Discovery of the First Highly M5-Preferring Muscarinic Acetylcholine Receptor Ligand, an M5 Positive Allosteric Modulator Derived from a Series of 5-Trifluoromethoxy *N*-Benzyl Isatins

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Received March 6, 2009

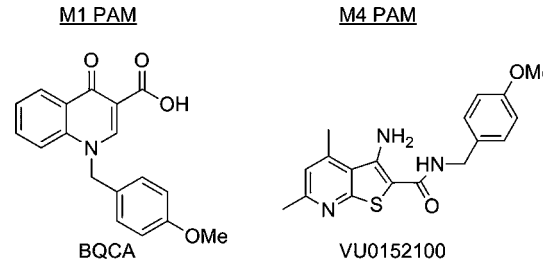
Abstract: This report describes the discovery and initial characterization of the first positive allosteric modulator of muscarinic acetylcholine receptor subtype 5 (mAChR5 or M5). Functional HTS, identified VU0119498, which displayed micromolar potencies for potentiation of acetylcholine at M1, M3, and M5 receptors in cell-based Ca^{2+} mobilization assays. Subsequent optimization led to the discovery of VU0238429, which possessed an EC_{50} of approximately 1.16 μM at M5 with >30-fold selectivity versus M1 and M3, with no M2 or M4 potentiator activity.

The five cloned muscarinic acetylcholine receptor subtypes (mAChR1-5 or M1-5[a]) are known to play highly important and diverse roles in many basic physiological processes including gastrointestinal, cardiovascular, motor, attention, learning, memory, pain, sleep, and other functions.[1−3] Correspondingly, muscarinic agonists and antagonists targeting one or more subtypes have been used preclinically and clinically for research and treatment of a wide range of pathologies.[3,4] Given the high sequence homology of the mAChRs across subtypes and particularly within the orthosteric acetylcholine (ACh) binding site, discovery of truly subtype-selective compounds has proven historically difficult. Because of the paucity of selective compounds, a detailed understanding of the precise roles of each subtype in neurobiology and in various central nervous system (CNS) disorders has thus remained challenging.[3,4]

Recently, a number of novel highly subtype-selective allosteric ligands for M1 and M4 have emerged from functional cell-based screening efforts.[5,6] However, no ligands have been reported to date as being highly M5-preferring or selective. Relative to the other mAChRs, little is known about M5, which is expressed at very low levels in the CNS and peripheral tissues.[2−4]

Interestingly, data from studies using mAChR5 knockout (M5-KO) mice suggest that M5 is the sole mediator of ACh-induced vasodilation in the cerebral vasculature and thereby may have therapeutic relevance for cerebrovascular diseases or acute ischemic stroke.[7,8] M5-KO mice have also been found to exhibit deficits in long-term potentiation (LTP) at the hippocampal mossy fiber-CA3 synapse and show deficits in hippocampal-dependent behavioral cognitive tests.[8]

Chart 1. M1 and M4 Positive Allosteric Modulators



In light of these and related findings, activation of M5 has been suggested as a potential target for treatment of Alzheimer's disease, perhaps in combination with M1 activation.[9] Consistent with the putative postsynaptic localization of M5 in the ventral tegmental area (VTA), other M5-KO data suggest this subtype plays an important role in regulation of mesolimbic dopamine transmission.[3,9] Indeed, M5-KO mice exhibit decreased reward responses to morphine, decreased self-administration of cocaine, and less pronounced drug withdrawal symptoms, suggesting that M5 antagonists or negative modulators may have therapeutic value in treatment of illicit drug addiction.[9−11] Further pharmacological exploration of these and related hypotheses greatly depends on the discovery of novel M5-preferring or selective small molecule tools.

We recently reported on a diverse group of novel mAChR positive allosteric modulators (PAMs), some of which were highly selective for M1 or M4 (Chart 1, Figure 1).[6,12,13] Other mAChR PAMs displayed mixed subtype-selectivity profiles.[12] These compounds enhanced receptor activation in response to ACh in Ca^{2+} mobilization assays and did not compete with the orthosteric antagonist [^{3}H]-*N*-methylscopolamine (NMS) in radioligand binding experiments performed with M1-CHO membranes, which strongly suggests an allosteric mechanism of modulation.[12] Interestingly, the *p*-bromobenzyl-substituted isatin screening hit VU0119498 (**113**) was found to exhibit allosteric potentiator activity at the natively G_q-coupled M1, M3, and M5 receptors with comparable potency and efficacy in Ca^{2+} mobilization assays but lacked potentiator effects at the natively G_i-coupled M2 and M4 receptors in cells cotransfected with

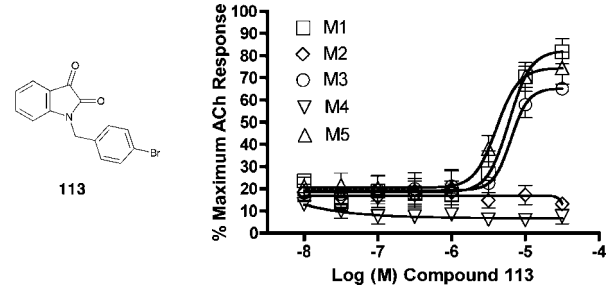


Figure 1. HTS hit **113** potentiates responses in M1, M3, and M5-expressing CHO cells. In the presence of a fixed submaximal concentration of ACh (~EC_{20}) in Ca^{2+}, assays performed with CHO cells stably expressing each of the five mAChR subtypes (M2 and M4 cotransfected with G_{qi5}). M1 EC_{50} = 6.04 μM, %max = 82.4; M3 EC_{50} = 6.38 μM, %max = 65.5; M5 EC_{50} = 4.08 μM, %max = 74.4; inactive at M2 and M4 (data shown are means ± SEM, $N \geq 3$).

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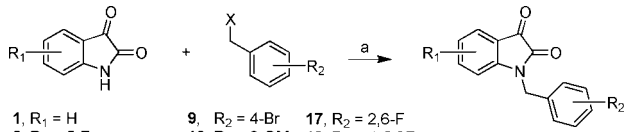
[a] Abbreviations: mAChR, muscarinic acetylcholine receptor; ACh, acetylcholine; CNS, central nervous system; KO, knockout; LTP, long-term potentiation; VTA, ventral tegmental area; PAM, positive allosteric modulator; NMS, *N*-methylscopolamine; AUC, area under curve; CRC, concentration−response curve.

10.1021/jm900286j CCC: $40.75
Published on Web 05/13/2009

Scheme 1. Library Synthesis of VU0119498 Analogues[a]



1, R_1 = H
2, R_1 = 5-F
3, R_1 = 7-F
4, R_1 = 5-Cl
5, R_1 = 7-Cl
6, R_1 = 5-Me
7, R_1 = 4,7-Cl
8, R_1 = 5-OCF_3

9, R_2 = 4-Br
10, R_2 = 3-OMe
11, R_2 = 4-OMe
12, R_2 = 2-CF_3
13, R_2 = 4-CF_3
14, R_2 = 2-Cl
15, R_2 = 2-F
16, R_2 = 2,4-F
17, R_2 = 2,6-F
18, R_2 = 4-OCF_3
19, R_2 = 2-Me
20, R_2 = 4-Br, 2-F

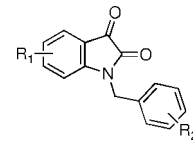
21-112

[a] Reagents and conditions: (a) K_2CO_3, KI, ACN, microwave 160°C, 10 min, 20−95%.

chimeric G_{qi5} protein, which facilitates coupling to the PLC/Ca^{2+} pathway (Figure 1).[12] In similar assays, a high fixed 30 μM concentration of **113** induced a 14-fold leftward shift of a full ACh concentration response curve (CRC), signifying a robust potentiation effect. This hit was therefore chosen as an attractive starting point for subsequent optimization efforts directed toward increasing M5 selectivity and potency while simultaneously decreasing M1 and M3 activity.

To rapidly explore SAR around **113**, we generated an analogue library in matrix format wherein each of eight commercially available isatins (**1**−**8**) was reacted with approximately 12 benzyl halides (**9**−**20**) under standard microwave alkylation conditions (Scheme 1).[14] Resulting analogues (**21**−**112**) were then screened in a single point format at a 30 μM final concentration in Ca^{2+} mobilization assays using M5 and M1 cells receiving a fixed submaximal concentration (~EC_{20}) of ACh (Figure 2, see full SAR table in Supporting Information).[14] This method efficiently triaged analogues displaying high M1 vs M5 or M5 vs M1 preference. Interestingly, some analogues displayed robust potentiation effects at M5 (i.e., elevation of ACh ~EC_{20} to >50−60% of maximum ACh response) with absent or weak potentiation at M1, thus exhibiting strong preference for M5 versus M1 activity.

Table 1. Structures and Activities at M1 and M5 of Analogues of Compound **113**



compd	R_1	R_2	M1 EC_{50} (μM)	M5 EC_{50} (μM)
22	H	4-OMe	>10	>30
25	7-F	4-Br	3.99	1.93
33	7-Cl	4-OMe	5.38	3.96
38	4,7-Cl	3-OMe	>30	7.15
41	5-OCF_3	3-OMe	>30	1.70
42	**5-OCF_3**	**4-OMe**	**>30**	**1.16**
44	H	4-CF_3	5.88	3.19
46	5-F	2-CF_3	6.21	4.09
47	5-F	4-CF_3	8.55	7.13
49	7-F	2-CF_3	>30	>30
50	7-F	4-CF_3	5.18	3.26
56	7-Cl	4-CF_3	3.20	2.11
63	5-OCF_3	4-CF_3	7.09	1.85
64	5-OCF_3	2-Cl	>30	4.07
103	7-Cl	2-F, 4-Br	7.14	4.66
110	5-OCF_3	4-OCF_3	>30	1.86
111	5-OCF_3	2-Me	>30	3.28
112	5-OCF_3	2-F, 4-Br	>30	>10

In terms of maximal potentiation efficacy, SAR from the initial 30 μM screen of the entire library suggested that 5-OCF_3 substitution of the isatin core (R_1) was generally favored for increased M5 versus M1 activity, while halogen substitutions at the 7-position conferred a more dual M1/M5 activity (Table 1).[14] Indeed, all 5-OCF_3 substituted compounds chosen from the initial screen possessed 1−5 μM potencies at M5 and >30 μM potencies at M1, with the exception of compunds **63** and **112**, which nonetheless possessed greater M5 versus M1 activities (Table 1).[14] Numerous benzylic substitutions (R_2) were tolerated for M1 and M5 activity, depending on the isatin core (R_1).[14] Methoxybenzyl was generally favored for M5 versus

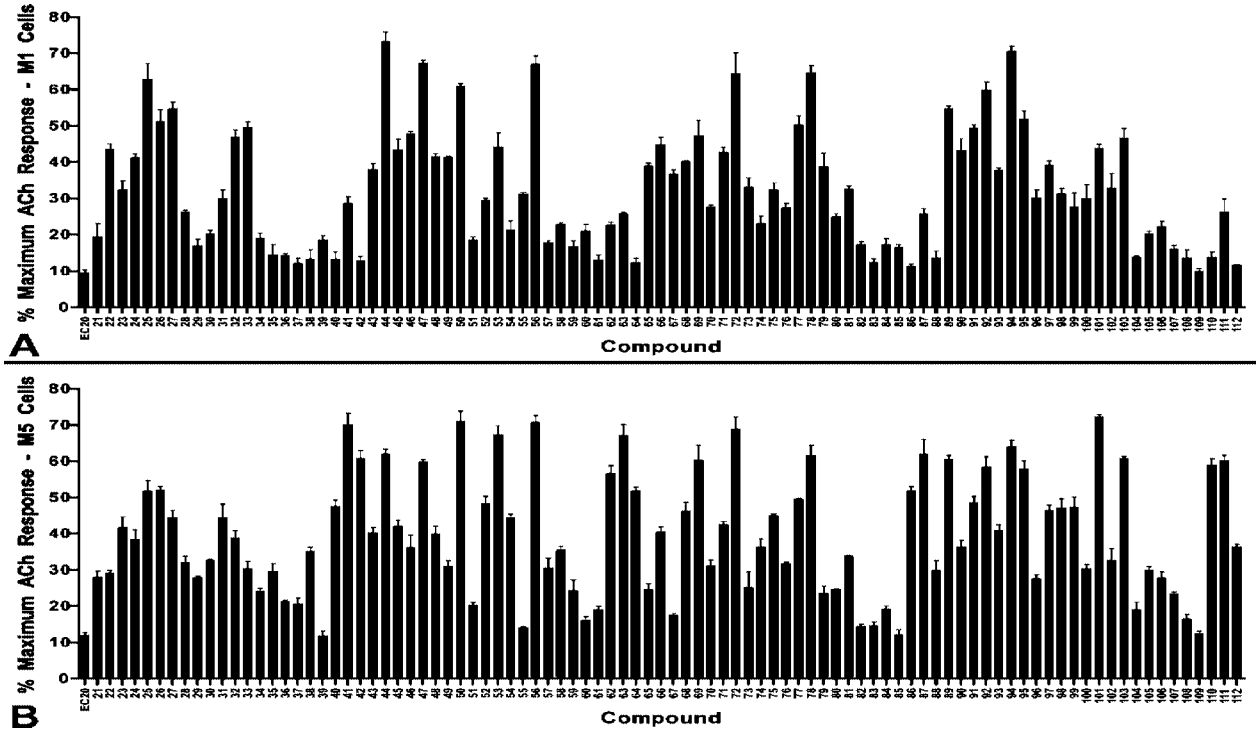


Figure 2. Screen of analogue library (compounds **21**−**112**) at 30 μM for potentiation of submaximal acetylcholine (~EC_{20}) in M1 (A) and M5 (B) cells by Ca^{2+} assay (data shown are means ± SEM, $N \geq 3$).[14]

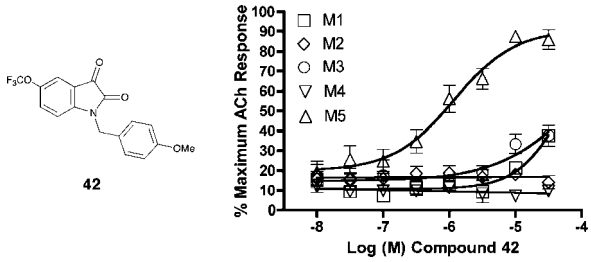


Figure 3. Concentration response curve for **42** performed in the presence of a fixed ACh ~EC_{20} in Ca^{2+} assays performed with CHO cells stably expressing each of the five mAChR subtypes (M2 and M4 cotransfected with G_{qi5}). M1 EC_{50} = >30 μM; M3 EC_{50} = >30 μM; M5 EC_{50} = 1.16 μM, %max = 91; inactive at M2 and M4 (data shown are means ± SEM, $N \geq 3$).

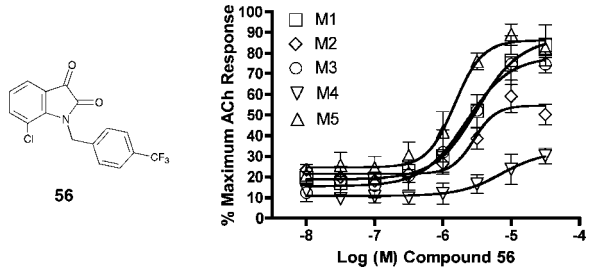


Figure 4. Concentration response curve for **56** performed in the presence of a fixed ACh ~EC_{20} in Ca^{2+} assays performed with CHO cells stably expressing each of the five mAChR subtypes (M2 and M4 cotransfected with G_{qi5}). M1 EC_{50} = 3.20 μM, %max = 87; M3 EC_{50} = 2.22 μM, %max = 78; M5 EC_{50} = 2.11 μM, %max = 86; M2 EC_{50} = 2.84 μM, %max = 55; M4 EC_{50} = >10 μM (data shown are means ± SEM, N ≥ 3).

M1 activity, whereas trifluoromethylbenzyl was generally favored for dual M1/M5 potentiation activity (Table 1).[14] Most other simple congeners, including those with various methyl substitutions at R_1 or R_2, displayed weak or no potentiation activity at either receptor.[14] In light of the high M5 versus M1 potentiation preference displayed by analogue **42** (1.16 μM M5 EC_{50} and >30 μM M1 EC_{50}) in these assays, the full subtype-selectivity profile of this compound was obtained in similar Ca^{2+} assays using M2, M3, and M4 cells. Likewise, full subtype-selectivity determination was also performed with compound **56**, which was the most potent dual M1/M5 potentiator in this initial evaluation at both receptors (2.11 μM M5 EC_{50} and 3.20 μM M1 EC_{50}).

Remarkably, **42** (VU0238429) had similarly poor potency at M3 as at M1, with potentiation of the ACh ~EC_{20} beginning to emerge only in the double-digit micromolar range (Figure 3). Gratifyingly, this compound maintained its lack of potentiation activity at M2 and M4 in cells cotransfected with G_{qi5} at up to 30 μM (Figure 3). Given these data, the approximately 1.16 μM M5 potency of compound **42** provides >30× selectivity for M5 versus the other four subtypes, thus representing the first highly M5-preferring muscarinic receptor ligand ever reported. Conversely, compound **56** (VU0238441) was found to retain the M1/M3/M5 potentiation profile of the parent HTS hit **113** and interestingly possessed modest potentiation at M2 and M4 at high concentrations (Figure 4).

To further evaluate the ability of **42** to potentiate ACh-induced activation of mAChR5, we obtained full CRCs for ACh in M5 cells in the presence of either vehicle or a fixed (30 μM) concentration of **42** (Figure 5, top right). In these assays, compound **42** induced a robust 14-fold left-shift of the ACh CRC, which was virtually identical to that found with the parent HTS

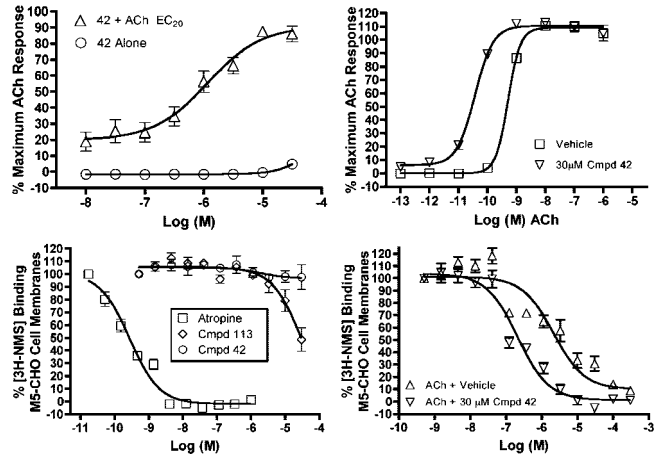


Figure 5. Compound **42** CRC alone or in the presence of fixed ACh EC_{20} demonstrating an absence of intrinsic agonist activity at M5 by Ca^{2+} assay (top left), effect of fixed 30 μM **42** on an ACh CRC demonstrating a 14-fold increase in ACh potency at M5 by Ca^{2+} assay (top right), lack of competition with [^{3}H-NMS] binding to M5 by **42** versus atropine control (K_i = 0.21 nM, bottom left), and the effect of fixed 30 μM **42** on ACh competition with [^{3}H-NMS] binding demonstrating a 10-fold increase in ACh affinity for M5 (ACh + vehicle K_i = 1.61 μM; ACh + **42** K_i = 159 nM; data shown are means ± SEM, $N \geq 3$).

hit **113** when evaluated in a similar assay at M1, as previously reported.[12] Moreover, **42** lacked intrinsic agonist activity at M5 when tested up to 30 μM in the absence of ACh (Figure 5, top left).

We also evaluated HTS hit **113** and the highly M5-preferring analogue **42** in competition-binding experiments with M5-CHO membrane preparations using the orthosteric radioligand [^{3}H]-*N*-methylscopolamine ([^{3}H]-NMS), a potent pan-mAChR antagonist. At up to 30 μM, **42** lacked competition with [^{3}H]-NMS, consistent with an allosteric mode of binding to M5 (Figure 5, bottom left). By contrast, **113** exhibited approximately 50% inhibition of radioligand binding at 30 μM (Figure 5, bottom left). Compound **42** also caused a 10-fold increase in ACh affinity for M5 at 30 μM, indicating that its mechanism of potentiation is in part due to enhancement of ACh binding to M5.

We then evaluated pharmacokinetics and brain penetration for the M5 PAM to evaluate its ability to serve as an in vivo probe. Unfortunately, **42** is characterized by poor systemic absorption after intraperitoneal administration with maximum concentration in plasma (161.7 ng/mL) being achieved within 1 h.[14] However, it is slowly eliminated from systemic circulation and has elimination half-life of 4.7 h.[14] Although quickly taken up in the brain, it exhibits poor brain penetration with AUC_{brain}/AUC_{plasma} value of 0.25.[14] Future lead optimization will focus on improving brain penetration.

Although a growing number of allosteric modulators have been recently documented for mAChR1 and mAChR4, to date no selective mAChR5 ligand has been reported. Compound **42**, a congener of **113** identified from an analogue library synthesis approach, serves as the first highly mAChR5-preferring small molecule. Despite the relatively limited scope of this analogue library in terms of structural modification, we found clear SAR associated with this novel mAChR chemotype, with 5-OCF_3 modification of the isatin core and 3- or 4- position OMe substitutions of the benzyl ring conferring strong M5-preference versus M1−M4. In contrast, virtually all modifications conferring increased M1 activity simultaneously increased M5 activity.[14] As exemplified in the case of **56**, 7-Cl substitution of the

isatin core carried similar and increased M1/M5 potency and efficacy but also caused degeneration into a pan-muscarinic potentiator when profiled at all five subtypes. Further chemical modification of compound **42** to improve M5 PAM potency and characterization in electrophysiology and in vivo experiments are underway and will be reported in due course.

Acknowledgment. We thank the NIH and NIMH for support of our programs. We specifically acknowledge the support of the Alzheimer's Association (IIRG-07-57131) and NIMH (1RO1MH082867-01 and 5R01MH073673). T.M.B. acknowledges an ITTD (T90-DA022873) predoctoral training grant.

Supporting Information Available: Experimental procedures and analytical data for compounds **113**, **42**, and **56** are provided as kinetics study. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM900286J