_2, 95% O_2 at 37 °C and tensioned to 1 g. The tracheal rings were left to equilibrate for 1 h, washed and re-tensioned to 1 g. The rings were 'primed' with methacholine (1 M), washed and left for a further 1 h. Then a cumulative methacholine concentration response curve was constructed (3×10^{-9} M to 3×10^{-5} M). Vehicle or test compound was then added to the baths and allowed to equilibrate for 1 or 4 h. Then, a second extended cumulative concentration response curve was constructed (3×10^{-9} M to 1×10^{-3} M final concentration). Changes in isometric force were recorded and results were expressed as % of maximum response in curve 1 for each individual tissue. A_{50} values were generated using calculated % increase in tension at each compound concentration. The A_{50} values for the vehicle control concentration effect curve and test concentration effect curve were determined and then used to calculate a potency pA2 value at each compound concentration used.
18. For pharmacokinetic studies, compounds were dosed to halothane anaesthetised guinea pigs by intratracheal instillation at 1 μg/kg. For terminal lung collection at 0.25, 0.5, 0.75, 1, 2, 4, 7, 12, 18, 24 h post-dosing, the animals were euthanised by an intravenous overdose of barbiturate (Euthatal 20 mL/kg) and the lungs removed and homogenized with phosphate buffer and added to ice cold methanol. Aliquots of the supernatant were analyzed by mass spectrometry/HPLC methodologies.
19. *In vivo guinea-pig bronchoconstriction:* The efficacy of compounds was measured in vivo by the inhibition of methacholine induced bronchoconstriction in Dunkin–Hartley guinea pigs. Dose solutions of test compounds were prepared in saline (0.9% (w/v) sodium chloride) and delivered to the lung by either

intratracheal or intranasal instillation using a Penn–Century microsprayer (Penn–Century Inc., Wyndmoor, PA, USA) under halothane anaesthesia. At various time points after dosing (2, 4, 24 or 48 h), guinea pigs were terminally anaesthetised with pentobarbitone, surgically prepared, ventilated (at 60 breaths/min at a tidal volume of 2 mL) and lung function measured by changes in respiratory resistance in response to the bronchoconstrictor agent methacholine (10 μg/kg, intravenous bolus), using the Flexivent lung function system (EMMS, UK). After each administration of methacholine, the peak resistance value was obtained and respiratory mechanics returned to baseline in between administrations of methacholine. At the end of the experiments animals were euthanized. Percentage inhibition of bronchoconstriction was calculated for each compound treated group in relation to the appropriate vehicle, time-matched control group.

20. *In vivo salivation:* The ability of compounds to inhibit salivation was measured in vivo by the reduction of pilocarpine induced salivation in guinea-pigs. Compounds or vehicle were administered intranasally in halothane-anaesthetised guinea-pigs. At 4 h after dosing, guinea-pigs were terminally anaesthetised (urethane). Once anaesthesia was established a gauze pad was inserted into the mouth for 5 min to dry the mouth of residual saliva. A pre-weighed pad was then inserted into the mouth for 5 min and weighed for a measurement of baseline saliva production. Pilocarpine (0.6 mg/kg at a dose volume of 2 mL/kg) was then administered subcutaneously. A new pre-weighed pad was inserted into the mouth immediately and replaced at 5 min intervals for 15 min. The difference in weight of the pads pre and post the 5 min sampling period was used to calculate the amount of saliva collected over a 15 min period.