## 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<sup>*a*</sup>) belong to the family A A2 subfamily of seven-transmembrane (7TM) receptors. Five distinct subtypes, denoted as M<sub>1</sub>, M<sub>2</sub>, M<sub>3</sub>, M<sub>4</sub>, and M<sub>5</sub> mAChRs, have been cloned from several species including human and mouse, exhibiting a very high sequence homology across species.<sup>1-3</sup> 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.<sup>3</sup> M<sub>1</sub>-M<sub>5</sub> mAChRs are widely distributed in mammalian organs where they mediate important neuronal and autocrine functions.<sup>4,5</sup>

In the mammalian lung, only  $M_1$ ,  $M_2$ , and  $M_3$  mAChRs have been recognized as playing important and functional roles.<sup>6</sup>  $M_3$ is predominantly expressed on airway smooth muscle and mediates smooth muscle contraction.<sup>7</sup> 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.<sup>8</sup> 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.<sup>9</sup> 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<sup>10</sup> that



Figure 1. In vitro profile of HTS hit 1.

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<sup>5</sup> by avoiding substantial systemic exposure.

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<sub>3</sub> receptor) led to the identification of pyrrolidine **1**, a mixture of two diastereoisomers, as an antagonist with a pIC<sub>50</sub> of 7.7 (Figure 1).<sup>11,12</sup> The compound was subsequently tested in M<sub>2</sub> and M<sub>1</sub> FLIPR assays and found to be about 10-fold selective for M<sub>3</sub> over M<sub>2</sub> and 100-fold selective for M<sub>3</sub> over M<sub>1</sub>.

To explore this novel HTS hit, an efficient and robust solidphase 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 2,6dimethoxy-4-polystyrenebenzyloxybenzaldehyde resin (DMHB resin)<sup>13</sup> 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 resinbound 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 (3S,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<sub>3</sub> over M<sub>2</sub> and 80-fold selective for M<sub>3</sub> over M<sub>1</sub>.

Lead optimization of this series led to the identification of quaternary ammonium salt **8a** with excellent potency in the  $M_3$  FLIPR assay (p $A_2 = 9.9$ ) and affinity in a  $M_3$  binding assay (p $K_i = 9.5$ ) (Table 2). In a kinetics studies using the  $M_3$  FLIPR assay, **8a** was found to be a competitive antagonist with a p $K_B$  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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<sup>&</sup>lt;sup>*a*</sup> 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.



<sup>*a*</sup> (a) 2-nitrobenzenesulfonyl chloride, pyridine, CH<sub>2</sub>Cl<sub>2</sub>, 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)<sub>3</sub>BH, 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<sub>2</sub>CO<sub>3</sub>, PhSH, NMP, room temp; (h) various benzaldehydes, Na(OAc)<sub>3</sub>BH, 10% of HOAc in NMP, room temp; (i) 50% of TFA in DCE, room temp; (j) MeI, CH<sub>3</sub>CN, room temp.

Table 1. Preferred Stereochemistry



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

In Vitro Potency	M <sub>3</sub>	$M_2$	$M_1$			
FLIPR pA <sub>2</sub>	9.9	9.0	7.8			
Binding pK <sub>i</sub>	9.5	8.5	7.5			
Rat PK Parametersª	$T_{1/2} = 1.1 h$ Cl = 15 mL/min/kg $V_{dss} = 0.55 L/kg$ F(%): plasma levels below lower limit					
Permeability CYP450 pIC <sub>50</sub>	5.4 (2D6)	< 3 nm/s 5.0 (3A4)	< 5.0 (1A2, 2C19 & 2C9)			
hERG binding	$\mathrm{pIC}_{50} = 5.6$					

<sup>*a*</sup> 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).

two targets in the panel. The compound was an antagonist of two ligand-gated ion channels,  $\alpha 1$  nicotinic acetylcholine receptor and  $\alpha 3$  nicotinic acetylcholine receptor, with pIC<sub>50</sub> 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<sub>3</sub> over five common cytochrome P450 (CYP450) isozymes (pIC<sub>50</sub> = 5.4 or less) and hERG (binding pIC<sub>50</sub> = 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.

Compound 8a was a mixture of (1S,3S,3'S) and (1R,3S,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 (1S,3S,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<sub>2</sub> of 10.6 in the M<sub>3</sub> FLIPR assay and showed about 60-fold selective for M<sub>3</sub> over M<sub>2</sub>, while the selectivity for M<sub>3</sub> over M<sub>1</sub> was 30-fold. Similarly, 13b had a pA<sub>2</sub> of 10.9 in the M3 FLIPR assay and was about 15-fold selective for M3 over M<sub>2</sub> and 25-fold selective for M<sub>3</sub> over M<sub>1</sub>. In addition, **13b** showed excellent general selectivity in CEREP (74% inhibition at 1  $\mu$ M 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 methyl-chloride **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	$ m FLIPR \ pA_2$		
		$M_3$	$\mathbf{M}_2$	$\mathbf{M}_{1}$
13a	Jol Ly	10.6	8.8	9.1
13b	$\bigcirc$ $\sim$	10.9	9.7	9.5

**Scheme 2.** Synthesis of Imidazothiazole-based Quaternary Ammonium Salts<sup>a</sup>



<sup>*a*</sup> (a) (i) NaBr, 1,3-dichloroacetone, EtOAc; (ii) AcOH, heat; (b) NaN<sub>3</sub>, DMSO; (c) H<sub>2</sub>, 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<sub>3</sub>, heat; (h) TFA; (i) Boc-Tyr(*t*Bu)-OH, HATU, Hunig's base, DMF; (j)  $R-NH_2$ , 4-NO<sub>2</sub>-PhOCOCl, DIEA, CH<sub>2</sub>Cl<sub>2</sub>.

yielded the primary amine **11**. Elaboration resulting in ureas **12** was affected by reaction with isocyantes or by *p*-nitrophen-



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 acetylcholineinduced bronchoconstriction in conscious guinea pigs at 4 h.



Figure 4. Effect of intratracheal administration of 13b on acetylcholineinduced bronchoconstriction in conscious guinea pigs at 24 h.

ylchloroformate 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.

Compounds **8a** and **13a** were evaluated in a methacholineinduced bronchoconstriction model in conscious mice. As shown in Figure 2, intranasal administration of **8a** and **13a** at a single dose (5  $\mu$ g/animal)<sup>14</sup> significantly inhibited methacholineinduced 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 Letters

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,<sup>15</sup> 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  $\mu$ g/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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