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## Development of a more highly selective M<sub>1</sub> antagonist from the continued optimization of the MLPCN Probe ML012

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### ABSTRACT

This Letter describes the continued optimization of an MLPCN probe molecule (ML012) through an iterative parallel synthesis approach. After exploring extensive modifications throughout the parent structure, we arrived at a more highly M<sub>1</sub>-selective antagonist, compound **131** (VU0415248). Muscarinic subtype selectivity across all five human and rat receptors for **131**, along with rat selectivity for the lead compound (ML012), is presented.

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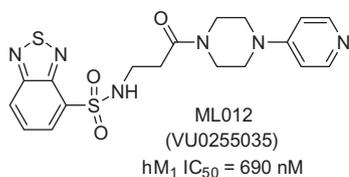
Acetylcholine (ACh) is a critical neurotransmitter with diverse functions both within the central nervous system (CNS) and in peripheral signaling pathways.<sup>1–4</sup> ACh carries out its functions by interacting with two very distinct groups of receptors; a set of ligand-gated ion channels—the nicotinic acetylcholine receptors (nAChRs)—and a set of family A, G protein-coupled receptors (GPCRs)—the muscarinic acetylcholine receptors (mAChRs). The muscarinic cohort of acetylcholine receptors are further divided into five subtypes (M<sub>1–5</sub>).<sup>5</sup> These five subtypes can be further classified into two subsets based on their G protein-coupling partners, with the M<sub>1, 3, 5</sub> receptors preferentially coupling to G<sub>q/11</sub> which stimulates PLC and calcium mobilization, and with the M<sub>2,4</sub> receptors preferentially coupling to G<sub>i/o</sub> to inhibit adenylate cyclase (AC), thereby decreasing cAMP production.<sup>4</sup> These mAChRs are widely expressed throughout the body with varying degrees of expression levels for specific subtypes based on the particular site or organ.<sup>6</sup> As a result, mAChRs play significant roles in a wide range of physiological functions such as memory and attention, motor control, nociception, regulation of sleep-wake cycles, cardiovascular function, secretory

functions, and mediators of inflammation, renal and GI function, among many others.<sup>6,7</sup>

While it is believed that the M<sub>1,4,5</sub> receptors are the most important for CNS targets, what remains unknown are the specific functions that are regulated by each individual receptor subtype.<sup>7</sup> This is a direct result of the highly conserved orthosteric binding site for the endogenous ligand (ACh), which is shared across all five mAChRs; this high conservation has severely confounded the development of muscarinic ligands with a high degree of selectivity for a single subtype.<sup>8</sup> A lack of subtype selectivity, however, has not precluded the use of mAChR-acting pharmaceuticals for a wide range of indications but many of the undesirable side effects for these non-selective medications can be attributed to activity at the other mAChRs (often M<sub>2</sub> and M<sub>3</sub>), limiting their clinical utility. Additionally, the limited selectivity displayed by earlier mAChR ligands has complicated the interpretation of both animal model studies as well as human clinical trials with respect to the specific mAChR subtype being responsible for the given desired (or undesired) result. For example, xanomeline, a reported M<sub>1</sub>- and M<sub>4</sub>-selective agonist, showed clinical efficacy in Phase II trials for Alzheimer's disease and schizophrenia,<sup>9,10</sup> but also has nearly equivalent agonist activity at M<sub>3</sub>.<sup>8</sup> Even in the absence of off-target mAChR activity, the debate

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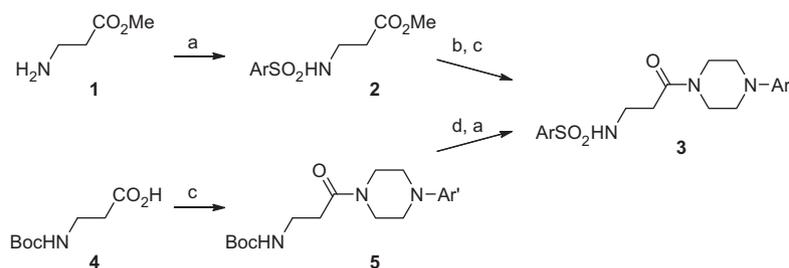
**Figure 1.** The MLPCN probe molecule ML012.

still remains as to whether a single mAChR subtype ( $M_1$  or  $M_4$ ) is responsible for the very encouraging positive outcomes, in these trials.

Single and double mAChR knock out (KO) mice for each of the muscarinic receptor subtypes have yielded a great deal of insight into the specific roles played by each receptor, but these results must always be viewed with the caveat that compensatory changes may have occurred during the development of the mutant KO mice.<sup>8</sup> More highly selective mAChR ligands would allow for a better understanding of the individual roles for each of the five mAChRs.

There are a number of ways to potentially engender mAChR subtype selectivity among synthetic ligands. First, the more classical approach, by simultaneously binding to the orthosteric site while at the same time extending beyond this highly conserved region of the receptor and into adjacent areas which may be less structurally conserved among the other mAChRs (vide infra).<sup>11</sup> Second, by binding to a completely distinct region of the mAChR (an allosteric site), which is distinct from the orthosteric binding site and which might also show less amino acid similarity between the five mAChRs. This allosteric approach has been highly successful for a number of the individual mAChRs:  $M_1$ ,<sup>12–14</sup>  $M_4$ ,<sup>15</sup> and  $M_5$ .<sup>16</sup>

In a previous publication from our laboratories, we described the highly selective  $M_1$  antagonist ML012 (VU0255035, Fig. 1).<sup>11</sup> Although acting at the orthosteric site on all five mAChRs, ML012 achieved  $M_1$  selectivity in the range of 45- to 159-fold over  $M_{2–5}$ . Additionally, ML012 reduced pilocarpine-induced seizures in rodent models at doses that did not induce deficits in contextual freezing, demonstrating that selective  $M_1$  antagonists may have greater therapeutic potential than the non-selective muscarinic antagonists presently available. Given the potential for  $M_1$  antagonists in such



**Scheme 1.** Reagents: (a)  $\text{ArSO}_2\text{Cl}$ ,  $\text{NEt}_3$ , DCM; (b)  $\text{NaOH}/\text{H}_2\text{O}/\text{THF}$ ; (c) amine, EDCI, HOBt,  $\text{NEt}_3$ ; (d) 4 M HCl in dioxane.

**Table 1**

Structures and activities of  $M_1$  antagonist analogs **6a–o**

| Compd     | Ar' | hM1 $\text{IC}_{50}$ ( $\mu\text{M}$ ) <sup>a</sup> |           |  |     |           |  |      |
|-----------|-----|---|-----------|--|-----|-----------|--|------|
| ML012     |     | 0.69  | <b>6e</b> |  | 4.5 | <b>6j</b> |  | 0.32 |
| <b>6a</b> |     | 2   | <b>6f</b> |  | >10 | <b>6k</b> |  | >10  |
| <b>6b</b> |     | >10   | <b>6g</b> |  | >10 | <b>6l</b> |  | >10  |
| <b>6c</b> |     | >10   | <b>6h</b> |  | >10 | <b>6m</b> |  | >10  |
| <b>6d</b> |     | >10   | <b>6i</b> |  | >10 | <b>6n</b> |  | >10  |
|           |     |   |           |  |     | <b>6o</b> |  | >10  |

<sup>a</sup> Average of at least three independent determinations. Standard deviations ranging from 9–19% of the value reported.

**Table 2**  
Structures and activities of M<sub>1</sub> antagonist analogs **13a–r**

Ar' = -Br      **13a-r**      Ar' =

| Compd      | Ar' | hM <sub>1</sub> IC <sub>50</sub> <sup>a</sup> (μM) | Compd      | Ar' | hM <sub>1</sub> IC <sub>50</sub> <sup>a</sup> (μM) |
|------------|-----|--|------------|-----|--|
| <b>13a</b> |     | 1.1  | <b>13b</b> |     | 2.6  |
| <b>13c</b> |     | >10  | <b>13d</b> |     | >10  |
| <b>13e</b> |     | >10  | <b>13f</b> |     | >10  |
| <b>13g</b> |     | 4.8  | <b>13h</b> |     | >10  |
| <b>13i</b> |     | >10  | <b>13j</b> |     | >10  |
| <b>13k</b> |     | 2.1  | <b>13l</b> |     | 0.4  |
| <b>13m</b> |     | >10  | <b>13n</b> |     | >10  |
| <b>13o</b> |     | >10  | <b>13p</b> |     | >10  |
| <b>13q</b> |     | >10  | <b>13r</b> |     | >10  |

<sup>a</sup> Average of at least three independent determinations. Standard deviations ranging from 5–23% of the value reported.

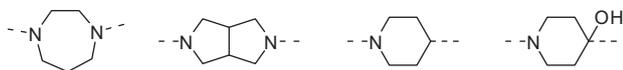
indications as Parkinson's disease, movement disorders, and Fragile X syndrome,<sup>17,18</sup> we have continued the optimization of ML012 and now detail those efforts in this Letter.

With ML012 as our starting point, we utilized an iterative parallel synthesis approach to explore structure–activity–relationships (SAR) at both ends of the molecule by employing the synthesis routes depicted in Scheme 1, while simultaneously making more speculative modifications within single compounds. To explore SAR around the Eastern portion, we started with β-alanine methyl ester **1**. Sulfonation to provide **2**, was then followed by saponification and amide coupling to provide amides (**3**) appearing in Table 1 containing numerous variations in aryl groups (Ar'). Alternatively, starting from the *N*-Boc protected β-alanine **4**, peptide coupling with a limited number of optimized Eastern portions provided compound **5**, which was then treated with HCl subsequent to sulfonation with a variety of sulfonyl chlorides to provide the sulfonamides (**3**) appearing in Table 2, which now display a wide range of Ar groups on the Western side.

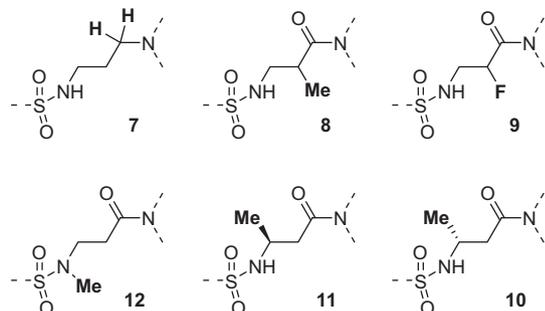
Table 1 shows the SAR developed around a variety of aryl groups attached to the piperazine. With ML012 as our benchmark (hM<sub>1</sub> IC<sub>50</sub> = 0.69 μM),<sup>19</sup> it can be seen that additional substitutions

on the 4-pyridyl ring only resulted in decreased antagonist activity (**6a–c**), as measured by their ability to inhibit the calcium mobilization induced by ACh. Very few analogs around a 3-pyridyl moiety (structures and data not shown) were prepared, but these compounds only served to confirm exploratory SAR demonstrating that a 3-pyridyl was the least tolerated.<sup>11</sup> Alternatively, substitutions around a 2-pyridyl scaffold were more fruitful. As a starting point, the unsubstituted 2-pyridyl analog **6d** possessed inhibitory activity roughly 14-fold less than ML012, but a modest improvement resulted from chlorination para to the nitrogen, as in compound **6e**. Halogenation at the *ortho*-position caused a decrease in activity (**6f** and **6g**), as did methylation at either the *para*- or the *ortho*-position (**6h** and **6i**). The largest improvement in activity resulted from the substitution of a bromine at the *meta*-position shown in compound **6j**. This M<sub>1</sub> antagonist displayed activity (IC<sub>50</sub> = 0.32 μM) 2-fold greater than ML012 and provided an alternative Eastern aryl group for additional rounds of SAR. Replacing the terminal pyridine with a variety of heterocycles met with similarly unforfeiting steep SAR (**6k–o**).

While not conducted in an exhaustive fashion, various replacements for the piperazine heterocycle were explored. However,



**Figure 2.** Piperazine replacements that did not maintain potency (hM<sub>1</sub> IC<sub>50</sub> >10 μM).



**Figure 3.** A collection of central linker modifications.

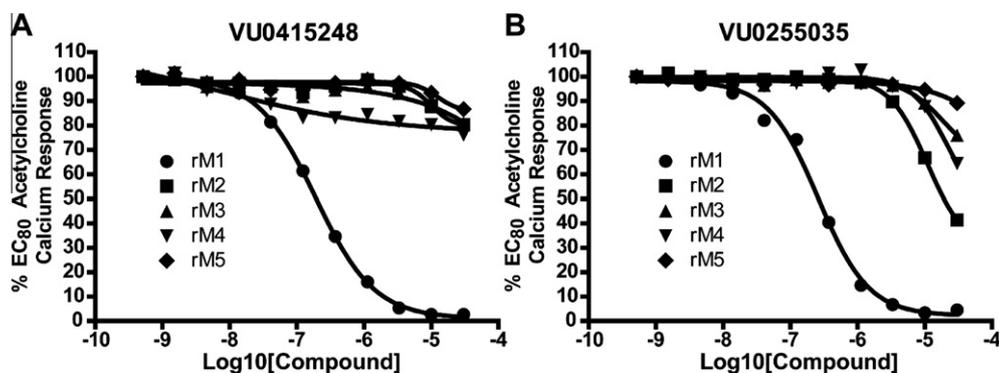
none of the more commonly employed surrogates shown in Fig. 2 provided antagonists with appreciable activity.

The β-alanine central linker was also explored with respect to the various modifications shown in Fig. 3. The reduced amide analog of ML012, compound **7** (Fig. 3), while still an antagonist, showed a greater than 14-fold loss in potency (hM<sub>1</sub> IC<sub>50</sub> >10 μM, but only 17% ACh activity remaining at the highest concentration tested (30 μM)). Methylation at the α-position of compound **13b** (See Table 2) provided a pair of inactive enantiomers (**8**). However, fluorination at this location did not remove all activity, but instead provided a racemic mixture with somewhat reduced inhibitory efficacy (**9**, 25% activity remaining at 30 μM) relative to the non-fluorinated analog, **13b** (hM<sub>1</sub> IC<sub>50</sub> = 4.5 μM). It should not be overlooked that compound **9** was a mixture of two enantiomers while compound **13b** was a single achiral molecule. Given the roughly 2-fold decrease in potency for **9**, there remains the possibility that one enantiomer of **9** had about the same potency as its non-fluorinated progenitor **13b**, while the other enantiomer of **9** could be inactive. Regardless, substitution by either of these groups was not obviously beneficial with respect to potency. The individual β-substituted enantiomers, **10** and **11**, were prepared from commercially available starting materials, and while both enantiomers were less potent than their unsubstituted analog **13b**, there was some evidence for enantiospecific activity between the two compounds. The (*R*)-enantiomer (**10**) was completely inactive at the highest

concentration tested (30 μM), while the (*S*)-enantiomer (**11**) demonstrated about 30% inhibition of the acetylcholine response at the same concentration. The free N–H of the sulfonamide was important for M<sub>1</sub> antagonist activity since N-methylation of ML012 to provide compound **12** resulted in a greater than 14-fold loss in potency (hM<sub>1</sub> IC<sub>50</sub> >10 μM).

Now focusing on the Western sulfonamide region, and employing our two best Eastern aryl groups, SAR was similarly restrictive (Table 2—Ar' = 4-pyridyl (analogs on the right) and 5-bromo-2-pyridyl (analogs on the left)). The slight change associated with replacing the benzothiadiazole with a benzoxadiazole in compounds **13a** and **13b** resulted in a 3-fold and 4-fold decrease in activity relative to their sulfur analogs **6j** and ML012, respectively. Substitution on either of these bicyclic heterocycles at the 5- or 7-positions resulted in weakly active or inactive compounds (**13c–g**). Moving the sulfonamide from the 4-position to the 5-position on the benzothiadiazole was also not tolerated, as can be seen with compound **13h**. Although it was not surprising that removing all heteroatoms from the sulfonamide aryl ring would result in a complete loss of activity (**13i**), it was somewhat surprising that the benzothiazole sulfonamide **13j** was also inactive. This was particularly unexpected considering the discovery that a single nitrogen atom reintroduced into the naphthalene sulfonamide, to arrive at the quinoline sulfonamides **13k** and **13l**, could provide compounds with activity on par with, or better than, their benzothiadiazole and benzoxadiazole analogs, and highlights the importance of this nitrogen's positioning. This nitrogen effect was very specific for mimicking the interactions presumably occurring with the N-1 nitrogen of the benzothiadiazole. Hence, the introduction of a nitrogen atom on the other side of the naphthalene, to mimic the N-3 nitrogen of the benzothiadiazole, resulted in a very weakly active compound (quinoline **13m**). Further attempts to present heteroatoms in this approximate location generally met with no success (**13n–r**).

Ultimately, **6j** and **13l** represented the only compounds with improved antagonist activity relative to ML012. The quinoline sulfonamide **13l** was deemed more attractive due to more pronounced improvements in its calculated and physical properties when compared to either ML012 or **6j**. Although not a major difference, **13l** possesses the lowest molecular weight among the three compounds (MW ML012 = 432, **6j** = 511 and **13l** = 425). Both **6j** and **13l** displayed more moderate *cLogP* values (1.88 and 1.04, respectively) relative to ML012 (*cLogP* = 0.37). The quinoline sulfonamide compound **13l** no longer contained the electron deficient benzothiadiazole found in both ML012 and **6j**, which subsequently reduced its polar surface area to 96 Å<sup>2</sup> and favorably compared to the other two compounds which both had a polar surface area of 108 Å<sup>2</sup>. With these properties in mind, compound **13l**



**Figure 4.** Compound **13l** (VU0415248) and ML012 (VU0255035) selectively antagonize the M<sub>1</sub> mAChR relative to the M<sub>2-5</sub> mAChRs. CRCs were performed in the presence of an EC<sub>80</sub> concentration of ACh for each receptor in a calcium mobilization assay. Data were normalized to the maximum response to 30 μM ACh and are presented as a percentage of the EC<sub>80</sub> ACh response. A, selectivity for **13l** across the rat mAChRs (rM<sub>1</sub> EC<sub>50</sub> = 0.18 μM). B, selectivity for ML012 across the rat mAChRs (rM<sub>1</sub> EC<sub>50</sub> = 0.24 μM).

was profiled against both the rat and human M<sub>1–5</sub> receptors in a functional assay for calcium mobilization. Figure 4A shows these results for the rat (r) M<sub>1–5</sub> receptors. Despite being presumably an orthosteric ligand, based on its close structural relationship to ML012, **131** shows very high selectivity for the M<sub>1</sub> receptor over the M<sub>2–5</sub> subtypes with a similar level of selectivity observed across the human subtypes (hM<sub>2–5</sub> IC<sub>50</sub>s >30 μM, data not shown). For comparison, ML012 was profiled against the complete set of rat M<sub>1–5</sub> receptors and these results appear in Figure 4B (data not available at the time of our initial publication on ML012). Gratifyingly **131** represented an improvement over ML012 with respect to muscarinic subtype selectivity at the rat receptor, and to a lesser extent a small improvement with respect to potency (rM<sub>1</sub> IC<sub>50</sub> = 0.18 μM for **131** and rM<sub>1</sub> IC<sub>50</sub> = 0.24 μM for ML012). Additionally, the quinoline nitrogen at its specific location indicated the presence of an important interaction between compound **131** and the M<sub>1</sub> receptor which does not appear to be present in the M<sub>2–5</sub> receptor subtypes. We next evaluated **131** for its potential as an improved in vivo tool, but were disappointed in the compound's poor pharmacokinetic properties. Consistent with in vitro DMPK predictions of high clearance, **131** displayed an IV clearance value of 77 mL/min/kg, roughly hepatic blood flow, and a shorter half life (t<sub>1/2</sub> = 40 min) relative to ML012 in male Sprague–Dawley rats.<sup>11</sup> In tandem, **131** was found to display very low estimated bioavailability and undetectable CNS exposure in the rat (% F <10 and undetectable CNS concentrations when dosed orally at 10 mg/kg).

In summary, we have expanded the SAR surrounding ML012, resulting in the development of **131** (VU0415248) as a more potent in vitro tool with improved selectivity for the M<sub>1</sub> receptor. On-going work which builds on the SAR described herein may provide compounds with both improved selectivity and PK parameters. This work will be reported in due course. ML012 is an MLPCN probe and freely available upon request.<sup>20</sup>

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19. Although the IC<sub>50</sub> for ML012 has been previously reported as 132 nM (Ref. 11), the inherent variability associated with functional assays supports that the most recent IC<sub>50</sub> = 690 nM for ML012 is equally valid and can serve as a baseline comparator between the two papers.
20. For information on the MLPCN and information on how to request probe compounds, such as ML012, see: <http://mli.nih.gov/mli/mlpcn/>.