MLSCN Probe Summary

VANDERBILT SCREENING CENTER FOR GPCRs, ION CHANNELS & TRANSPORTERS

Screening Center: Vanderbilt Screening Center for GPCRs, Ion Channels, and Transporters

Principal Investigator (PI) Name: P. Jeffrey Conn

Project Title: Discovery of Novel Allosteric Modulators of the M1 Muscarinic Receptor

Grant Number: 1 X01 MH077606-01

Specific Aim: To identify small molecule modulators of M1 muscarinic receptor that are cell permeable, exhibit micromolar potency, and show greater than 10 fold selectivity over other muscarinic family members M2, M3, M4 and M5.

Significance: Previous attempts to develop compounds that are highly selective for M1 or other specific mAChR subtypes have failed because of the high conservation of the Ach binding site and difficulty in developing truly specific compounds. The lack of highly selective compounds has made it impossible to definitively determine the behavioral and clinical effects of these receptors. Probes developed from these efforts will greatly advance the current state of the art by aiding in the understanding of M1's role in cell-based physiology and may extend the clinical understanding of psychotic and cognitive symptoms associated with neurodegenerative disorders like Alzheimer's Disease.

Rationale: In recent years, major advances have been made in the discovery of highly selective antagonists of other G protein-coupled receptors (GPCRs) that act at an allosteric site rather than the orthosteric binding site (May, L.T. and A. Christopoulos, *Allosteric modulators of G-protein–coupled receptors*. Curr Opin Pharmacol, 2003. **3**(5): p. 551-6.). By screening for compounds that act at an allosteric site on the receptor, it is anticipated that compounds that selectively regulate M1 versus the other muscarinic subtypes may be identified.

PubChem Bioassay Identifier (AID): 626, 628, 677 and 678

PubChem Bioassay Name: Discovery of novel allosteric modulators of the M1 muscarinic receptor: Antagonist

PubChem Primary Assay Description:

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1. Hamster Ovary (CHO) cells containing M1 receptor (ATCC#CRL-1985) were plated at 10,000 cells/well in assay media (F12 (Ham), 10% FBS, 2 millimolar GlutaMAX (Invitrogen), 20mM HEPES) in 384 well plates.
2. The plates were incubated overnight at 37 degrees C in 5% CO2.
3. Media was removed and assay buffer (Hanks Balanced Salt Solution, 20 millimolar HEPES, 2.5 millimolar Probenecid, pH 7.4) containing 4.0 micromolar Fluo4-AM dye (Invitrogen) was added.
4. Cells were incubated for 45 minutes (37 degrees C, 5% CO2) for dye loading.
5. Cell plates were loaded into the Hamamatsu FDSS equipped with 480 nanometer excitation and 540 nanometer emission filters.
6. 10 micromolar test compound in assay buffer + 0.1% DMSO was added at 5 seconds; simultaneously, the plate was kinetically imaged.
7. Subsequently, 8 nanomolar acetylcholine (EC80) in assay buffer was added at 197 seconds and imaging continued for a total of 4 minutes acquisition time.
8. 0.1% DMSO, compound vehicle, and 80nM acetylcholine (ECMAX) were added to each plate as controls.

Center Summary of Screen: For the discovery of novel allosteric modulators of the M1 muscarinic receptor, we completed the primary screen using a real-time cell-based assay against the full 65K library. The assay performed very well (Z' averaged 0.7) and we identified approximately 2000 agonist and 2000 antagonist primary hits. The compounds clustered nicely into different structural classes with multiple representative analogs in each cluster. The antagonist compounds were chosen as the focus of program. 1754 antagonist hits were reordered from Biofocus-DPI and 1665 were received. Duplicate testing yielded 752 confirmed hits (45%). A counter-screen for specificity against mGluR4/CHO cells was performed followed by concentration-response testing. 743 compounds were tested in triplicate in 10 point concentration-response curves against M1/CHO and M4/CHO cells and we identified two lead compounds (Pubchem_SID 4248988 and 7967528) that showed selectivity for M1 over M4 with low micromolar potency. Compound 4248988 was the more potent of the two leads and was selected for resynthesis in parallel with a 23-member analog library. Each of the newly synthesized compounds was tested in 11-point dose-response curves in triplicate against the family of rat muscarinic receptors in a CHO cell background: M1, M2, M3, M4 and M5. The parent compound as well as 4 analogs showed low micromolar potency and M1 selectivity (Figure 1). The parent compound exhibited an IC50 of 1.2 micromolar and the best analog, CL29-8, inhibited M1-mediated calcium transients with an IC50 of 65 nanomolar.

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The selectivity ranges from greater than 100 fold for the parent to greater than 800 fold for the analog. (Figure 2).

Center Comments: These probes are the first M1-specific antagonists identified to date and have huge potential for impacting the current understanding of M1's physiological role. A large number of animal and human studies suggest that cholinergic projections within the brain play a critical role in memory and attention mechanisms and that the clinical syndrome associated with Alzheimer's disease (AD) results, at least in part, from failed neurotransmission at cholinergic synapses [1] for review; [2-8]. Evidence suggests that cholinergic transmission in the forebrain is mediated primarily by muscarinic acetylcholine receptors (mAChRs) and mAChRs are likely to mediate the cholinergic involvement in learning and memory [5, 9, 10]. These studies suggest that agents that enhance cholinergic transmission could be clinically useful for ameliorating the loss of cognitive function in patients with AD and other memory disorders. This hypothesis has been partially substantiated by clinical trials with tacrine and other acetylcholinesterase (AChE) inhibitors, establishing dose-related improvements in measures of cognitive performance and quality of life [11-13] [14-17]. In addition, the compound M1/M4-preferring agonist xanomeline has shown robust therapeutic effects on psychotic symptoms and behavioral disturbances associated with AD [18, 19] such as vocal outbursts, suspiciousness, delusions, hallucinations, mood swings, and other behavioral disturbances. This is consistent with a growing body of clinical evidence that compounds that increase cholinergic neurotransmission have efficacy in treating behavioral disturbances and psychotic symptoms in patients suffering from AD as well as Lewy body dementia, Parkinson's disease dementia, and vascular dementia [20-26]. Furthermore, [27] performed a double blind, placebo-controlled clinical study showing that xanomeline induced robust improvements in positive and negative symptoms in schizophrenic patients. Interestingly, the response was superior to that seen with traditional antipsychotics and therapeutic effects were seen in less than one week as opposed to the multi week delay for traditional antipsychotics. Consistent with these findings with cholinergic agonists, antimuscarinics induce psychosis, confusion and cognitive deficits in humans [28]. Together, these studies provide strong clinical validation of mAChR agonists as novel therapeutic agents used for treatment of psychosis and behavioral disturbances in patients suffering from a broad range of disorders including schizophrenia, AD, and other neurodegenerative disorders.

Due to the lack of specificity of both AChE inhibitors and xanomeline, it has been unclear as to which mAChR subtype is responsible for the antipsychotic and cognitive effects of these compounds. A number of genetic, pharmacological, and anatomical studies, however, suggest that M1 is a likely candidate [29-32]. Consistent with this, M1 knockout (KO) mice exhibit a phenotype that is similar to that seen in animal models of psychosis including

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hyperactivity, increased dopamine release, enhanced response to amphetamine [33, 34], and selective deficits in cognitive function involving hippocampal function and activation of the prefrontal cortex [35]. However, M4 KO mice have a similar, though milder, phenotype and some evidence also points to M4 as playing a role in the antipsychotic actions of mAChR activation [36, 37]. Also, xanomeline is equally active at M1 and M4 subtypes. Thus, it will be important to rigorously test the hypothesis that M1 is the most important receptor for these effects and to determine the potential role of M4. The development of an antagonist that shows 800-fold selectivity for M1 versus the other muscarinic subtypes will now allow testing of the hypothesis that M1 is the receptor responsible for the beneficial effects of AChE inhibitors and xanomeline.

In addition to the critical role that this tool will play in advancing the determination of which muscarinic receptor subtype is responsible for beneficial antipsychotic and cognitive effects in Alzheimer's disease and schizophrenia, it is also hypothesized that antagonists of M1 and/or M4 may have important therapeutic effects in diseases such as Parkinson's disease and other movement disorders such as dystonia. Both of these receptors are expressed in the basal ganglia and striatum and, in rodent models that mimic parkinsonian tremors, muscarinic antagonists have been shown to have beneficial effects [38-39]. As discussed above, it has been noted that muscarinic antagonists can induce confusion and psychosis but, again, it is unclear which subtypes are responsible for this effect. It is therefore anticipated that selective tools will now aid in defining the subtypes responsible for beneficial versus adverse effects of muscarinic antagonists in various movement disorders. It will now be of interest to use this new M1 antagonist in both electrophysiology and in vivo models to study the function of M1 in basal ganglia physiology and to explore the therapeutic potential of M1 in movement disorders.

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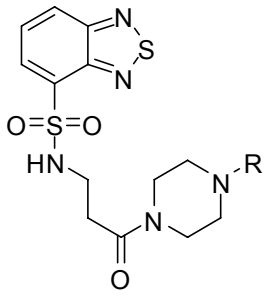


1

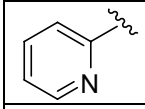
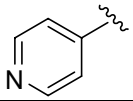
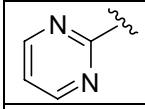
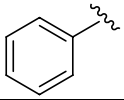
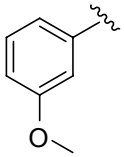
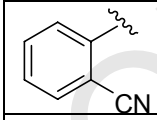
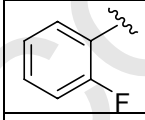
R	rM1 EC_{50} (□M)	rM2 EC_{50} (□M)	rM3 EC_{50} (□M)	rM4 EC_{50} (□M)	rM5 EC_{50} (□M)
	1.2	>150	>150	>150	>150
	0.065	112	>150	55	>150
	18.9	>150	>150	>150	>150
	6.5	>150	>150	>150	>150
	2.4	>150	>150	>150	>150
	4.5	5.3	32.9	6.8	31
	34	>150	>150	>150	>150
	>150	>150	>150	>150	>150

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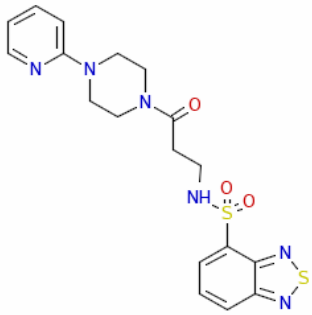
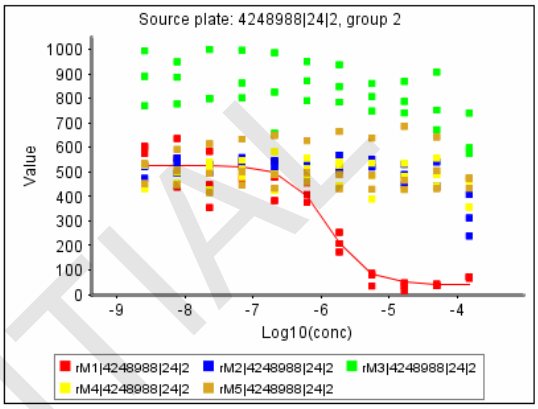
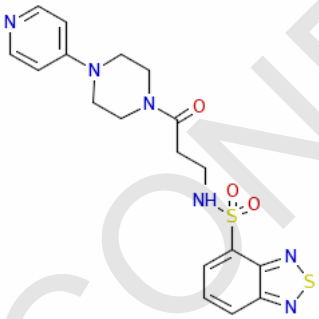
Figure 2: Structure activity relationships for members of the technology enabled synthesis (TES) analog library. The optimization strategy was to use commercially available sulfonyl chloride treated with a β-amino acid ester, followed by saponification to afford the acid **1** followed by standard parallel synthesis using PS-DCC, HOBt and one equivalent of 24 amines, and scavenging with MP-carbonate delivered a library of 24 compounds.



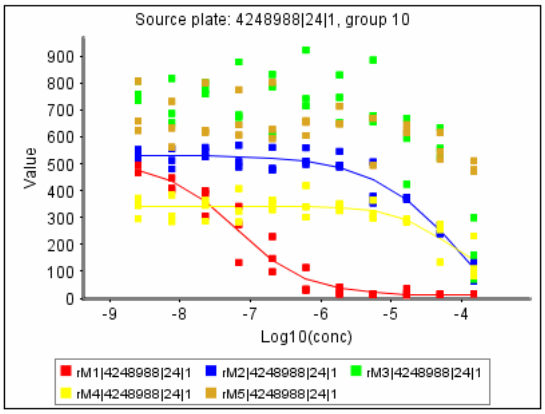
CL29-9



Barcode	Bottom	Top	Slope	LogEC50	EC50
rM1|4248988|24|2	41 (+/- 20, P=0.05808)	524 (+/- 17, P=0.00000)	-1.57 (+/- 0.38, P=0.00028)	-5.912 (+/- 0.078, P=0.00000)	1.225 (+/- 0.000)
rM2|4248988|24|2					
rM3|4248988|24|2					
rM4|4248988|24|2					
rM5|4248988|24|2					



CL29-8



Barcode	Bottom	Top	Slope	LogEC50	EC50
rM1|4248988|24|1	10 (+/- 12, P=0.43578)	505 (+/- 36, P=0.00000)	-0.86 (+/- 0.16, P=0.00001)	-7.189 (+/- 0.115, P=0.00000)	0.065 (+/- 0.000)
rM2|4248988|24|1	-237 (+/- 398, P=0.55644)	534 (+/- 11, P=0.00000)	-0.66 (+/- 0.20, P=0.00276)	-3.950 (+/- 0.670, P=0.00000)	112.121 (+/- 0.000)
rM3|4248988|24|1					
rM4|4248988|24|1	84 (+/- 137, P=0.54198)	340 (+/- 10, P=0.00000)	-1.23 (+/- 0.84, P=0.15324)	-4.260 (+/- 0.475, P=0.00000)	54.893 (+/- 0.000)
rM5|4248988|24|1					

Figure 2: Concentration response curves tested with each of the muscarinic family members M1, M2, M3, M4 and M5 demonstrate that the newly synthesized parent compound 4248988 (top panel) and analog CL29-8 (bottom panel) are potent and selective modulators of M1 muscarinic receptor.

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