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Probe Report

Title: Development of the First Highly Selective mAChR 5 (M_5) Positive Allosteric Modulator (PAM)

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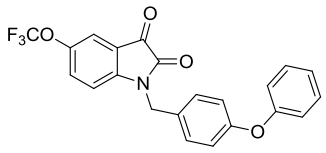
PubChem Summary Bioassay Identifier (AID): 2416

Abstract

This probe (ML172, CID 44602489), which possesses improved muscarinic selectivity over our previous M_5 positive allosteric modulator (PAM) probe (ML 129, CID 42633508), can be used for *in vitro* molecular pharmacology and electrophysiology experiments to study the role of selective M_5 receptor activation. This probe possesses unprecedented selectivity versus M_1, M_2, M_3 and M_4. The probe (ML172, CID 44602489) is not readily CNS penetrant, and so would need to be administered i.c.v. to study the role of central M_5 activation *in vivo*.

Probe Structure & Characteristics

1-(4-phenoxybenzyl)-5-(trifluoromethoxy)indoline-2,3-dione, MW = 413.4, logP = 5.8, tPSA = 107.0 Å^2



ML172

CID/ML#	Target Name	IC_{50}/EC_{50} (nM) [SID, AID]	Anti-target Name(s)	IC_{50}/EC_{50} (μM) [SID, AID]	Fold Selective	Secondary Assay(s) Name: IC_{50}/EC_{50} (nM) [SID, AID]§
CID 44602489 / ML172	M_5	1900 [SID 87352032, AID 2416]	M_1, M_2, M_3, M_4	> 30 μM [SID 87352032, AID 2204]	> 30	ACh Fold-Shift (5-fold) [SID 87352032, AID 2186]

Recommendations for Scientific Use of the Probe

This probe (ML172, CID 44602489), which possesses improved muscarinic selectivity over our previous M_5 PAM probe (ML 129, CID 42633508), can be used for *in vitro* molecular pharmacology and electrophysiology experiments to study the role of selective M_5 receptor activation. This probe possesses unprecedented selectivity versus M_1, M_2, M_3 and M_4. The probe (ML172, CID 44602489) is not readily CNS penetrant, and so would need to be administered i.c.v. to study the role of central M_5 activation *in vivo*.

1 Introduction

Specific AIM: To identify small molecule positive allosteric modulators (PAMs) and/or allosteric agonists of the M_5 muscarinic acetylcholine receptor that are cell permeable, possess low- to sub-micromolar potency and show greater than 10-fold selectivity over the other mAChRs (M_1, M_2, M_3 and M_4) employing a functional HTS approach. Out of this effort primarily aimed at M_1, which afforded a highly selective M_1 allosteric agonist (CID 25010775) and two highly selective M_1 PAM probes (CID 44251556, and CID 44475955), we also identified and optimized the first selective M_5 PAM (CID 42633508), and now report the next-generation M_5 PAM described herein. Another MLSCN screening effort identified a highly selective M_4 PAM (CID 864492) and a subsequent highly selective M_4 PAM endowed with improved human potency (ML 173, CID 45142486); thus, a toolkit containing highly selective, allosteric mAChR ligands are available from the MLPCN to study individual mAChR function.

Significance: The five cloned muscarinic acetylcholine receptor subtypes (mAChR1-5 or M_{1-5}) are known to play highly important and diverse roles in many basic physiological processes.[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] Based on 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. Due to the scarcity 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] A number of novel highly subtype-selective allosteric ligands for M_1 and M_4 have emerged from functional cell-based screening efforts.[5,6] Only recently have the first M_5 selective ligands been disclosed from these laboratories.[13] Relative to the other mAChRs, little is known about M_5, which is expressed at very low levels in the CNS and peripheral tissues.[2-4]

Rationale: Data from studies using mAChR5 knock-out (M_5-KO) mice suggest that M_5 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] M_5-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] In light of these and related findings, activation of M_5 has been suggested as a potential target for treatment of Alzheimer's disease, perhaps in combination with M_1 activation.[9] Consistent with the putative post-synaptic localization of M_5 in the ventral tegmental area (VTA), other M_5-KO data suggest this subtype plays an important role in regulation of mesolimbic dopamine transmission.[3,9] Indeed, M_5-KO mice exhibit decreased reward responses to morphine, decreased self-administration of cocaine, and less pronounced drug withdrawal symptoms, suggesting that M_5 antagonists or negative allosteric modulators may have therapeutic value in the treatment of illicit drug addiction.[9-11] Further pharmacological exploration of these and related hypotheses greatly depends on the discovery of novel M_5-preferring or selective small molecule tools.

2 Materials and Methods

2.1 Assays

PubChem Primary Assay Description: Chinese hamster ovary (CHO K1) cells stably expressing rat (r)M_1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured according to their recommendations. CHO cells stably expressing human (h) M_2, hM_3, and hM_5 were generously provided by A. Levey (Emory University, Atlanta, GA); rM_4 cDNA provided by T. I. Bonner (National Institutes of Health, Bethesda, MD) was used to stably transfect CHO-K1 cells purchased from the ATCC using Lipofectamine 2000. To make stable hM_2 and rM_4 cell lines for use in calcium mobilization assays, cell lines were

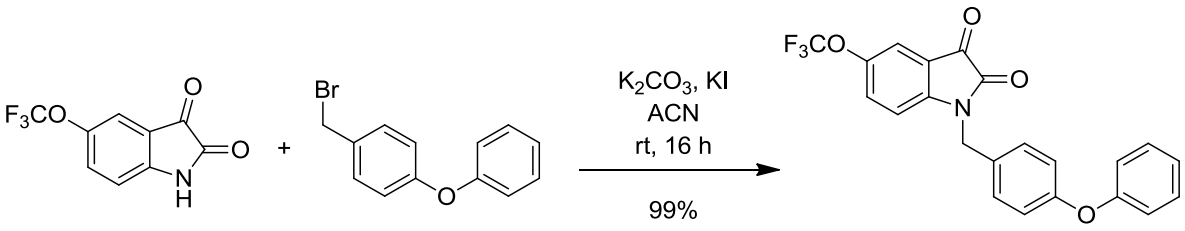
cotransfected with a chimeric G protein (G_{qi5}) using Lipofectamine 2000. rM_2, hM_3, and hM_5 cells were grown in Ham's F-12 medium containing 10% heat-inactivated fetal bovine serum, 2 mM GlutaMax I, 20 mM HEPES, and 50 µg/mL G418 sulfate. hM_2-G_{qi5} cells were grown in the same medium supplemented with 500 µg/mL hygromycin B. Stable rM_4 cells were grown in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 2 mM GlutaMax I, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 20 mM HEPES, and 400 µg/mL G418 sulfate; rM_4-G_{qi5} cells were grown in the same medium supplemented with 500 µg/mL hygromycin B. CHO cells stably expressing rM_1, hM_3, or hM_5 were plated at a seeding density of 50,000 cells/100 µL/well. CHO cells stably coexpressing hM_2/G_{qi5} and rM_4/G_{qi5} were plated at a seeding density of 60,000 cells/100 µL/well. For calcium mobilization, cells were incubated in antibiotic-free medium overnight at 37 °C/5% CO_2 and assayed the next day.

Calcium Mobilization Assay: Cells were loaded with calcium indicator dye [2 µM Fluo-4 acetoxymethyl ester (50 µL/well) prepared as a stock in DMSO and mixed in a 1:1 ratio with 10% Pluronic acid F-127 in assay buffer (1xHanks' balanced salt solution supplemented with 20 mM HEPES and 2.5 mM probenecid, pH 7.4)] for 45 min at 37 °C. Dye was removed and replaced with the appropriate volume of assay buffer. All compounds were serially diluted in assay buffer for a final 2x stock in 0.6% DMSO. This stock was then added to the assay plate for a final DMSO concentration of 0.3%. Acetylcholine (EC_{20} concentration or full dose-response curve) was prepared at a 10x stock solution in assay buffer before addition to assay plates. Calcium mobilization was measured at 25 °C using a FLEXstation II (Molecular Devices, Sunnyvale, CA). Cells were preincubated with test compound (or vehicle) for 1.5 min before the addition of the agonist, acetylcholine (ACh). Cells were then stimulated for 50 s with a submaximal concentration (EC_{20}) or a full dose-response curve of acetylcholine. The signal amplitude was first normalized to baseline and then as a percentage of the maximal response to acetylcholine.

List of PubChem bioassay identifiers generated for this screening project (AID 626, AID 2186, AID 2192, AID 2194, AID 2198, AID 2204, AID 2206, AID 2416 and AID 2665.

2.2 Probe Chemical Characterization

Synthetic procedure (large scale) and spectral data for ML172 (CID 44602489).



ML 172
(CID 44602489)

Probe compound ML172 (CID 44602489) was prepared according to the above scheme and provided the following characterization data: LCMS (>98%) *m/z* = 414 [M+H$^+$] (1.65 min retention, 214 nm),^{1}H NMR (>95%) (400 MHz, $CDCl_3$) δ =7.52 (s, 1H), 7.36 (m, 6H), 7.15 (t, *J* = 7.2 Hz, 1H), 7.10 (t, *J* = 5.2 Hz, 2H), 6.86 (d, *J* = 8.8 Hz, 2H), 4.93 (s, 2H). ^{13}C-NMR (100MHz, d_6 - DMSO) δ 182.2, 157.8, 157.6, 156.4, 145.3, 130.9, 129.8, 129.0, 128.3, 123.7, 121.6, 120.4, 119.2, 119.0, 118.5, 118.1, 111.9, 43.7, HRMS (Q-TOF): *m/z* calc for $C_{22}H_{15}NO_4F_3$ [M + H]: 414.0953, found 414.0959.

Solubility. Solubility in PBS (at pH = 7.4) was determined to be less than 0.10 µM although alternative formulations could potentially improve this number. For example, ML172 shows good solubility in DMSO (>100 µM), but this is not a universally acceptable vehicle.

Stability. Stability (at room temperature = 23 °C) for ML172 in PBS (no antioxidants or other protectorants and DMSO concentration below 0.1%) is shown in the table below. After 48 hours, the percent of parent compound remaining was 134%, but the assay variability over the course of the experiment ranged from a low of 84% (at 24 hours) to a high of 167% (at 30 minutes). It is important to point out that ML172 showed very poor LCMS sensitivity and exhibited high levels of non-specific binding. Given these caveats, it is difficult to draw any firm conclusions with respect to ML172's stability from this experiment.

	Percent Remaining (%)						
Compound	**0 Min**	**15 Min**	**30 Min**	**1 Hour**	**2 Hour**	**24 Hour**	**48 Hour**
ML172	100	164	167	154	114	84	134

Reactivity. As assessed through a glutathione (GSH) trapping experiment in phosphate buffered saline (with a substrate concentration of typically 5-50 µM and a GSH concentration of 5 mM, at t = 60 minutes) ML172 was found to not form any detectable GSH adducts.[21]

Compounds added to the SMR collection (MLS#s): 003108019 (ML172, CID 44602489, 500 mg), 003108020, 003108021, 003108022, 003108023, 003108024.

2.3 Probe Preparation

1-(4-phenoxybenzyl)-5-(trifluoromethoxy)indoline-2,3-dione (ML172, CID 44602489): To a flask containing acetonitrile (20 mL) was added 5-trifluoromethoxyisatin (750 mg, 3.245 mmol), potassium carbonate (897 mg, 6.490 mmol), 4-phenoxybenzyl bromide (897 mg, 3.407 mmol), and potassium iodide (54 mg, 0.325 mmol). The reaction was stirred overnight at room temperature and then judged complete by LCMS analysis. The solution was partitioned

between CH_2Cl_2 and H_2O, the organics were dried over $MgSO_4$, and then the solution was filtered and concentrated *in vacuo* to afford the crude product. Purification by silica gel plug (1:3 EtOAc:Hexanes fixed solvent gradient) afforded the title compound as a bright red-orange sticky solid (1.340 g, 3.242 mmol, 99%)

3 Results

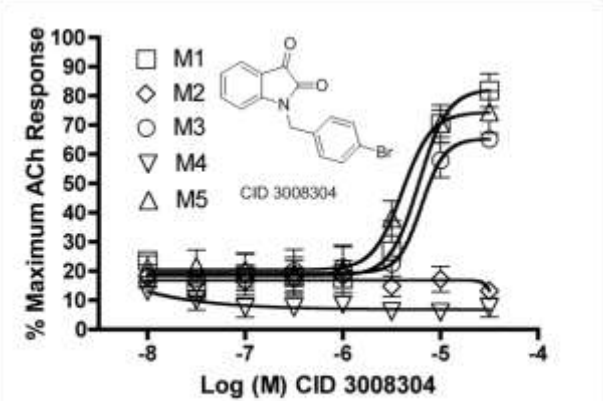
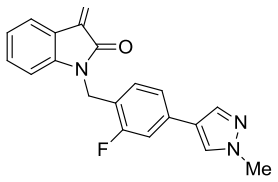


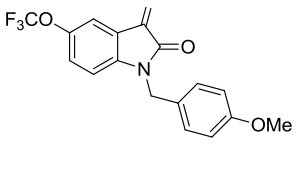
Figure 1. CRCs at M1-M5 for HTS lead CID 3008304. CID 3008304 is a pan G_q-M1, M3, M5 PAM

Center Summary of Screen: This screen was performed in the pilot phase, the MLSCN, when the MLSMR compound collection at Vanderbilt only contained 65K compounds. From the primary M_1 screen of 65K compounds, ~12 putative M_1 PAMs were identified giving an average Z' score of 0.70±0.09. The confirmation screen (singles at 10 µM) produced two lead compounds, one of which was recently developed into a structurally novel M_1 PAM (CID 44475955). The other, CID 3008304, represented a unique, and never before seen, pharmacological profile (**Figure 1**) in that it was a PAM of all the G_q-coupled mAChRs (M_1 EC_{50} = 6.1 µM, M_3 EC_{50} = 6.4 µM and M_5 EC_{50} = 4.1 µM), but devoid of activity at the $G_{i/o}$-coupled M_2 and M_4.[12] This led us to predict it would be possible to dial-in or dial-out different mAChR sub-types and potentially develop an M_1 selective PAM, an M_3 selective PAM and/or an M_5 selective PAM from this non-selective lead through chemical optimization.[13a] As predicted, CID 3008304 served as the common starting point for the development of the two highly selective probe molecules shown in **Figure 2**: the M_1 PAM (CID 44251556) and the M_5 PAM (CID 42633508, EC_{50} = 1.1 µM, 91% ACh Max). Although very selective for M_5, CID 42633508 did retain a detectable amount of activity at the M_1 and M_3 receptors at concentrations of 30 µM and above. Therefore we chose to explore the possibility of further optimizing CID 42633508 as described next.

Figure 2. Two MLPCN probe molecules derived from CID 3008304

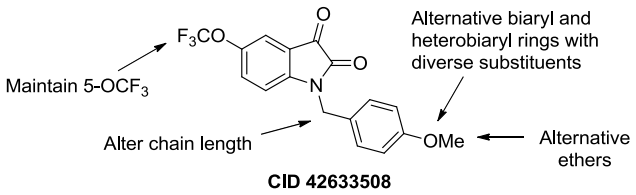


CID 44251556
(M_1 PAM)

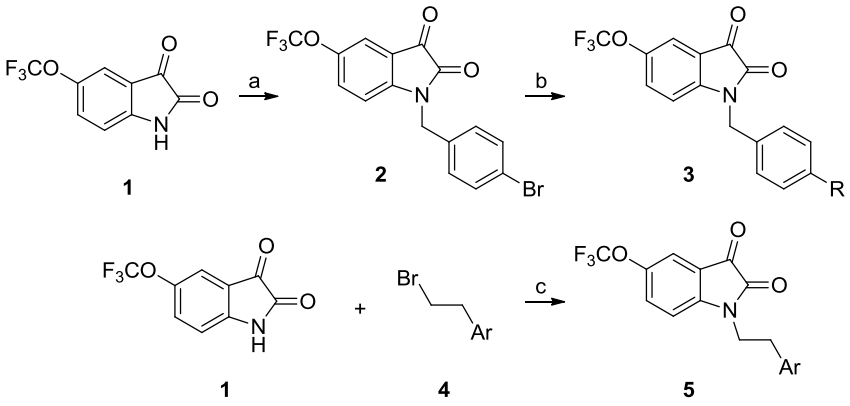


CID 42633508
(M_5 PAM)

Figure 3. Optimization strategy for CID 42633508, a highly M_5-preferring PAM.



Probe Optimization Strategy: For the continued optimization of CID 42633508, we chose the strategy depicted in **Figure 3**, and as SAR with allosteric ligands is often shallow, we employed an iterative parallel synthesis approach. From the development of our initial M_5 probe, we were confident that the 5-OCF_3 group was essential for M_5-preferring activity and selectivity, so this moiety was universally retained. Libraries were prepared according to **Scheme 1**, wherein the commercially available indoline-2,3-dione **1** was alkylated with 4-bromobenzylbromide to deliver intermediate **2**. An eleven-membered Suzuki library was then prepared to explore the



Scheme 1. Reagents and conditions: (a) *p*-bromobenzylbromide, K_2CO_3, KI, CAN, rt, 16 h (99%); (b) R-$B(OH)_2$, Pd(*t*-$Bu_3P)_2$, Cs_2CO_3, THF:H_2O, mw, 120 ^oC, 20 min (10-90%); (c) K_2CO_3, KI, CAN, rt, 16 h (50-90%)

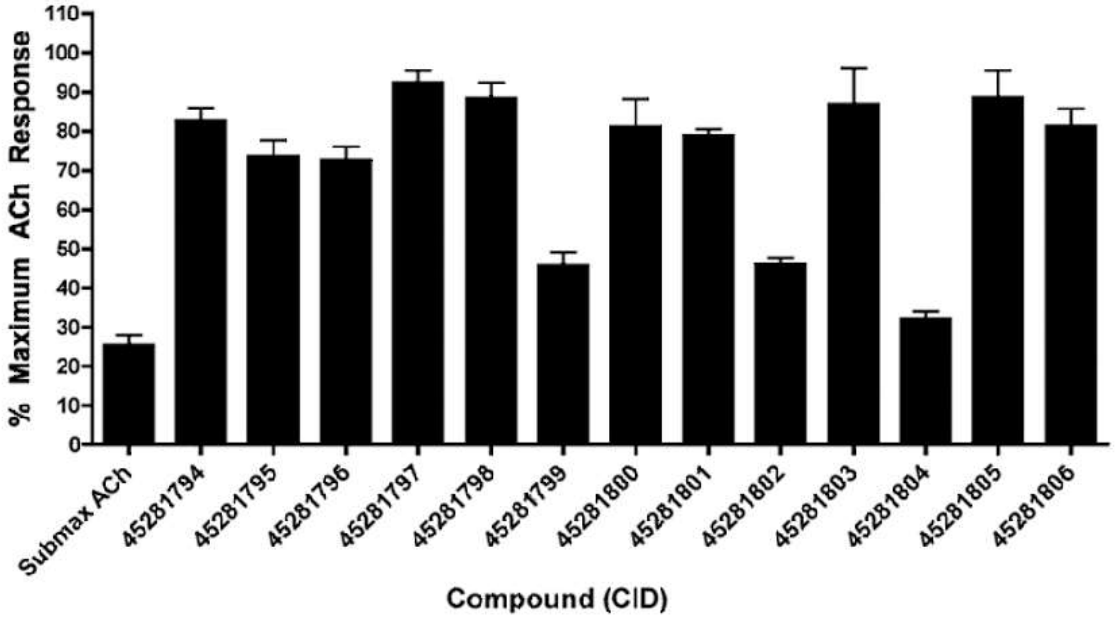
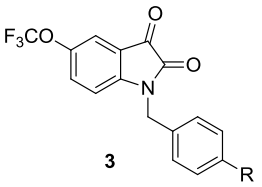


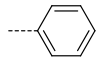
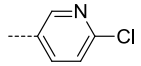
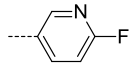
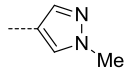
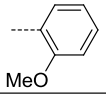
Figure 4. Ca2+ mobilization screen with M_5-CHO cells using 10 µM test compound in the presence of a submaximal concentration of Ach (~EC_{20}) to triage libraries 3 and 5 for PAM activity. Data represent means ± SEM from 3 independent determinations

biaryl and heterobiaryl replacements for the methyl ether of CID 42633508, thus providing analogs **3**. Concomitantly, **1** was alkylated with functionalized phenethyl bromides **4** to explore the effect of chain homologation present in analogs **5**. Compounds from these libraries were then triaged by a single point (10 µM) screen for their ability to potentiate an EC_{20} of ACh in M_5-CHO cells with those results appearing in **Figure 4**.

Table 1. Structures and activities of analogs 3.



3

CID	R	M_5 EC_{50}[a] (µM)	%ACh Max[a]
45281794		2.7	85
45281797		2.8	85
45281798		4.8	85
45281801		3.6	80
45281803		3.3	85
45281796		3.9	70

[a] Average of at least 3 independent determinations. All compounds displayed M_1 EC_{50} > 30 µm.

In general, chain homologation in analogs **5** did not produce improved profiles relative to the benzyl analogs **3**. Although M_5 PAM efficacy was maintained it came at the cost of right-shifted EC_{50} values. For example, CID 45281805, the direct phenethyl analog of CID 42633508 possessed an M_5 EC_{50} of 4.9 and an 80% ACh maximum response (data not shown). Biaryl and heterobiaryl analogs **3** proved far more productive, affording a number of M_5 PAMs with high selectivity versus M_1 (M_1 EC_{50}s >30 µM) and low micromolar M_5 EC_{50}s (**Table 1**). The remaining 5 analogs of **3** displayed M5 EC_{50}s > 10 µM. A wide variety of R- groups could be tolerated, both five- (CIDs 45281797 and 45281803) and six-membered heterocycles (CIDs 45281798 and 45281801) provided active compounds, as did bare phenyl (CID 45281794) and substituted phenyl (CID 45281796). Potency was virtually identical for all of the active analogs **3** (M_5 EC_{50}s 2.7 – 4.8 µM) with similar ACh Max values (70-85%). Shallow SAR was again noted for these allosteric ligands, with compounds either being active in the micromolar potency range or inactive as M_5 PAMs.

The two most potent analogs from **Table 1** (CIDs 45281794 and 45281797) were selected for additional follow-up. **Figure 5** depicts only the G_q mAChR (M_1, M_3 and M_5) concentration response curves (CRCs) for CID 45281794 and CID 45281797. Note that CID 45281794 possesses improved M_5 selectivity versus our initial M_5 probe CID 42633508, with almost negligible activation of M_3 at 30 µM (**Figure 5A**). Both analogs CID 45281794 and CID 45281797 elicit significant leftward shifts (> 50-fold) of the ACh CRC at M5 (**Figure 5C**), as compared to the 14-fold shift of our initial M_5 probe CID 42633508. As seen with the M_1 PAM BQCA[14-16] and other ago-potentiators for class C GPCRs,[17-19] **Figure 5C** indicates moderate intrinsic allosteric agonism at 30 µM for both analogs at the M_5 receptor.

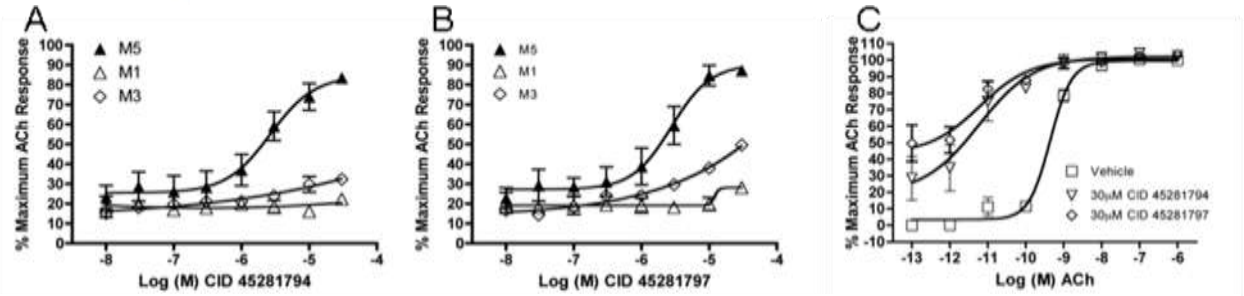
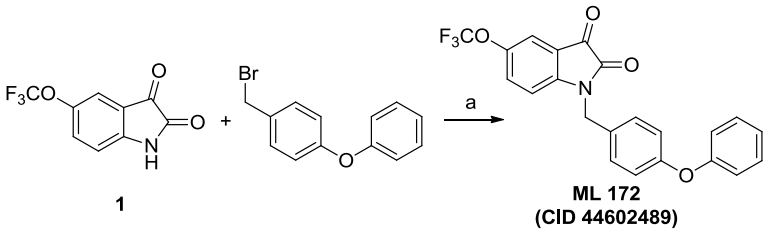


Figure 5. Concentration-response curves in Ca^{2+} mobilization assays with M_1-,M_{2Gqi5}-, M_3, $M_{4/Gqi5}$-, and M_5-CHO cells for (A) CID 45281794 (M_5 EC_{50} = 2.7 μM) and (B) CID 45281797 (M5 EC50 = 2.8 μM). (C) Ach concentration-response fold-shift Ca2+ mobilization assay with M5-CHO cells for CID 45281794 and 45281797 (Both >50x shift versus vehicle control). Data represent means ± SEM from 3 independent determinations

Encouraged by the potency and mAChR selectivity of the phenyl-containing CID 45281794 and the ether-containing CID 42633508 (**Figure 2**), a hydrid analog was prepared (**Scheme 2**) that possessed a biphenyl ether moiety, CID 44602489 (ML 172), and displayed an M_5 EC_{50} of 1.9 μM with a 75% ACh Max. More Importantly, CID 44602489 (ML 172) was completely selective versus M_{1-4}, affording no elevation of an ACh EC_{20} at M_{1-4} at concentrations as high as 30 μM (**Figure 6**). Notably, CID 44602489 (ML 172) represents the most selective M_5 PAM described to date; however, unlike CID 45281794 and CID 45281797, this hybrid analog only afforded a ~5-fold shift of the M_5 ACh CRC at 30 μM and did not display intrinsic allosteric agonism (graph not shown). While subsequent compounds were prepared in an effort to improve this fold shift, potency and fold shift do not always track in the same direction, and in this case our attempts were unfruitful. Therefore CID 44602489 (ML172), the most highly M_5 muscarinic selective PAM known to date, was chosen as our next-generation M_5 probe molecule.



Scheme 2. Reagents and conditions: (a) K_2CO_3, KI, CAN, mw, 160 ^oC, 10 min (68%).

Subsequent follow up demonstrated that many of these analogs displayed moderate to poor PK in rats with limited brain exposure (AUC_{Brain} /AUC_{Plasma} ~ 0.25), presumably due to the bis-carbonyl of the isatin moiety. However, these are important tools to study M_5 function in cells, in electrophysiology and by i.c.v. injection. We did not examine the brain exposure when a DMSO-containing vehicle was employed, but using a DMSO-containing vehicle has been shown to improve brain levels.[17-18]

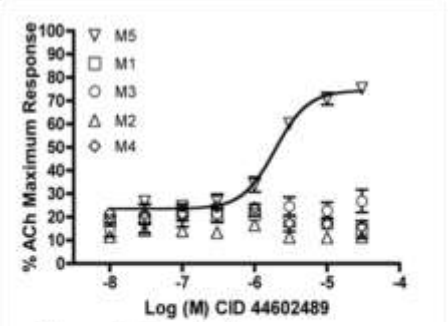


Figure 6. Concentration-response curves in Ca^{2+} mobilization assays with M_1-,M_{2Gqi5}-, M_3, $M_{4/Gqi5}$-, and M_5-CHO cells for CID 44602489 (M_5 EC_{50} = 1.9 μM). Data represent means ± SEM from 3 independent determinations

The calculated physical properties appearing in **Table 2** for the in initial M_5 probe molecule (CID 42633508) and this current probe ML172 (CID 44602489) were generated using TRIPOS software. Also included in **Table 2** are the averages from the MDDR database of compounds both entering Phase I and launched drugs. These numbers indicate that both probes are within the average values for Phase I compounds and launched compounds with respect to MW, hydrogen bond donors/acceptors and number of rotatable bonds. However, both molecules have relatively high cLogP values, which may contribute to their moderate to poor PK, and is also supported by the calculated low LogS (Solubility) values of -4.94 and -6.67 (CID 42633508 and CID 44602489, respectively). Similarly, the greater than 100 square angstroms of polar surface area for each molecule, although not necessarily high enough to preclude entry into the CNS, might be contributing to their less than ideal CNS exposure.

Table 2: Calculated Property Comparison with MDDR Compounds

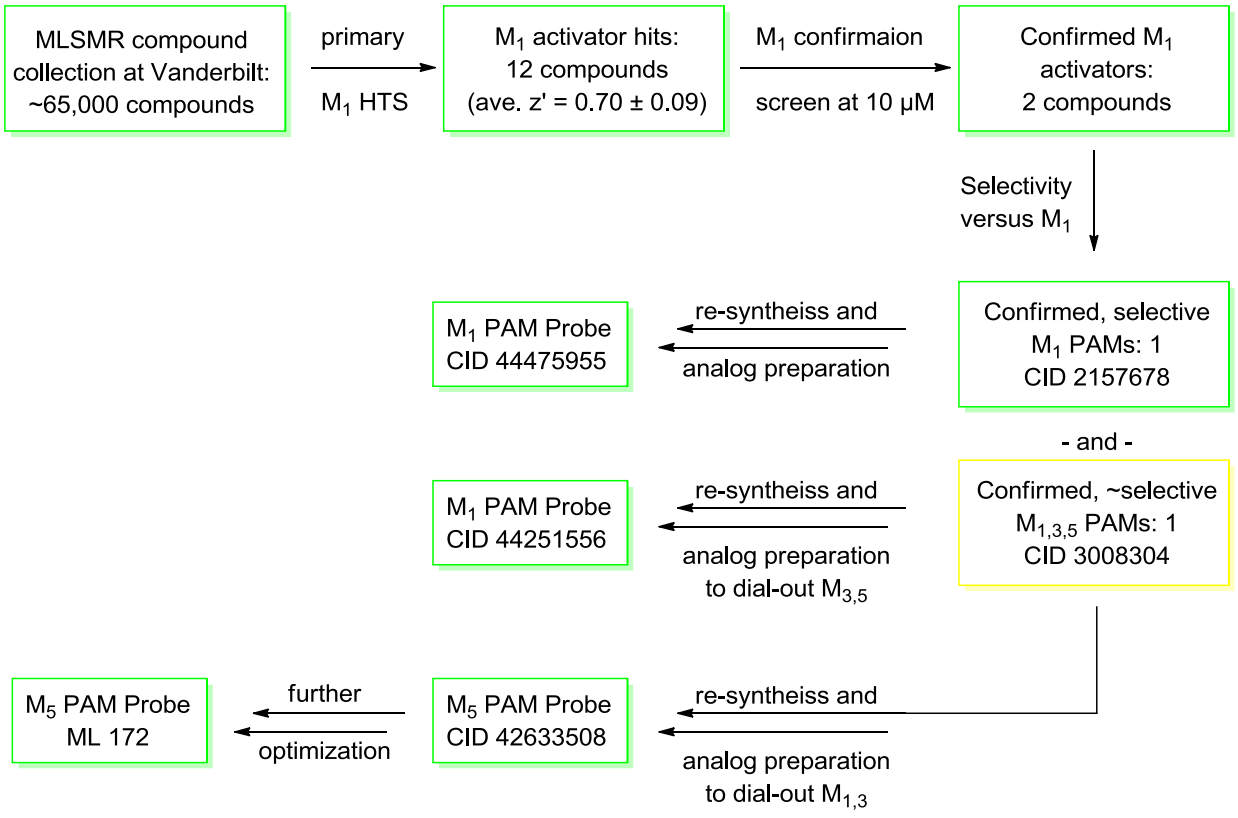
Property	CID 42633508	CID 44602489 (ML172)	MDDR Phase I	MDDR Launched
MW	351.3	413.4	438.98	415.20
cLogP	3.64	5.82	3.21	2.21
TPSA	103.9	107.0	97.06	91.78
Hdon	0	0	2.12	2.13
Hacc	5	5	7.06	6.47
LogS	-4.94	-6.67	-4.96	-3.73
NrotB	5	6	7.08	5.71

To more fully characterize this novel M_5 PAM probe molecule, ML172 (CID 44602489) was tested at MDS Pharma's (now Ricerca's) Lead Profiling Screen (binding assay panel of 68 GPCRs, ion channels and transporters screened at 10 μM), and was found to not significantly interact with 36 out of the 68 assays conducted (no inhibition > 50% at 10 μM).[20] ML172 (CID 44602489) did have activity at the following 32 targets (human targets at 10 μM, unless stated as rat): Adenosine A_1 (50%), Adenosine A_3 (97%), rat Adrenergic α_{1A} (79%), Adrenergic α_{1D} (66%), Adrenergic α_{2A} (100%), Adrenergic β_1 (89%), NET (93%), Bradykinin B_1 (52%), rat Calcium Channel L-Type, Dihydropyridine (74%), CB_1 (109%), D_1 (99%), D_{2S} (85%), D_3 (79%), DAT (90%), rat GABA transporter (56%), H_1 (99%), H_2 (101%), rat Imidazoline, central (50%), Leukotriene, Cysteinyl $CysLT_1$ (83%), MT_1 (60%), Muscarinic M_1 (98%), Muscarinic M_2 (96%), Neuropeptide Y Y_1 (83%), KOP (98%), MOP (99%), PAF (58%), hERG (81%), EP_4 (66%), 5-HT_{1A} (69%), 5-HT_{2B} (90%), SERT (80%) and rat Sodium Channel, site 2 (97%). However it

should be pointed out that these are only single-point values and that functional selectivity may be significantly better than suggested by these "% activities." This is best illustrated by the fact that we see no functional activity for ML172 (CID 44602489) at either the M_1 or M_2 receptor at concentrations as high as 30 µM (**Figure 6**), while the lead profiling screen indicates 98% and 96% inhibition of radio ligand binding at M_1 and M_2, respectively, at just 10 µM.

In summary, ML172 (CID 44602489) represents a marked improvement over our initial M_5 probe with respect to muscarinic selectivity, however ancillary pharmacology, at the binding level, appears to have suffered. CNS penetration is poor, using DMSO-free vehicles, possibly restricting ML172 (CID 44602489) to use as an *in vitro* or i.c.v. probe.

3.1 Summary of Screening Results



3.2 Dose Response Curves for Probe

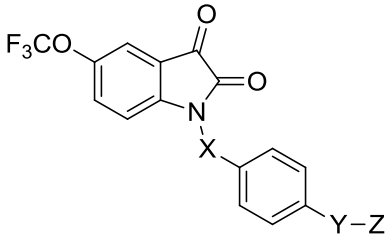
See **Figure 6** (*vide supra*) for M_5 PAM potency and selectivity against M_{1-4}.

3.3 Scaffold/Moiety Chemical Liabilities

No chemical liabilities for the probe molecule have been identified at the present time; however its high lipophilicity may be contributing to a less than ideal profile in the Lead Profiling Screen (Ricerca).[20] Additionally, the isatin core could be viewed as a liability given historical data. To address this potential issue one could envision various isatin replacements, but given the steep SAR generally associated with allosteric modulators it would be a significant undertaking to embark on such an effort. As such, the replacement of the isatin core might be more adequately undertaken as part of an extended probe characterization proposal.

3.4 SAR Table

Table 3: SAR Analysis for M_1 Positive Allosteric Modulators.



Entry	CID	SID	VU Number	*	X	Y	Z	M1 PAM EC50 (μM)**	M1 ACh fold-shift***
1	45281794	92845376	VU0365114-1	S	methylene	phenyl	-	2.7	> 50
2	45281795	92845377	VU0365115-1	S	methylene	3,5-dichlorophenyl	-	~ 10	ND
3	45281796	92845378	VU0365116-1	S	methylene	phenyl	2-methoxy	3.9	ND
4	45281797	92845379	VU0365117-1	S	methylene	thiophen-3-yl	-	2.8	> 50
5	45281798	92845380	VU0365118-1	S	methylene	pyridin-3-yl	6-chloro	4.8	ND
6	45281799	92845381	VU0365119-1	S	methylene	phenyl	4-chloro	~ 10	ND
7	45281800	92845382	VU0365120-1	S	methylene	2,5-difluorophenyl	-	~ 10	ND
8	45281801	92845383	VU0365121-1	S	methylene	pyridin-3-yl	6-fluoro	3.6	ND
9	45281802	92845384	VU0365122-1	S	methylene	phenyl	4-methoxy	~ 10	ND
10	45281803	92845385	VU0365123-1	S	methylene	1H-pyrazol-4-yl	1-methyl	3.3	ND
11	45281804	92845386	VU0365138-1	S	methylene	phenyl	4-propoxy	> 10	ND
12	45281805	92845387	VU0365124-1	S	ethyl	4-methoxy	-	4.9	ND
13	45281806	92845388	VU0365125-1	S	ethyl	4-bromo	-	~10	ND
14	44602489	87352032	VU0400265-1	S	methylene	O	phenyl	1.9	5

* S = synthesized, P = purchased. ** Data represent the mean values from at least 3 experiments with similar results [AID 2416]. *** Leftward shift of an ACh CRC in the presence of 30 μM compound relative to ACh CRC control [AID 2186].

3.5 Cellular Activity

This series of positive allosteric modulators displayed functional activity (Ca^{+2} mobilzation) in CHO cells stably expressing M_{1-5} receptors [AID 2416 and 2204]. Many of these analogs were tested in rat PK, but were found to give moderate to poor exposure (low absolute levels detected) and limited brain exposure (AUC_{Brain}/AUC_{Plasma} ~ 0.25) demonstrating that at least some level of CNS exposure was obtained.

4 Discussion

4.1 Comparison to existing art and how the new probe is an improvement

Presently there are no known selective M_5 PAMs with the exception of those disclosed from our labs, which include the two MLPCN probe molecules (ML 129 and ML172). Relative to our initial M_5 PAM probe (ML 129, CID 42633508) this second generation probe (ML172, CID 44602489) possesses dramatically improved selectivity relative to the other M_{1-4} receptors. The previous probe, although very selective, did begin to show a trend towards off-target receptor activation at the highest doses, ML172 (CID 44602489) is clearly improved in this context (**Figure 6**). Lastly, this probe (ML172, CID 44602489), along with our initial M_5 probe (ML 129, CID 42633508), is not encumbered with patent restrictions and rests firmly in the public domain.

4.2 Mechanism of Action Studies

It is believed that this probe (ML172, CID 44602489) is functioning by positive allosteric modulation of the muscarinic acetylcholine M_5 receptor given its lack of activity on the other muscarinic receptor subtypes (M_{1-4}) and its dependence on the presence of ACh to elicit functional activity.

4.3 Planned Future Studies

Future work will focus on developing a more centrally penetrant M_5 PAM with improved physical properties, which maintains this unprecedented level of muscarinic selectivity and possesses an improved ancillary pharmacology profile. Ultimately it would be highly desirable to develop such a selective M_5 PAM for use as an *in vivo* tool to better understand the biological processes associated with the M_5 receptor.

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