Discovery of Novel and Long Acting Muscarinic Acetylcholine Receptor Antagonists

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Abstract: High throughput screening and subsequent optimization led to the discovery of novel quaternary ammonium salts as highly potent muscarinic acetylcholine receptor antagonists with excellent selectivity. Compounds **8a**, **13a**, and **13b** showed excellent inhibitory activity and long duration of action in bronchoconstriction in vivo models in two species via intranasal or intratracheal administration. The novel inhaled muscarinic receptor antagonists are potentially useful therapeutic agents for the treatment of chronic obstructive pulmonary disease and other bronchoconstriction disorders.

Muscarinic acetylcholine receptors (mAChRs[a]) belong to the family A A2 subfamily of seven-transmembrane (7TM) receptors. Five distinct subtypes, denoted as M_1, M_2, M_3, M_4, and M_5 mAChRs, have been cloned from several species including human and mouse, exhibiting a very high sequence homology across species.[1−3] The five subtypes share a common orthosteric ligand-binding site with an extremely high sequence homology, which explains why it has been difficult historically to identify subtype selective ligands.[3] M_1-M_5 mAChRs are widely distributed in mammalian organs where they mediate important neuronal and autocrine functions.[4,5]

In the mammalian lung, only M_1, M_2, and M_3 mAChRs have been recognized as playing important and functional roles.[6] M_3 is predominantly expressed on airway smooth muscle and mediates smooth muscle contraction.[7] Blockade of M_3 on airway smooth muscle reduces excess airway smooth muscle contraction. M_2 is primarily found on postganglionic nerve termini and functions to limit acetylcholine release from parasympathetic nerves.[8] Blockade of the M_2 function would be expected to enhance bronchoconstriction. M_1 is found in parasympathetic ganglia and facilitates neurotransmission through ganglia, thus enhancing cholinergic reflexes.[9] Blockade of M_1 may help to reduce bronchoconstriction. In chronic obstructive pulmonary disease (COPD) and asthma, inflammatory conditions lead to loss of neuronal inhibitory activity mediated by M_2 on parasympathetic nerves, causing excess acetylcholine reflexes[10] that result in airway hyperreactivity and hyperresponsiveness mediated by increased acetylcholine release and thus excess stimulation of M_3. Therefore, potent mAChR antagonists, particularly directed toward the M_3 subtype, would be useful as therapeutics in these mAChRs-mediated disease states. Inhaled delivery of such antagonists could potentially prevent side effects mediated by peripheral and/or central M_1, M_2, or M_3 antagonism[5] by avoiding substantial systemic exposure.

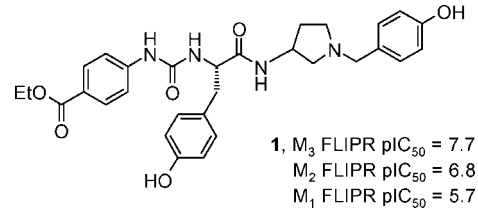

Figure 1. In vitro profile of HTS hit **1**.

High throughput screening (HTS) of the corporate compound collection using a fluorometric imaging plate reader (FLIPR) assay (measuring inhibition of acetylcholine-mediated $[Ca^{2+}]_i$-mobilization in Chinese hamster ovary (CHO) cells stably expressing human recombinant M_3 receptor) led to the identification of pyrrolidine **1**, a mixture of two diastereoisomers, as an antagonist with a pIC_{50} of 7.7 (Figure 1).[11,12] The compound was subsequently tested in M_2 and M_1 FLIPR assays and found to be about 10-fold selective for M_3 over M_2 and 100-fold selective for M_3 over M_1.

To explore this novel HTS hit, an efficient and robust solid-phase synthesis was developed (Scheme 1). Commercially available Boc-protected 3-aminopyrrolidine and piperidine (**2**) were converted to nosyl-protected diamines **3** in two steps. The diamines **3** were loaded onto commercially available 2,6-dimethoxy-4-polystyrenebenzyloxybenzaldehyde resin (DMHB resin)[13] via reductive amination to afford resin-bound amines **4**. Amines **4** were coupled with Fmoc-protected *tert*-butyltyrosine, followed by Fmoc removal, to produce resin-bound amines **5**. Urea formation from intermediates **5**, nosyl-group removal, and subsequent reductive amination afforded resin-bound intermediates **6**. Resin cleavage and simultaneous removal of the *tert*-butyl group of **6** produced the targeted tertiary amines **7** in good yields and purity. Alkylation of tertiary amines **6**, followed by resin cleavage and *tert*-butyl group removal afforded the desired quaternary ammonium salts **8**.

The preferred stereochemistry was determined by preparing all four possible diastereoisomers starting from optically pure 3-aminopyrrolidine and protected tyrosine. As shown in Table 1, **7b**, the (3*S*,3′*S*) diastereoisomer, was the most potent diastereoisomer, 100-fold more potent than the other three diastereoisomers. Compound **7b** also had the best subtype selectivity, about 10-fold selective for M_3 over M_2 and 80-fold selective for M_3 over M_1.

Lead optimization of this series led to the identification of quaternary ammonium salt **8a** with excellent potency in the M_3 FLIPR assay ($pA_2 = 9.9$) and affinity in a M_3 binding assay ($pK_i = 9.5$) (Table 2). In a kinetics studies using the M_3 FLIPR assay, **8a** was found to be a competitive antagonist with a pK_B of 10.1, consistent with its binding affinity. **8a** was also a potent M_2 and M_1 antagonist and maintained the same level of subtype selectivity (10-fold selective for M_3 over M_2 and 100-fold selective for M_3 over M_1) compared with **1** and **7b**. Compound

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[a] Abbreviations: mAChRs, muscarinic acetylcholine receptors; 7TM, seven-transmembrane; COPD, chronic obstructive pulmonary disease; HTS, high throughput screening; CYP450, cytochrome P450; PK, pharmacokinetic; Penh, enhanced pause.

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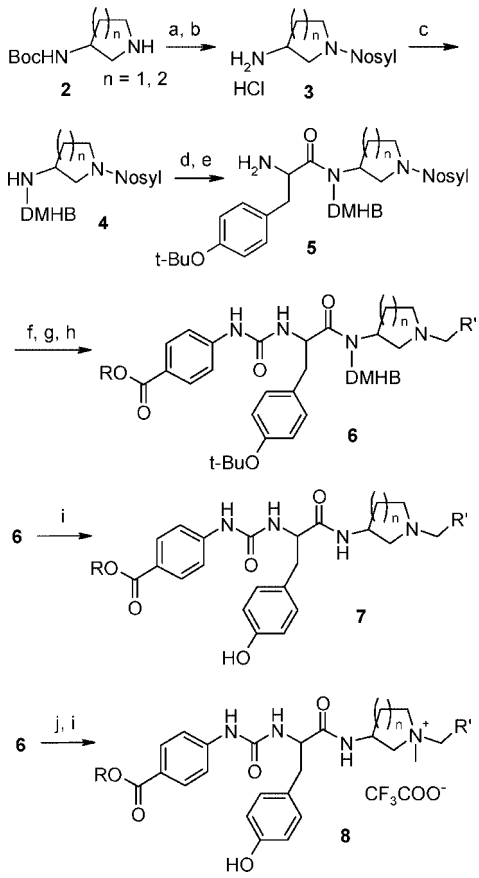
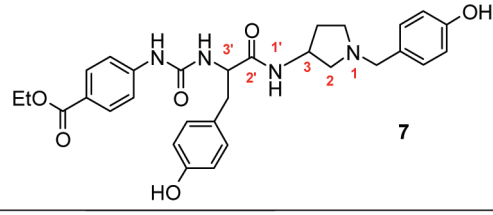
Scheme 1. Synthesis of Pyrrolidine/Piperidine-Based Tertiary Amines and Quaternary Ammonium Salts[a]



[a] (a) 2-nitrobenzenesulfonyl chloride, pyridine, CH_2Cl_2, 0 °C to room temp; (b) 4 M HCl in 1,4-dioxane, MeOH, room temp; (c) 2,6-dimethoxy-4-polystyrenebenzyloxybenzaldehyde (DMHB-resin), $Na(OAc)_3BH$, DIEA, 1% of HOAc in NMP, room temp; (d) Fmoc-Tyr(*t*Bu)-OH, DIC, HOAt, NMP, room temp; (e) 20% of piperidine in NMP, room temp; (f) 4-isocyanatobenzoates, DCE, room temp; or 4-aminobenzoates, 1,1′-(oxomethanediyl)bis-1*H*-pyrrole, DIEA, DCE, room temp; (g) K_2CO_3, PhSH, NMP, room temp; (h) various benzaldehydes, $Na(OAc)_3BH$, 10% of HOAc in NMP, room temp; (i) 50% of TFA in DCE, room temp; (j) MeI, CH_3CN, room temp.

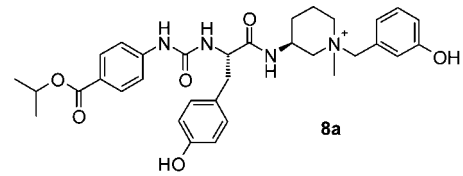
Table 1. Preferred Stereochemistry



Compound	Diastereoisomer	FLIPR pIC_{50}		
		M_3	M_2	M_1
7a	(3*S*, 3'*R*)	5.5	5.3	4.9
7b	(3*S*, 3'*S*)	7.8	6.9	6.0
7c	(3*R*, 3'*R*)	4.7	4.7	4.7
7d	(3*R*, 3'*S*)	5.8	5.8	5.4

8a was profiled in more than 100 in-house 7TM, ion channel, enzyme, transporter, and nuclear hormone receptor selectivity assays and was found to have excellent general selectivity displaying less than a 30,000-fold selectivity margin against only two targets in the panel. The compound was an antagonist of two ligand-gated ion channels, α1 nicotinic acetylcholine receptor and α3 nicotinic acetylcholine receptor, with pIC_{50} values of 6.1. In addition to the excellent general selectivity, **8a** had good developability properties. For example, **8a** was more than 10,000-fold selective for M_3 over five common cytochrome P450 (CYP450) isozymes (pIC_{50} = 5.4 or less) and hERG (binding pIC_{50} = 5.6). In a rat iv/po pharmacokinetic (PK) studies, **8a** had low−moderate clearance (Cl = 15 (mL/min)/kg), short half-life ($T_{1/2}$ = 1.1 h), and no appreciable oral bioavailability—suitable for inhaled delivery. The low oral bioavailability of **8a** was consistent with its extremely low artificial membrane permeability (less than 3 nm/s), which was suitable for targeting membrane-bound receptors such as mAChRs.

Table 2. Profiles of 3-Aminopiperidinium Salt **8a**



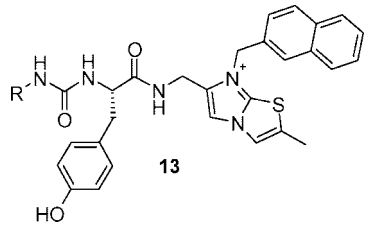
In Vitro Potency	M_3	M_2	M_1
FLIPR pA_2	9.9	9.0	7.8
Binding pK_i	9.5	8.5	7.5
Rat PK Parameters[a]	$T_{1/2}$ = 1.1 h Cl = 15 mL/min/kg V_{dss} = 0.55 L/kg F(%): plasma levels below lower limit of quantitation		
Permeability	< 3 nm/s		
CYP450 pIC_{50}	5.4 (2D6)	5.0 (3A4)	< 5.0 (1A2, 2C19 & 2C9)
hERG binding	pIC_{50} = 5.6		

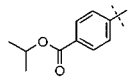
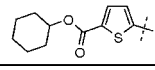
[a] Rat PK parameters are from an iv/po study in Sprague−Dawley rats dosed at 2.2 mg/kg (iv) and 4.8 mg/kg (oral).

Compound **8a** was a mixture of (1*S*,3*S*,3′*S*) and (1*R*,3*S*,3′*S*) diastereoiosmers as a result of forming a new chiral center: the quaternary ammonium nitrogen. It was determined that the ratio of the two diastereoiosmers was about 8:1 with the (1*S*,3*S*,3′*S*) diastereoisomer being the major, using extensive 2D and NOE NMR analysis. Separation of the two diastereoisomers via chromotagraphy was difficult. In the course of further lead optimization of the series to reduce the number of chiral centers, imidazothiazole based quaternary ammonium salts **13a** and **13b** were identified (Table 3). Compound **13a** had outstanding potency with a pA_2 of 10.6 in the M_3 FLIPR assay and showed about 60-fold selective for M_3 over M_2, while the selectivity for M_3 over M_1 was 30-fold. Similarly, **13b** had a pA_2 of 10.9 in the M_3 FLIPR assay and was about 15-fold selective for M_3 over M_2 and 25-fold selective for M_3 over M_1. In addition, **13b** showed excellent general selectivity in CEREP (74% inhibition at 1 μM against NK2 receptor, less than 25% inhibition against other targets in the panel except muscarinic receptors).

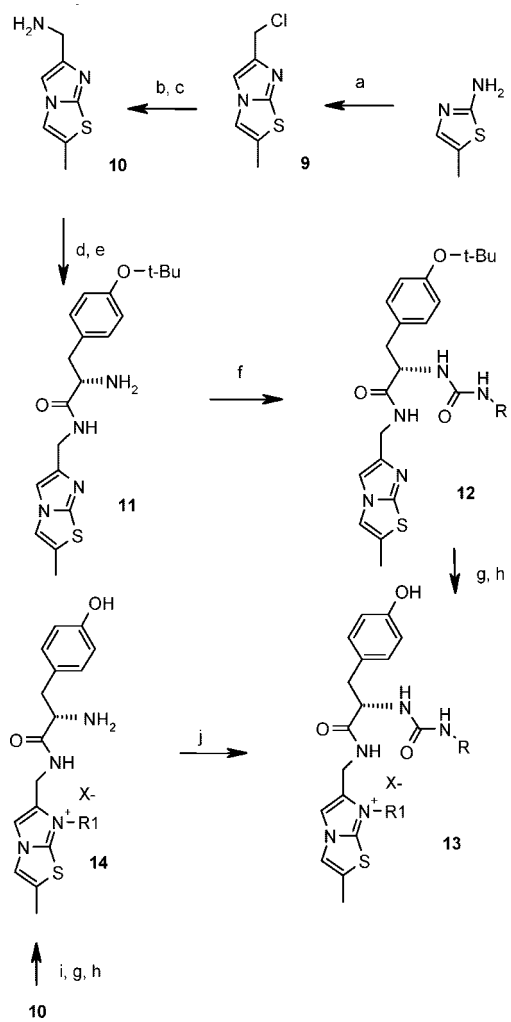
Imidazothiazolium salts **13** were synthesized according to the route outlined in Scheme 2. The imidazothiazole methylamine **10** was prepared by condensation of 2-amino-5-methylthiazole with 1,3-dichloroacetone to give the imidazothiazole methylchloride **9**, followed by displacement of the chloride by azide and reduction to give the primary amine. Amide formation of **10** with *N*-Fmoc-*O*-*t*Bu-Tyr and subsequent Fmoc deprotection

Table 3. Potency of Imidazothiazolium Salts **13a** and **13b**



Compound	R	FLIPR pA2		
		M_3	M_2	M_1
13a		10.6	8.8	9.1
13b		10.9	9.7	9.5

Scheme 2. Synthesis of Imidazothiazole-based Quaternary Ammonium Salts[a]



[a] (a) (i) NaBr, 1,3-dichloroacetone, EtOAc; (ii) AcOH, heat; (b) NaN_3, DMSO; (c) H_2, Pd/C, MeOH; (d) Fmoc-Tyr(*t*Bu)-OH, HATU, Hunig's base, DMF; (e) piperidine, DMF; (f) R−N=C=O, EtOAc, dichlorobenzene; (g) R1−X, MeCN/$CHCl_3$, heat; (h) TFA; (i) Boc-Tyr(*t*Bu)-OH, HATU, Hunig's base, DMF; (j) R−NH_2, 4-NO_2−PhOCOCl, DIEA, CH_2Cl_2.

yielded the primary amine **11**. Elaboration resulting in ureas **12** was affected by reaction with isocyantes or by *p*-nitrophenylchloroformate mediated urea formation from the corresponding arylamine. The former method was used for the preparation of **13a**. The final compounds **13** were then prepared by quaternization with the appropriate halide (in the case of **13a**, 2-bromomethylnaphthalene) followed by cleavage of the *tert*-butyl ether under acidic conditions. Alternatively, the amine **10** could be coupled with *N*-Boc-*O*-*t*Bu-Tyr followed by quaternization and acid mediated deprotection to give **14**. The final compounds **13** were then obtained using the above-mentioned procedures for urea formation. This method was used for the preparation of **13b**. The cyclohexyl 5-amino-2-thiophenecarboxylate used in the preparation of **13b** was prepared from 2-nitrothiophene-5-carboxylic acid ester formation with cyclohexanol and subsequent reduction of the nitro group under standard hydrogenation conditions.

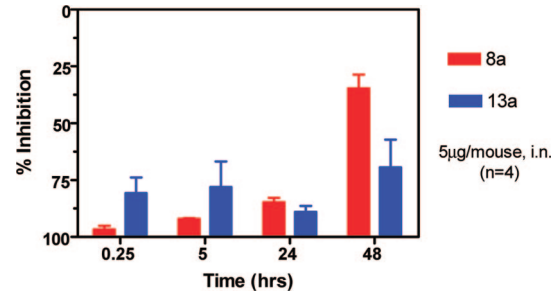


Figure 2. Effect of intranasal administration of **8a** and **13a** on methacholine-induced bronchoconstriction in conscious mice.

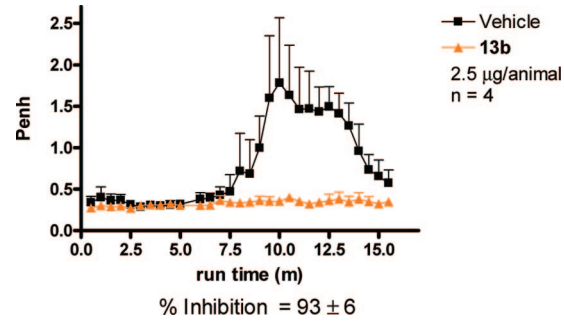


Figure 3. Effect of intratracheal administration of **13b** on acetylcholine-induced bronchoconstriction in conscious guinea pigs at 4 h.

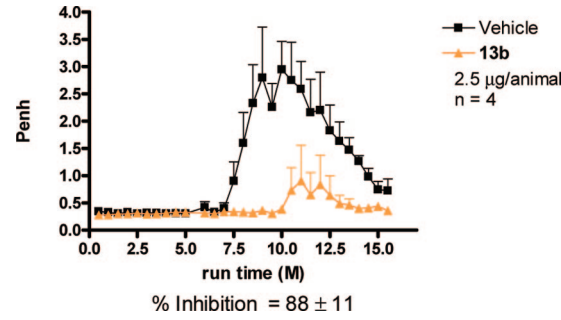


Figure 4. Effect of intratracheal administration of **13b** on acetylcholine-induced bronchoconstriction in conscious guinea pigs at 24 h.

Compounds **8a** and **13a** were evaluated in a methacholine-induced bronchoconstriction model in conscious mice. As shown in Figure 2, intranasal administration of **8a** and **13a** at a single dose (5 μg/animal)[14] significantly inhibited methacholine-induced bronchoconstriction at 15 min and 5 h after dosing. Excellent inhibitory activity (greater than 80%) was maintained at 24 h. Even at 48 h after the single low dose, **8a** and **13a** still

exhibited over 30% and 60% of bronchoprotection, respectively, thus demonstrating excellent in vivo efficacy and duration of action.

In addition, **13b** was evaluated in an acetylcholine-induced bronchoconstriction model in conscious guinea pigs, measuring enhanced pause (Penh), an indicator of bronchoconstriction,[15] using barometric plethysmography (Figures 3 and 4). **13b** showed significant inhibitory activity at 4 h (93% inhibition) and 24 h (88% inhibition) in the guinea pig model following a single low dose (2.5 μg/animal, intratracheal dosing). The excellent in vivo efficacy and duration of action results of **13b** were similar to those of **8a** and **13a** in the in vivo mouse model, demonstrating that these novel muscarinic receptor antagonists are potentially useful therapeutic agents for the treatment of COPD and other bronchoconstriction disorders.

In conclusion, novel, very potent, and highly selective mAChR antagonists were discovered. Quaternary ammonium salts **8a**, **13a**, and **13b** with 10- to 100-fold subtype selectivity for M_3 over M_2 and M_1 significantly inhibited methacholine/acetylcholine-induced bronchoconstriction in two species with excellent duration of action. Full accounts on this novel series including detailed SAR will be the subject of future publications.

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Supporting Information Available: Synthetic procedures and characterization data for all compounds, procedures for M_3, M_2, and M_1 FLIPR and binding assays, in vivo bronchoconstriction mouse and guinea pig models, and specta of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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