DISCOVERY, OPTIMIZATION, AND CHARACTERIZATION OF NOVEL

SUBTYPE-SELECTIVE MUSCARINIC ACETYLCHOLINE RECEPTOR

M4 AND M5 POSITIVE ALLOSTERIC MODULATORS

By

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To my parents Vicki and Allen Bridges

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# LIST OF ABBREVIATIONS

°C                      Degrees celsius

$[^3H]$-NMS             $[^3H]$-$N$-methylscopolamine

α                       Cooperativity factor

AC                      Adenylyl cyclase

Acetyl-CoA              acetyl-coenzyme A

ACh                     Acetylcholine

AChE                    Acetylcholinesterase

AChEI                   Acetylcholinesterase inhibitor

AD                      Alzheimer's disease

Aβ                      Amyloid-β

APP                     Amyloid precursor protein

ATCM                    Allosteric ternary complex model

AUC                     Area under the curve

β                       Efficacy modulation factor

BBB                     Blood-brain barrier

BQCA                    Benzyl quinolone carboxylic acid

BuChE                   Butyrylcholinesterase

CAA                     Cerebral amyloid angiopathy

cAMP                    Cyclic adenosine monophosphate

CAR                     Conditioned avoidance responding

CCh                     Carbachol

cGMP                    Cyclic guanosine monophosphate

CBF                     Cerebral blood flow

ChAT                    Choline acetyltransferase

| | |
|---|---|
| CHO-K1 | Chinese hamster ovary-K1 |
| CNS | Central nervous system |
| CPP | Conditioned place preference |
| CRC | Concentration-response curve |
| CSF | Cerebrospinal fluid |
| $D_1$ | Dopamine $D_1$ receptor |
| $D_2$ | Dopamine $D_2$ receptor |
| DA | Dopamine |
| DMPK | Drug metabolism and pharmacokinetic |
| DMSO | Dimethyl sulfoxide |
| DOS | Diversity-oriented synthesis |
| EDTA | Ethylenediamine tetra-acetic acid |
| ELSD | Evaporative light scattering device |
| EPSC | Excitatory post-synaptic current |
| ERK | Extracellular signal regulated kinase |
| GABA | γ-amino-butyric acid |
| $GABA_A$ | γ-amino-butyric acid receptor A |
| GlyT1 | Glycine type-1 transporter |
| GIRK | G-protein coupled inward-rectifying $K^+$ channel |
| GPCR | G-protein coupled receptor |
| GRK | G-protein coupled receptor kinase |
| GTPγS | Guanosine trisphosphate-γS |
| HPLC | High-performance liquid chromatography |
| HTS | High-throughput screening |
| Hz | coupling constant |
| IHC | Immunohistochemistry |
| IP | Immunoprecipitation |

| | |
|---|---|
| I.P. | Intraperitoneal |
| IP$_3$ | Inositol trisphosphate |
| $K_{A/a}$/$K_{B/b}$/$K_{D/d}$ | Equilibrium dissociation constant |
| $K_{I/i}$ | Equilibrium dissociation constant from competition binding |
| KO | Knockout |
| LCMS | Liquid chromatography mass spectrometry |
| logP | Log partition coefficient |
| LTP | Long-term potentiation |
| M$_1$-M$_5$ | Muscarinic acetylcholine receptor subtypes 1-5 |
| mAChR | Muscarinic acetylcholine receptor |
| MAPK | Mitrogen-activated protein kinase |
| MCH | Melanin-concentrating hormone |
| mGluR | Metabotropic glutamate receptor |
| MHz | Megahertz |
| MLSCN | Molecular libraries screening center network |
| MLPCN | Molecular libraries probe center network |
| mPFC | Medial pre-frontal cortical/cortex |
| NAc | Nucleus accumbens |
| nAChR | Nicotinic acetylcholine receptor |
| NAM | Negative allosteric modulator |
| NDMC | *N*-desmethyl clozapine |
| NFT | Neurofibrillary tangle |
| NMDA | *N*-methyl-D-aspartic acid |
| NMDAR | *N*-methyl-D-aspartic acid receptor |
| NMR | Nuclear magnetic resonance |
| NO | Nitric oxide |
| PAM | Positive allosteric modulator |

| | |
|---|---|
| PBL | Plasma-brain levels |
| PCP | Phencyclidine |
| PEG | Polyethylene glycol |
| PET | Positron emission tomography |
| P-gp | P-glycoprotein |
| PI3K | Phosphatidyl inositol-3 kinase |
| PK | Pharmcokinetic |
| PKA | Protein kinase A |
| PKG | Protein kinase G |
| PLA | Phospholipase A |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PNS | Peripheral nervous system |
| PPI | Prepulse-inhibition |
| ppm | Parts per million |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SAR | Structure-activity relationship(s) |
| S.C. | Subcutaneous |
| SEM | Standard error of the mean |
| SNc | Substantia nigra pars compacta |
| TCM | Ternary complex model |
| TLC | Thin-layer chromatography |
| UV | Ultra-violet |
| VAChT | Vesicular ACh transporter |
| VHTSC | Vanderbilt high-throughput screening center |
| VTA | Ventral tegmental area |
| WT | Wild-type |

# Chapter I

# INTRODUCTION

## Muscarinic acetylcholine receptors

*Acetylcholine biosynthesis and receptors*

Acetylcholine (ACh) is the only known non- amino acid or non- amino acid-derived small molecule neurotransmitter and is vitally important to a wide range of basic physiological processes and functions throughout the mammalian body[1-5]. ACh is synthesized endogenously from dietary choline, which is acetylated within the cell by the enzyme choline acetyltransferase (ChAT) using acetyl-coenzyme A (acetyl-CoA) as substrate to furnish the acetyl group (**Fig. 1**)[1]. Subsequent intraneuronal packaging of cytoplasmic ACh into synaptic vesicles occurs via the vesicular ACh transporter (VAChT) (**Fig. 1**)[6,7]. Following ACh release into the synapse, the enzyme acetylcholinesterase (AChE) rapidly terminates ACh signaling by hydrolyzing the ester of ACh to produce acetate and choline, the latter of which is then subject to local neuronal reuptake by the high-affinity choline transporter (**Fig. 1**)[8,9]. Such reuptake represents the rate-limiting step in the ACh synthetic cycle[9]. In addition to AChE, which is encoded by a single gene but occurs in multiple forms via alterative mRNA-splicing, butyrylcholinesterase (BuChE) serves as a second enzyme responsible for degradation of ACh[10-12]. However, BuChE is mainly present in plasma and in non-neuronal tissues where it contributes to termination of ACh signaling[11,12].

1

**Cholinergic Neuron Terminal**
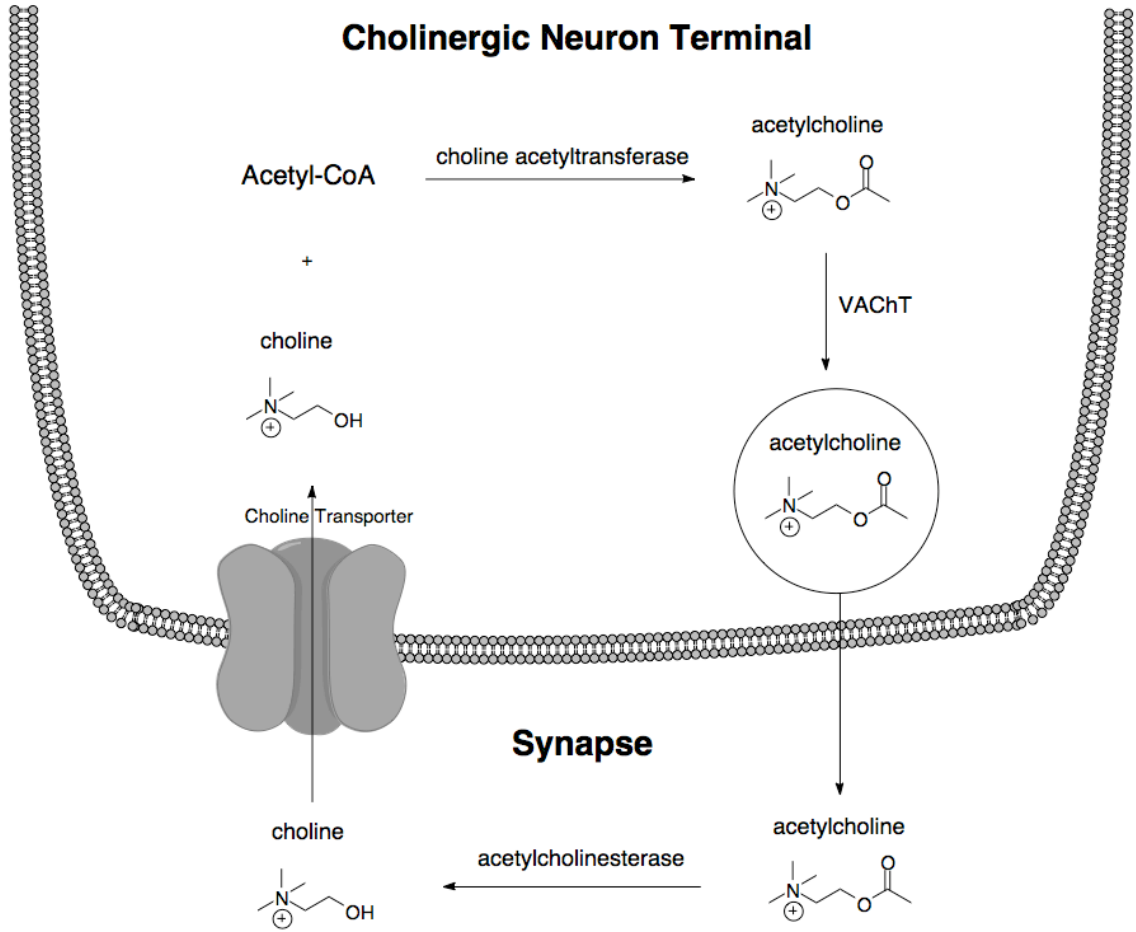


**Figure 1**. Generalized diagram of cholinergic biosynthesis, packaging, degradation, and reuptake.

As a neurotransmitter, ACh plays essential roles in both the central nervous system (CNS) and peripheral nervous system (PNS)[3,13]. Within the autonomic arm of the PNS, cholinergic signaling occurs at the pre-ganglionic sympathetic and parasympathetic synapses, the post-ganglionic parasympathetic synapses, and at a subset of post-ganglionic sympathetic synapses in skin and sweat glands[3,13]. ACh is also released at the skeletal neuromuscular junction, where it is responsible for initiation of muscle contraction[3,13]. In the CNS, ACh is released from cholinergic neurons arising from six main brain regions (denoted as Ch. I – Ch. VI) and is responsible for a multitude of

integral brain functions and processes such as regulation of motor control, reward, sleep/wake cycles, metabolism, learning and memory, attention, and more (**Fig. 2**)[3,13,14].
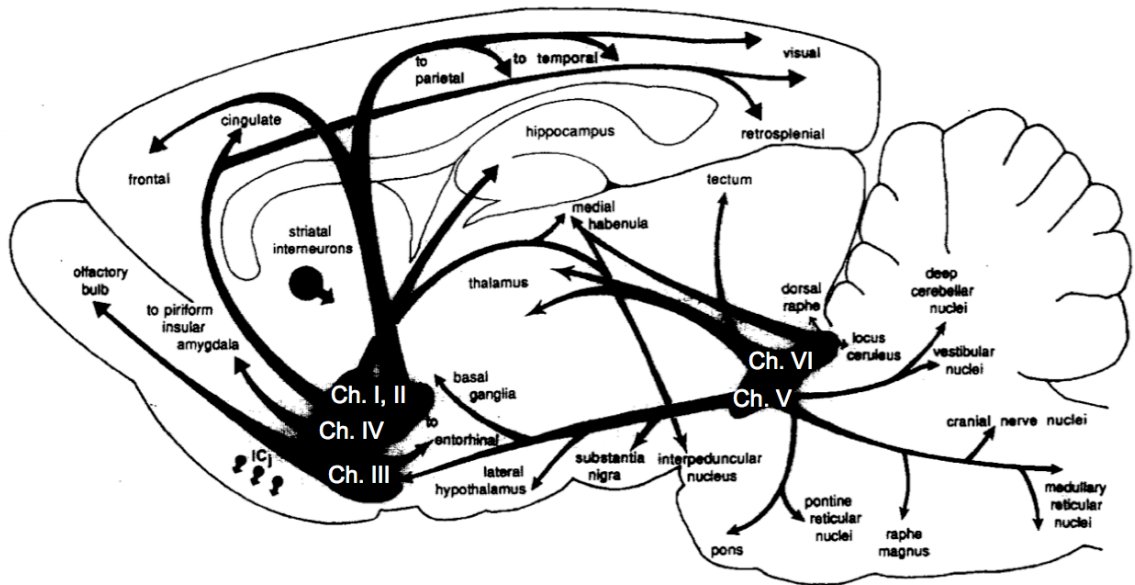


**Figure 2**. Diagram of six main central cholinergic neuron projection pathways in the rat brain (adapted from Woolfe 1991).

There exist two classes of ACh receptors, the ionotropic receptors activated by exogenous nicotine and the metabotropic receptors activated by exogenous muscarine, termed nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs), respectively[15,16]. These two classes can also be delineated by their sensitivity to antagonism by either tubocurarine (selective for nAChRs) or atropine (selective for mAChRs). Of the nAChRs, muscular ($N_m$) nAChRs mediate the actions of ACh at the skeletal neuromuscular junction while neuronal ($N_n$) nAChRs are present in the autonomic ganglia and CNS[15]. All nAChRs are pentameric ion channels comprised of various combinations of subunits ($\alpha_{1\text{-}10}$, $\beta_{1\text{-}4}$, as well as the less common $\gamma$, $\delta$, and $\varepsilon$ subunits), which confer distinct structures and functions[15]. In general, nAChRs in the

3

CNS, such as those comprised of $\alpha_4$ and $\beta_2$ subunits or all $\alpha_7$ subunits, are present at both pre- and post-synaptic membranes where they enhance neuronal excitation by increasing $Na^+$ and $Ca^{2+}$ permeability[15]. In contrast, mAChRs are G-protein coupled receptors (GPCRs) of which there exist five distinct subtypes (mAChR$_{1-5}$ or $M_1$-$M_5$), each encoded by a separate intron-less gene (CHRM1-5)[2,5,16]. These mAChR subtypes are expressed differentially in both central and peripheral tissues, where they mediate the metabotropic signaling of ACh.

*Structure and signaling of mAChR subtypes*

Following cloning of the rat and human mAChR genes, a large body of research has focused on elucidating the molecular structure and function of these receptors. mAChRs are Type I (or Class A) rhodopsin-like GPCRs ranging in size from 460 ($M_1$) to 590 ($M_3$) amino acids and sharing a high degree of homology among their respective orthosteric ACh-binding sites[2,5,16-18]. $M_1$, $M_3$, and $M_5$ exhibit higher structural homology to each other than to $M_2$ and $M_4$, which themselves share highest homology to each other[2,5]. Correspondingly, the pre- versus post- synaptic localization patterns and associated downstream signaling pathways are generally similar for $M_1$, $M_3$, and $M_5$ versus $M_2$ and $M_4$[17,18]. The former three are often considered stimulatory or excitatory given their predominantly post-synaptic localization and coupling to the $G\alpha_q$/phospholipase-C (PLC)/inositol triphosphate ($IP_3$)/$Ca^{2+}$ pathway, while the latter two are typically regarded as inhibitory with predominantly pre-synaptic localizations as autoreceptors or heteroreceptors with coupling to the $G\alpha_i$/adenylyl cyclase (AC)/cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) effector system[17,18].

4

It is important to note that many of the intracellular effects of mAChR activation are also mediated by Gβγ, such as the Gβγ-induced opening of inward rectifying $K^+$ channels (GIRKs) in the case of $M_2$ and $M_4$ or Gβγ-mediated phosphatidyl inositol-3 kinase (PI3K)/Src signaling in the case of $M_1$[17,19-24]. Furthermore, mAChRs have been found to activate many other downstream effectors including the phospholipases PLA and PLD, and all mAChRs have been shown to activate mitogen-activated protein kinase (MAPK)-signaling as measured by extracellular signal regulated kinase (ERK) phosphorylation[17,25]. Like other Type I GPCRs, additional mAChR signaling occurs via β-arrestin1/2 (Arrestin2/3) binding following phosphorylation of the receptor by G-protein coupled receptor kinases (GRKs)[17]. In addition to their role(s) in receptor desensitization and internalization, Arrestins can also serve as protein scaffolds and signal-transduction nodes themselves[26]. Thus, mAChR signaling within the cell can involve a broad and complex network of pathways, which are highly dependent upon the specific cell type and its protein composition.

More recently, pharmacological studies have revealed that different small molecule mAChR ligands are capable of exerting drastically different downstream signaling effects (through the same receptor subtype), presumably due to stabilization of distinct receptor structure conformations[27,28]. Such phenomena, often referred to as ligand-induced receptor trafficking/signaling-bias, represent an important area of study for neuropharmacology, as ligands with the same mode of pharmacology (e.g. agonists) but of different chemical classes/chemotypes may cause divergent intracellular signaling through the same mAChR. This has obvious implications for use of novel (and particularly allosteric) ligands in basic neurobiological research and in therapeutic drug

discovery because different compounds targeting the same receptor may yet cause different electrophysiological, neurochemical, and behavioral effects[28].

Activation of mAChRs occurs via structural rearrangement and subsequent G-protein activation in response to ACh-binding to the orthosteric site deep within the transmembrane-spanning $\alpha$-helical domain of the receptor[29]. Within this site, a number of key residues form reversible non-bonding interactions with ACh[29]. For example in the case of $M_1$, tyrosine-381, which is located in the 6th-transmembrane $\alpha$-helix, forms a $\pi$-cation interaction with the positively charged ACh quaternary amine that is crucial to potent receptor activation by ACh[30]. When this residue is mutated to an alanine, ACh potency at $M_1$ is right-shifted by several orders of magnitude[30]. This $M_1$ mutant is thus particularly useful for characterization of novel $M_1$ activators because it can provide evidence for or against an orthosteric mode of binding[31].


*Localization of mAChR subtypes*

A number of molecular biology techniques, including *in-situ* mRNA hybridization, reverse transcriptase PCR (RT-PCR), immunohistochemistry (IHC), immunoprecipidation (IP), and radioligand-binding have been used to investigate the neuroanatomical localization patterns of each mAChR[32-40]. Due to differences inherent among these various techniques, the fact that different investigators have used different antibody preparations (in the case of IHC/IP studies), and the observation that presence of mRNA for a given receptor in a particular tissue does not directly demonstrate equivalent functional protein levels in that tissue, has prevented widespread quantitative agreement on mAChR subtype density and localization. Nonetheless, relative distributions and

general localization of each subtype is known. At the mRNA level, transcripts for all five mAChR subtypes are found throughout the body in most tissues with detection levels ranging from high to near-baseline. At the protein level, the breadth of mAChR expression appears lower; however, there is generally good agreement between receptor mRNA levels and protein levels in most brain regions of primary interest to neurobiological research.

In the CNS, $M_1$ represents the predominant subtype, with high levels of expression throughout the cortex, striatum, hippocampus, and to a lesser extent the olfactory bulb and amygdala[33,35,40]. $M_1$ is also present at low levels in peripheral smooth muscle[33]. Similar to $M_1$, the $M_3$ subtype is found in the brain cortex, striatum, thalamus/hypothalamus, hippocampus, and olfactory bulb; though, levels of $M_3$ in the CNS are generally lower than those of $M_1$ across each shared brain region[33,35,40,41]. Peripheral $M_3$ is predominately found throughout cardiovascular and gastrointestinal smooth muscles as well as in numerous glandular tissues, and to a lesser extent in the heart[33]. The structurally related $M_5$ receptor initially proved the most difficult subtype to localize due to its extremely low levels of expression. Indeed, it has been estimated that this subtype represents less than 2% of all mAChR protein in the brain[38]. Ultimately, $M_5$ has been shown to be the only mAChR present on midbrain dopaminergic cell bodies originating in the ventral tegmental area (VTA) and substantial nigra pars compacta (SNc)[34,37]. These midbrain regions contain the highest levels of $M_5$ in the CNS, with cortical and hippocampal tissues containing $M_5$ at levels far below those of $M_1$ or $M_3$[33,35,40]. Importantly, non-neuronal $M_5$ is found throughout the cerebrovascular system, including within the microvessels and epithelial cells comprising the blood-brain barrier

(BBB), and in the larger arterioles and arteries of the brain[36,42]. The $M_2$ subtype is expressed largely on brainstem and basal forebrain cholinergic neurons (Ch. V-VI and Ch. IV, respectively) as well as in the thalamus, hippocampus, and striatum[33,35,40]. $M_2$ is also highly expressed in cardiac tissues and to a lesser extent in peripheral smooth muscles[33]. The $M_4$ subtype is most heavily expressed in the midbrain, striatum, and other areas of the basal ganglia, with lower levels present in the hippocampus and cortex[32,33,35,37,40,43]. Peripherally, $M_4$ may be present in mainly in lung tissue[33].

*Genetic deletion of mAChR subtypes*

Given the high structurally homology of the orthosteric ACh-binding site across the five mAChR subtypes, discovery of small molecule ligands possessing high binding and/or functional selectivity for an individual subtype has proven challenging[44]. As discussed in greater detail in a subsequent section of this Chapter, a number of potent orthosteric mAChR agonists and antagonists have been discovered, which have provided utility in certain pharmacological research contexts but most have suffered from poor subtype-selectivity. Due to the longstanding paucity of selective pharmacological tools, insight into the specific functions of each mAChR at the cellular, neurocircuitry, neurochemical, and behavioral levels have relied largely on studies using mice genetically engineered to lack one or more mAChR subtype[45]. These receptor knockout (KO) mice have provided insight to the role(s) of each mAChR in basic physiology, neurobiology, and within the context of various CNS disorders[45]. Overall, the multitude of findings from mAChR-KO mice agree well with the predicted functions of each subtype based on their localization and known signaling.

Given the high density of $M_1$ in brain regions known to be important to cognitive function, such as the cortex and hippocampus, it is not surprising that $M_1$-KO mice exhibited deficits in multiple biochemical and behavioral studies related to learning and memory. Specifically, $M_1$-KO mice have been shown to exhibit cognitive deficits in behavioral tests such as the eight-arm radial maze, fear-conditioning (under conditions requiring extra-hippocampal mechanisms), and social discrimination[45-47]. Interestingly, no deficits were found in other tests of cognition such as the Morris water maze, a common paradigm for assessing impairments in hippocampal-dependant spatial reference memory[45,47]. These and other related behavioral findings have contributed to the hypothesis that $M_1$ is not required for initial formation of memory in the hippocampus but is likely important to long-term memory consolidation and to interactions between the hippocampus and cortex[46]. Electrophysiological experiments using $M_1$-KO mice have suggested that $M_1$ activation enhances *N*-methyl-D-aspartic acid receptor (NMDAR) currents in hippocampal slices[48]. Likewise, in related electrophysiology experiments investigating long-term potentiation (LTP) in $M_1$-KO mice, selective hippocampal impairments were found compared to wild-type (WT) under certain conditions[45,49,50]. Biochemical evidence from $M_1$-KO mice also supports a role for $M_1$ in certain learning and memory processes. For instance, activation of MAPK signaling, which is believed to be highly important to synaptic plasticity, was absent in cultured cortical cells from $M_1$-KO mice (versus WT) following muscarinic agonist stimulation[51,52]. Similar results were also found in analogous experiments using hippocampal pyramidal neurons from $M_1$-KO mice[52].

Taken together, these and other related findings support the notion that $M_1$ plays a role in learning and memory; however, it is likely that other mAChRs are also important to specific cognitive functions. Indeed, pan-mAChR antagonists such as scopolamine induce substantial cognitive deficits beyond those observed with genetic deletion of $M_1$ alone, and other mAChRs have been implicated in various electrophysiological and biochemical roles highly relevant to learning and memory functions[45,53,54]. Moreover, $M_1$-KO mice exhibit hyperlocomotor activity, which complicates other behavioral assessments and may be related to the expression of striatal $M_1$ receptors in WT animals[45,47,55]. Such hyperlocomotion is likely due to elevated striatal dopamine (DA) levels in $M_1$-KO mice, which were found to be approximately 2-fold greater than in WT controls[55]. Lastly, WT mice given high doses of the muscarinic agonist pilocarpine will fall into seizures that most often culminate in death. This effect was completely abolished in $M_1$-KO mice, suggesting that $M_1$-mediates pilocarpine-induced seizure activity, possibly via the enhancement of NMDAR signaling[49].

Genetic deletion of $M_3$ in mice has suggested that this subtype plays an important role in central regulation of metabolism, which is consistent with $M_3$ localization in the thalamus/hypothalamus. Specifically, mice lacking $M_3$ are hypophagic and exhibit significantly reduced total body weight (~25% decrease versus WT), reduced fat pad mass (~50% decrease versus WT), and reduced serum levels of leptin and insulin (~5-10x decrease versus WT)[45,56]. These effects have been hypothesized to occur due to absence of $M_3$ that is normally expressed on melanin-concentrating hormone (MCH)-positive neurons located in the hypothalamus[56]. Given the overlapping CNS expression pattern and similar signaling properties between $M_3$ and $M_1$, it is likely that cortical and/or

hippocampal $M_3$ contributes to certain aspects of learning and memory. Indeed, recent behavioral studies with $M_3$-KO mice revealed profound deficits in contextual fear-conditioning but none in auditory cue fear-conditioning, suggesting that $M_3$ plays a role in hippocampal-dependent learning and memory[57]. In agreement with localization data, deletion of $M_3$ has also been shown to substantially reduce ACh-induced smooth muscle contraction in the periphery[58-60]. Likewise, muscarinic agonist-induced salivation was decreased but not abolished in $M_3$-KO mice, suggesting that $M_3$ as well as other mAChRs play a role in salivary secretion[61].

In the case of $M_5$, consistent with its highest expression in midbrain dopaminergic neurons where it is the only mAChR present, $M_5$-KO mice have suggested that this subtype plays an important role in mediating reward-responses through the mesolimbic DA pathway and in regulation of striatal DA levels (**Fig. 3**)[34,37,45]. Specifically, muscarinic-agonist induced elevations in striatal DA release were reduced (~5-10x versus WT), but not abolished in $M_5$-KO mice brain slices, which has led to the hypothesis that lack of pre-synaptic $M_5$ heteroreceptors located on dopaminergic terminals innervating the striatum are responsible for this effect[62]. However, based on numerous localization studies, a virtual absence of $M_5$ expression in the striatum is at odds with this hypothesis, and the slices used in this particular study would not have contained the distal DA cell bodies that originate from the midbrain, where $M_5$ is most likely excitatory and post-synaptic[37,62]. Additional evidence that $M_5$ plays a vital role in the mesolimbic DA pathway comes from microdialysis experiments with $M_5$-KO mice. In WT mice, electrical stimulation of hindbrain cholinergic nuclei (Ch. V and Ch. VI) leads to a prolonged and significant rise in DA levels of the nucleus accumbens (NAc), the primary

forebrain target of VTA dopaminergic projections, which was completely abolished in

M$_5$-KO mice[63]. Behaviorally, M$_5$-KO mice exhibit substantially reduced sensitivity to

the rewarding effects of drugs such as cocaine and opiates (both of which act as strong

activators of mesolimbic DA signaling)[45,64,65]. These mice displayed large decreases in

self-administration of cocaine and decreased conditioned place-preference (CPP) in

response to training with cocaine or morphine place-pairing[64,65]. Additionally, morphine-

induced elevations of DA levels in the NAc and in Fos-B expression were reduced in M$_5$-

KO mice[64]. Furthermore, withdrawal-induced anxiety behaviors were less pronounced in

M$_5$-KO[64]. Interestingly, morphine retained its analgesic efficacy in M$_5$-KO mice,

suggesting that if M$_5$ antagonists proved useful in the treatment of opiate addiction, the

pain-alleviating effects of such drugs may be retained in clinical settings[64].
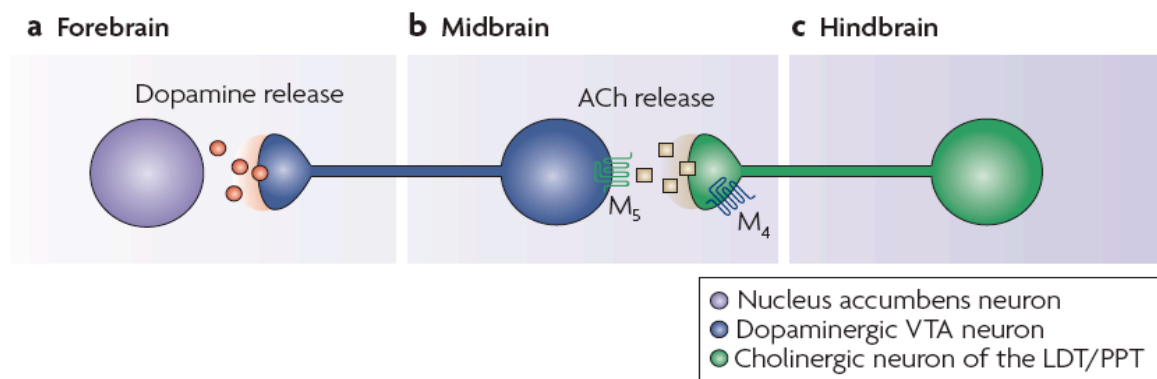


**Figure 3**. Abbreviated diagram of the putative roles of M$_4$ and M$_5$ in the central mesolimbic DA pathway (Wess 2007).

Taken together, this large body of data supports the hypothesis that the primary

role of neuronal M$_5$ is in regulation of central DA signaling/pathways. Although less

research has focused on nigrostriatal DA signaling, it is likely that M$_5$ plays a similarly

important and parallel role. However, other studies with M$_5$-KO mice have found subtle

deficits in cognitive function and in neuroanatomical/biochemical correlates of cognition. Specifically, atrophy of cortical pyramidal neurons from $M_5$-KO mice was observed along with modest behavioral deficits in spatial and non-spatial memory functions (as assessed by Y-maze and novel object recognition tests, respectively)[66]. LTP at the mossy fiber-CA3 synapse of the hippocampus was also impaired in brain slices from $M_5$-KO mice[66]. Some or all of these deficits may be indirect results of decreased cerebral blood flow (CBF) and oxygen delivery resulting from absence of $M_5$ in the cerebrovascular system. It is well established that decreased blood flow to the brain can disrupt cognitive functions and alter brain histology[67,68]. In WT animals, ACh-induces a pronounced vasodilatory effect across multiple cerebrovascular structures, which is completely abolished in $M_5$-KO mice[62]. Furthermore, mice lacking $M_5$ exhibit a tonic/constitutive constriction of cerebral blood vessels that results in decreased cerebral blood flow, which may underlie the cognitive effects[66]. Interestingly, ACh-induced vasodilation in peripheral vascular tissues was unaffected by genetic $M_5$ deletion, consistent with the hypothesis that the $M_3$ receptor mediates the peripheral vasodilatory effects of ACh[62]. Cholinergic neurons, particularly those originating in basal forebrain structures, provide direct innervation to cerebral blood vessels, where ACh release induces vasodilation likely via a nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)-dependent mechanism[69-72]. Thus, some of the cognitive symptoms of CNS disorders, including Alzheimer's disease (AD) and cerebrovascular dementia, may be related to decreased CBF resulting from loss of cholinergic nuclei of the basal forebrain and consequently decreased activation of cerebrovascular $M_5$[66,67]. The potential

therapeutic relevance of these findings is discussed in more detail in a subsequent section of this Chapter.

High doses of muscarinic agonists such as oxotremorine induce tremor and hypothermia in rodents, which was completely abolished and reduced, respectively, in $M_2$-KO mice[73]. Likewise, analgesic efficacy of muscarinic agonist administration was reduced in these mice, consistent with related studies that found electrophysiological deficits in spinal ganglion neurons of $M_2$-KO mice[73,74]. In isolated heart atria, absence of $M_2$ resulted in a complete lack of carbachol-induced bradycardia, which agrees with previous localization and pharmacological studies suggesting that $M_2$ is the predominant mAChR mediating the parasympathetic actions of ACh in cardiac tissue[75]. In the case of central $M_2$, mice lacking this receptor were devoid of oxotremorine-induced inhibition of ACh-release in both cortical and hippocampal brain slices, which supports the hypothesis that $M_2$ is mainly present as a pre-synaptic autoreceptor governing ACh release[76]. *In vivo*, behavioral studies with $M_2$-KO mice found impairments in passive avoidance as well as elevated ACh efflux in the hippocampus[77].

Consistent with high striatal $M_4$ expression and principally pre-synaptic auto/heteroreceptor role(s) for $M_4$ in the CNS, $M_4$-KO mice were found to lack oxotremorine-mediated inhibition of ACh and DA release in striatal slices[76,78]. These mice also exhibited increased basal locomotor activity, increased sensitivity to dopamine $D_1$ receptor ($D_1$) agonist-induced hyperlocomotion, and increased sensitivity to phencyclidine (PCP)-mediated disruptions in pre-pulse inhibition (PPI)[79,80]. These results are not surprising given the purported interplay between central $M_4$ activation and decreased DA signaling[81]. Futhermore, they suggest that activation of $M_4$ may have

therapeutic relevance to treatment of the positive symptoms of schizophrenia, given the established role of elevated DA as a large component underlying this symptom cluster[44].

Despite these pronounced phenotypic effects of selective mAChR genetic deletion, all $M_1$-$M_5$-KO mice are devoid of overt abnormalities in general development, reproductive ability, or lifespan[45]. Furthermore, compensatory up-regulation of other mAChR subtypes in these KO mice appears to be absent, as expression levels for all remaining mAChR subtypes in every individual KO mice strain are approximately equal to those of WT mice[45]. Nonetheless, genetic approaches to studying mAChR function suffer from several disadvantages and caveats, such as differences in genetic background between multiple strains, unseen compensatory effects beyond the context of the cholinergic system, and the generally challenging technical aspects of mouse neurobiological research.

*Therapeutic potential of mAChRs as drug targets*

In light of the large body of data obtained from receptor localization and mAChR-KO mice studies, it is evident that selective activation or inhibition of signaling through one or more mAChR subtype may provide therapeutic utility for treatment of a wide range of diseases and disorders. Numerous pharmaceutical companies and research organizations have thus sought to discover and develop novel small molecule mAChR-targeted drugs[44]. However, due to the difficulty in obtaining truly selective ligands for a particular subtype that arises from high orthosteric binding site structural homology across all mAChRs, few muscarinic drugs have reached the market[44]. Internationally, current clinical use of approved muscarinic drugs is generally limited to antagonists for

the treatment of urinary incontinence, gastrointestinal and respiratory disorders, and in some cases Parkinson's disease. Nevertheless, great potential remains for drug discovery and development projects aimed at identifying novel subtype-selective mAChR ligands, particularly in the treatment of numerous CNS disorders based on the following hypotheses. For purposes of relevance to this thesis and in order to maintain focus on $M_1$, $M_4$, and $M_5$, which comprise the most important subtypes to treatment of CNS disorders, discussion of the therapeutic potential of selective $M_2$- or $M_3$- targeting is limited in the sections below.

One of the most well-known therapeutic potentials of selective $M_1$ activation is in the treatment of Alzheimer's disease, the most common cause of dementia, which is characterized by the two pathophysiological hallmarks of amyloid plaques comprised of amyloid-β (Aβ) and neurobrillary tangles (NFTs) comprised of hyperphosphorylated tau[44,82]. In general, progressive neurodegeneration with increased oxidative stress, inflammation, and decreased cerebral blood flow accompany these two hallmarks[82]. As is the situation for many CNS disorders, obtaining a clear and comprehensive understanding of the cause, progression, and complex molecular characteristics of AD has proven challenging, but will likely be vital to development of newer, more effective therapies. With the exception of the NMDA receptor antagonist Memantine, the only drugs currently approved for treatment of AD are AChE inhibitors (AChEIs), such as Donepezil and Rivastigmine, which are only moderately efficacious in addressing cognitive symptoms and cause numerous side effects[83,84]. The rationale for this AChE inhibition approach stems from a longstanding cholinergic hypothesis of AD[83,84].

Degeneration of basal forebrain cholinergic neurons was observed as an early event in AD, and it is now believed that this contributes to the impairments in cognitive function[85-87]. Furthermore, numerous links between decreased ACh, Aβ deposition, and tau NFTs, have been found[88-91]. This may provide a connection between the historic cholinergic hypothesis and more recent hypotheses that center on Aβ or tau as the primary factor in AD. Although a wide group of potential therapeutic strategies continue to emerge, many focus solely on reducing Aβ via inhibition of β-secretase, inhibition of γ-secretase, immunization to promote Aβ clearance, or prevention of Aβ aggregation[92]. However, cholinergic treatments directly targeting muscarinic receptors such as $M_1$ have also been explored preclinical and clinically as a means to overcome the limitations of AChE inhibitors. Although no $M_1$ agonists have reached the market, many have provided preclinical evidence supporting the $M_1$ receptor as a putative molecular target for AD[44].

In the cortex and hippocampus, $M_1$ is believed to mediate the post-synaptic actions of ACh released from basal forebrain neurons such as the nucleus basalis of Meynert (Ch. IV) and the diagonal band of Broca (Ch. II)[41,53,93]. Early and selective loss of these cholinergic neurons is a frequent feature of AD, and the degree of this degeneration correlates well with degree of dementia[94,95]. In light of this, lesioning of these neurons by molecular neurotoxins such as 192 IgG-saporin has been used in rats as a model for AD, which has been found to recapitulate many aspects of the disease[96]. Depending on specifics of the behavior test parameters, such as toxin dose and corresponding degree of lesioning, impairments in the learning of an attention task were observed in this model; however, performance defects in the Morris water maze were

only moderate, consistent with the results from studies using $M_1$-KO mice in this behavioral paradigm[96]. In agreement with *in vitro* experiments demonstrating that activation of $M_1$ or $M_3$ (but not $M_2$ or $M_4$) in recombinant cells drives amyloid precursor protein (APP) processing towards the non-amyloidogenic pathway, basal forebrain cholinergic lesioning has been shown to increase cortical APP expression and amyloidogenic processing[97-99]. Interestingly, these effects can be partially reversed by treatment of lesioned animals with a muscarinic agonist, further implicating the connection between the muscarinic system and APP regulation[99]. However, whether the observed non-amyloidogenic APP processing (i.e. reduction in Aβ secretion) *in vivo* is mediated by $M_1$ or $M_3$ and what downstream signaling pathways underlie this regulation remains to be determined, as the absence of subtype-selective ligands has hampered such investigation.

Many $M_1$ agonists have been evaluated in human AD trials, such as talsaclidine, sabcomeline, and xanomeline[100]. Although some of these compounds displayed efficacy in reducing behavioral symptoms or in decreasing Aβ levels in the cerebrospinal fluid (CSF), all such candidates to date have failed in development largely due to side effects arising from lack of subtype-selectivity, such as those mediated by peripheral $M_2$ and $M_3$ activation[44]. Efficacy of mAChR agonists in tests of cognitive function in AD trials was absent or moderate; however, it is not known whether this was due to lack of selectivity for $M_1$, poor CNS penetration or other drug metabolism and pharmacokinetic (DMPK) factors, dose-limitations from peripheral side effects, or if $M_1$ is simply not a clinically valid target for AD (or perhaps some combination of these possibilities)[44].

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In cortical and hippocampal samples obtained post-mortem from human AD patients, the protein levels of $M_1$ generally remain unaltered; however, $M_2$ levels are significantly reduced[101,102]. This is likely due to the pre-synaptic localization of $M_2$ on basal forebrain cholinergic neurons, which would be expected to reflect decreased $M_2$ levels as degeneration occurs. This decrease in $M_2$ is also found in basal forebrain cholinergic lesioned animals[96]. Given the pre-synaptic role of $M_2$ in regulating ACh release from these nuclei, selective targeting of this mAChR by small molecule antagonists has been considered in pharmaceutical development for AD[44]. *In vivo* antagonism of $M_2$ has been shown to increase ACh release; although, this approach suffers from potential drawbacks associated with the likely diminished $M_2$ receptor protein levels in already-diagnosed AD patients as well as the serious cardiovascular liabilities inherent to $M_2$ antagonism[44,102]. The fact that post-synaptic $M_1$ levels in the cortex remain approximately the same throughout AD suggests that selective activation of this subtype may be a more optimal approach if truly selective drugs were available. These findings and related considerations based on  links between $M_1$, cognition, APP processing, and tau phosphorylation have been used to construct proposals that integrate putative signaling relationships in AD (**Fig. 4**)[44].
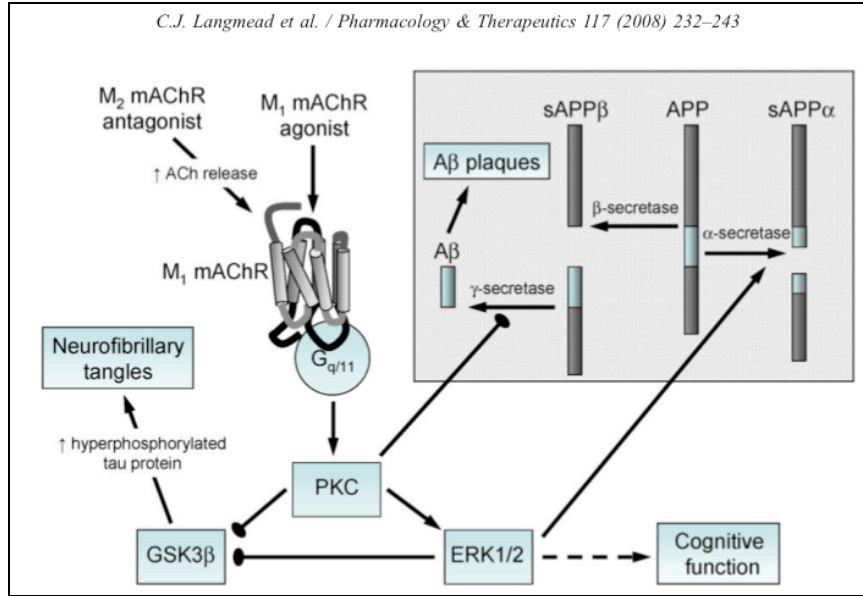
**Figure 4**. Proposed signaling relationships between $M_1$, APP processing, tau NFTs, and cognition, within the context of Alzheimer's disease. (Adapted from Langmead *et al.* 2008)

However, decreased functional activity of $M_1$ in brain tissue from AD patients may still limit the utility of this strategy, which may have contributed to the failure of muscarinic agonists in clinical trials for AD. Interestingly, decreases in high affinity agonist binding, agonist-stimulated PLC activity, agonist-stimulated guanosine trisphosphate-γS (GTPγS)-binding, and GTPase activity provide evidence that G-protein coupling downstream of $M_1$ is disrupted in AD[101,103]. The reason for this uncoupling is not known, nor is it established that restoration of G-protein signaling downstream of $M_1$ could be achieved via receptor agonism or potentiation by allosteric modulators known to increase G-protein coupling efficiency in recombinant systems.

In addition to AD, $M_1$ activation may prove useful in the treatment of schizophrenia[44]. Although a thorough discussion of the current understanding of the underlying mechanisms responsible for producing each of the positive, negative, and cognitive symptom clusters observed in schizophrenic patients lies beyond this scope of

this Chapter, it is generally believed that hyperactivity of the forebrain DA signaling contributes heavily to the positive symptom cluster[104]. This belief arose from numerous lines of evidence, including the observation that chronic administration of dopaminergic drugs of abuse such as cocaine and amphetamine can lead to emergence of positive symptom-like behaviors in non-schizophrenic individuals as well as the fact that most currently used antipsychotic drugs with positive symptom efficacy possess high-affinity DA receptor antagonist properties[104]. In light of the high expression of $M_1$ and $M_4$ in the striatum, the pronounced elevation in forebrain DA levels as well as the pro-psychotic phenotype (i.e. increased locomotion and increased sensitivity to dopamine agonists in behavioral models of psychosis) observed in mice lacking these receptors, it is possible that drugs targeting central $M_1$ and/or $M_4$ may prove efficacious in the alleviation of the positive symptoms of schizophrenia.

Interestingly, administration of NMDAR antagonists such as PCP can recapitulate all symptom clusters of schizophrenia in normal individuals and can also exacerbate symptoms in previously stable psychotic patients[105-107]. Correspondingly, enhancement of NMDAR signaling by increasing the synaptic concentration of its co-agonist glycine via inhibition of the central glycine type-1 transporter (GlyT1) has exhibited broad and robust efficacy in numerous preclinical models of psychosis[108,109]. These and other related findings have led to a NMDA hypofunction hypothesis of schizophrenia[106]. Given the importance of $M_1$ to certain aspects of learning and memory, including potentiation of NMDAR signaling, it is possible that $M_1$ activation may also prove efficacious in treatment of the cognitive symptom cluster. Although less established, $M_4$ activation may affect certain cognitive aspects of schizophrenia as well, given its

hippocampal expression and putative autoreceptor-mediated regulation of ACh release in multiple brain regions.

Perhaps the strongest evidence that $M_1$ and/or $M_4$ activation represents a viable treatment strategy for schizophrenia comes from clinical trials with the $M_1/M_4$-preferring orthosteric agonist xanomeline. Although lacking adequate subtype-selectivity and ultimately failing to successfully emerge from clinical development due to $M_2/M_3$-mediated dose-limiting peripheral side effects and DMPK-related issues, this compound proved effective in reducing a broad range of psychotic-like behavioral disturbances in a large phase III AD trial[100]. A subsequent phase II trial performed to directly explore the utility of xanomeline in treatment of schizophrenia revealed improvements in measures of cognitive function and in some positive symptoms, and a multitude of preclinical animal studies found similarly promising results[110]. However, it has remained a longstanding question as to what degree activation of $M_1$ and $M_4$ has contributed to these anti-psychotic effects of xanomeline.

Given the relationships between $M_1$- and $M_4$-mediated regulation of striatal DA signaling and motor function, antagonism of $M_1$ and/or $M_4$ has been proposed as an attractive therapeutic strategy for treatment of various motor-related disorders such as Parkinson's disease and dystonia[44,81]. Orthosteric anticholinergic drugs, such as procylidine and trihexyphenidyl, are presently used in the clinical management of such disorders; however, they suffer from poor-subtype selectivity and carry numerous side effects. Because $M_1$ activation is likely to be more important to cognitive function than $M_4$ activation in general, and because midbrain autoreceptor $M_4$ expression on cholinergic neurons projecting to the SNc and VTA (where it is believed to regulate ACh

release and thus subsequent excitation of dopaminergic projection neurons) renders $M_4$ antagonism particularly suited to reduction of forebrain DA levels, it has been suggested that selective $M_4$ antagonists would prove superior to $M_1$ antagonists in the treatment of motor dysfunction[44,111]. However, to date no highly selective $M_4$ antagonists have passed through clinical development.

In the case of $M_5$, selective antagonism could be hypothesized to provide efficacy in treating the positive symptoms of schizophrenia due to its associated but opposite relationship to $M_4$ in regulation of mesolimbic DA release (**Fig. 3**). Whereas activation of $M_4$ in the VTA would purportedly cause decreased DA release in the NAc, antagonism of $M_5$ would be required to exert the same effect, and vice-versa for increasing NAc DA levels, due to the putative post-synaptic excitatory role of $M_5$ compared to the pre-synaptic inhibitory role of $M_4$, in the VTA. Similarly, given the importance of activation of the mesolimbic DA pathway to the rewarding and addictive properties of many illicit drugs such as cocaine and opiates, similar pharmacological approaches may be useful in the treatment of drug addiction[44,111]. This represents perhaps the most well recognized therapeutic hypothesis for selective $M_5$ antagonism and lies in close agreement with studies using $M_5$-KO mice in models of drug addiction and reward. However, given the role of $M_5$ in mediating ACh-induced cerebrovasodilation and the various neuroanatomical and behavioral cognitive defects observed in $M_5$-KO mice, antagonism of $M_5$ for treatment of drug addiction may carry cerebrovascular and/or cognitive side effects.

Conversely, selective $M_5$ activation using a non-CNS penetrant drug would presumably produce the sole result of cerebrovasodilation, without impacting cholinergic

or dopaminergic neuronal activity and thereby avoiding unwanted side effects and recreational abuse potential. This possibility presents $M_5$ as a virtually ideal target for treatment of cerebrovascular disorders such as cerebrovascular dementia and prevention and/or treatment of ischemic stroke. If $M_5$ activation is found to regulate APP processing toward the non-amyloidogenic pathway in a manner analogous to that of $M_1$ or $M_3$, which would be predicted based on the close relationship between $M_1$, $M_3$, and $M_5$ intracellular signaling profiles, selective $M_5$ agonists may also prove useful for treating cerebral amyloid angiopathy (CAA). Furthermore, given that impaired cerebral blood flow and associated decreased cholinergic tone at cerebrovascular structures due to loss of cholinergic basal forebrain neurons has been observed in AD, it is possible that $M_5$ agonism alone or in combination with agonism of $M_1$ could address multiple aspects of AD pathology and thereby provide greater therapeutic benefit. However, no subtype-selective or even –preferring agonists or antagonists for the $M_5$ receptor have been reported, and therefore discovery of such ligands will clearly be necessary in order to pharmacological test these various therapeutic hypotheses.

## Allosteric modulation of GPCRs

*Introduction to allosteric modulation of GPCRs*

Small molecules that bind to cell-surface receptors at sites topographically distinct from the orthosteric binding site and thereby modulate receptor activity were discovered long ago for ligand-gated ion channels such as the γ-amino-butyric acid (GABA) receptor A (GABA$_A$)[112]. Numerous drugs of the benzodiazepine class bind to such allosteric

("other") sites on $GABA_A$, where they potentiate receptor activation in response to binding by orthosteric agonist (e.g. GABA)[113]. More recently, a number of small molecule chemotypes have been found to bind to allosteric sites on various GPCRs, such as the Class C metabotropic glutamate receptors (mGluRs) as well as numerous members of GPCR Class A including mAChRs (**Fig. 5**) and various chemokine receptors[112,114]. Relatively fewer allosteric ligands have been identified for Class B GPCRs, which could related to their lower abundance compared to Class A GPCRs[115]. These allosteric ligands may exhibit positive or negative modulatory effects on the target receptor, wherein the affinity and/or efficacy of the orthosteric probe (often the endogenous agonist) may be increased or decreased, termed positive allosteric modulation (PAM) and negative allosteric modulation (NAM), respectively[115]. Such modulation is fully dependant upon presence of an orthosteric ligand; however, some series of allosteric modulators may also exhibit intrinsic receptor activity on their own in the absence of orthosteric ligand, which is typically observed at higher concentrations[116]. Alternatively, an allosteric modulator may bind to a receptor but exhibit a pharmacologically neutral/silent effect, wherein binding affinity and functional efficacy of the orthosteric ligand remains unchanged[114,116]. Increasingly, evidence for probe-dependent allosteric modulation has demonstrated that a given PAM or NAM may cause dramatically different modulatory effects on the same receptor, depending on what orthosteric ligand is present[115].
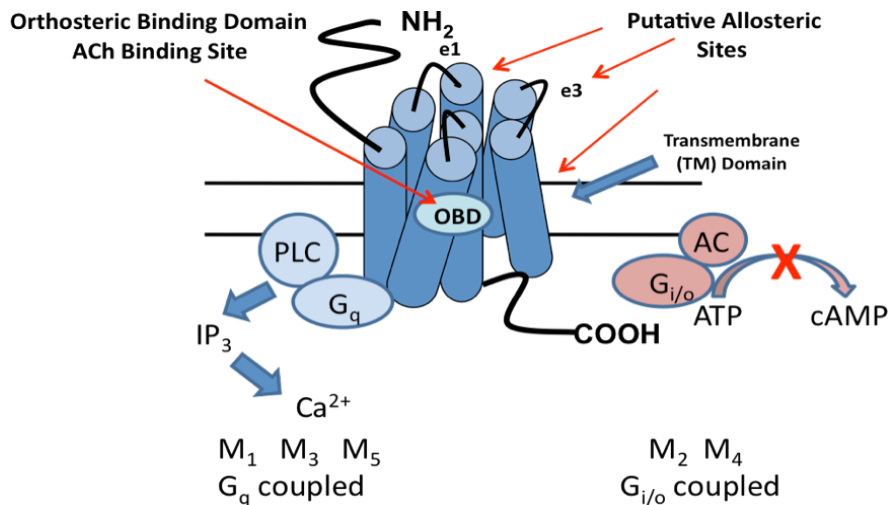
**Figure 5**. Generalized cartoon model of mAChR allosteric modulation depicting putative allosteric sites in relation to the orthosteric binding site.

Because receptor structure is often less conserved at allosteric binding sites across a given receptor subfamily (e.g. mAChRs), allosteric modulators often represent a means to achieve increased subtype-selectivity within a particular GPCR subfamily. However, given the possibility for probe-dependence, evidence is mounting that some purportedly highly-selective allosteric modulators only exhibit functional selectivity in the presence of a particular orthosteric agonist while actually binding to multiple subtypes of the target receptor subfamily with neutral modulation. In the presence of a different orthosteric probe, positive or negative modulatory effects at the other receptor subtypes may emerge, which underlines the importance of exploring functional selectivity with multiple orthosteric ligands. Nonetheless, allosteric modulators provide a number of possible therapeutic advantages over traditional orthosteric ligands beyond their often-superior selectivity for a given receptor[115]. Allosteric modulators provide a more subtle and physiologically-relevant approach to increasing or decreasing target activity because receptor activation will still only occur in the presence of the endogenous agonist that

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remains under normal regulation[115,116]. This may confer greater therapeutic tolerability and benefit for different CNS disorders. Furthermore, allosteric modulation may in some cases avoid receptor desensitization and/or down-regulation that often occurs following chronic administration of a traditional agonist[115,116]. Target/mechanism-mediated side effects that could arise from accidental overdose may also be avoided with allosteric modulator drugs because they confer a 'ceiling-effect' where maximal receptor occupancy does not directly translate to a maximal effect of receptor activation that would normally result from a high dose of an orthosteric drug[115].

Beyond the positive or negative modulation of a GPCR via an allosteric site, direct activation of the receptor in the absence of orthosteric ligand may also occur via allosteric ligand binding[115]. However, such allosteric agonists may also exhibit distinct subtype-selectivity via binding or functional propensities, positive or negative cooperativity with certain orthosteric ligands, and other pharmacological properties similar to those of PAMs or NAMs. Furthermore, different allosteric chemotypes may appear to be pure PAMs, NAMs, or agonists when a single receptor activation readout is used (e.g. mobilization of intracellular $Ca^{2+}$) but in reality possess various degrees of potency and efficacy for modulation of multiple downstream signaling cascades through the same receptor[117]. Such phenomena represent an additional layer of complexity in the study and use of allosteric modulators, which underlines the importance of thorough pharmacological characterization of a given compound prior to initiation of *in vivo* studies so that results may be properly interpreted.

Within the context of receptor theory, a variety of mathematical models have been presented to describe the relationships between allosteric modulator, orthosteric ligand,

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and receptor. These range in complexity from the simpler four-point allosteric ternary complex model (ATCM, **Fig. 6**) to intricate multidimensional sixteen-point models[114,115]. The former is based on the original ternary complex model (TCM) used to describe interactions between a ligand, receptor, and G-protein but is used here to describe interactions between two ligands and a receptor. In this model, each ligand is assigned an equilibrium dissociation constant ($K_a$ and $K_b$) as well as a cooperativity factor ($\alpha$) that denotes the mutual effect of the two ligands' on each other's affinity for the receptor. The value of $\alpha$ for two ligands can be determined empirically by measuring the affinity of one ligand (e.g. an orthosteric agonist) in the absence and presence of a saturating concentration of the second ligand (e.g. allosteric modulator) and then dividing the latter affinity by the former. Thus, values for $\alpha$ that are > 1.0 reflect positive cooperativity and those < 1.0 reflect negative cooperativity. An $\alpha = 1.0$ describes a cooperatively neutral relationship. Furthermore, efficacy modulation can be included in the ATCM by addition of the $\beta$ variable, to reflect the positive or negative effect of one ligand (e.g. PAM) on another's efficacy (e.g. orthosteric agonist) (**Fig. 6**).
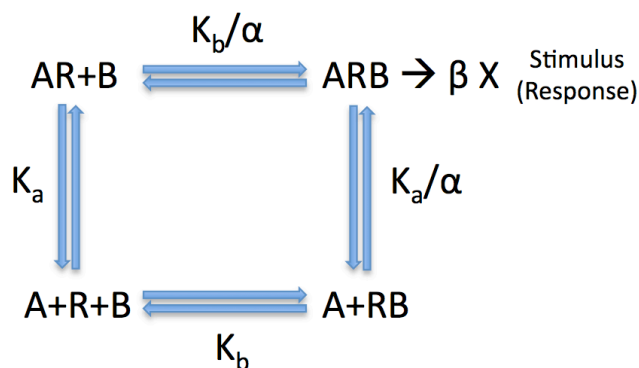


**Figure 6**. Allosteric ternary receptor complex model describing interaction between two ligands (e.g. allosteric modulator and orthosteric agonist) and a receptor. $K_a$ and $K_b$ reflect equilibrium dissociation constants (i.e. affinity values) for two ligands A and B that both bind simultaneously to receptor R. Binding cooperativity between A and B is denoted by $\alpha$. Modulation of efficacy is denoted by $\beta$.

*Characterization of allosteric modulators of GPCRs*

A variety of functional and binding assays are often used to identify and characterize allosteric modulators. In the case of cell-based functional high-throughput screening (HTS) campaigns aimed at discovering novel allosteric modulators, which often utilize recombinant stably-transfected receptor expression systems with a straight-forward readout for receptor activation such as fluorometric determination of intracellular $Ca^{2+}$ mobilization, 'double-add' or 'triple-add' assay formats are most often employed[116]. For example, under a double-add format (**Fig. 7**), test compound is added to the cells at a fixed concentration after baseline has been established (i.e. first add). After a 2-5 minute equilibration period, a submaximal (e.g. ~$EC_{20}$) concentration of agonist is added (i.e. second add). If the test compound is a PAM, it will enhance or potentiate the response to the submaximal agonist concentration (**Fig. 7**). Similar formats can be used to identify NAMs or antagonists by searching for compounds that decrease receptor activation in response to a near maximal (e.g. ~$EC_{80}$) concentration of agonist.
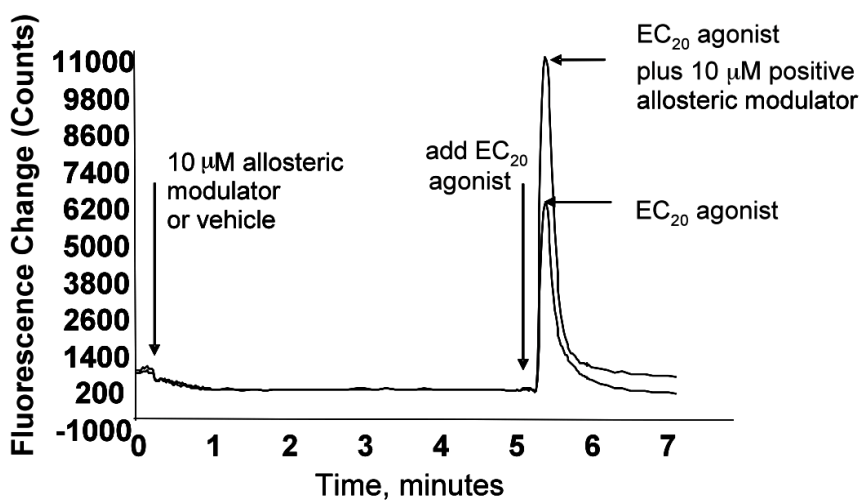


**Figure 7**. Example of a 'double-add' assay format designed to identify PAMs by functional fluorometric $Ca^{2+}$ mobilization readout.

Once hits from such HTS have been identified, similar functional counter-screening and secondary screening is often performed using a test compound CRC assay format in the presence of a fixed submaximal concentration of orthosteric agonist (**Fig. 8a**).  This allows determination of PAM (or NAM) potency values.  Related functional assays using CRCs of orthosteric agonist in the absence and presence of increasing fixed concentrations of test compound can be used to assess a modulator's maximum efficacy in terms of 'fold-shift' of the agonist $EC_{50}$ induced by the test compound (**Fig 8b**).  Together, these two basic assays can be used to characterize allosteric modulators' functional effects, which are typically used as the primary parameters in SAR descriptions and for driving potency/efficacy optimization efforts.
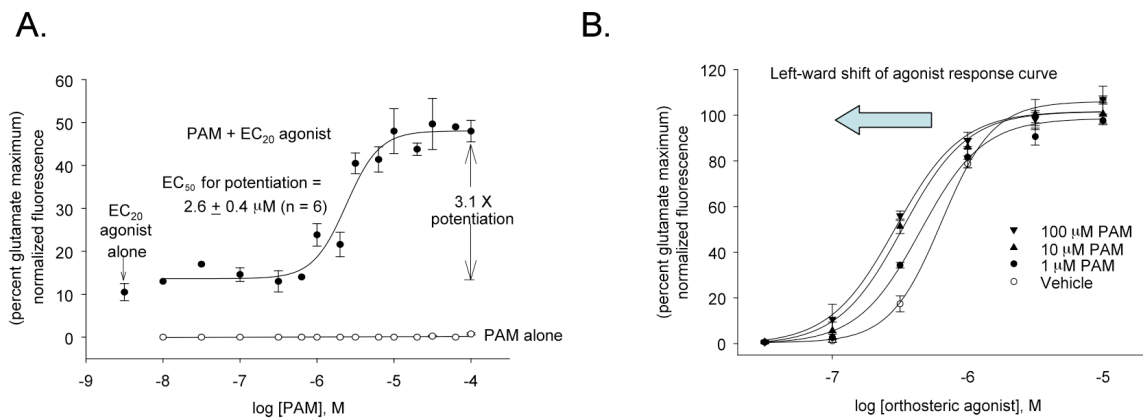


**Figure 8**.  Example of generic PAM CRC in the presence of a fixed submaximal concentration of orthosteric agonist to determine PAM potency (A) and agonist CRC in the absence and presence of increasing fixed concentrations of PAM to determine fold-shift (B).

Radioligand binding experiments are useful for assessing the location and mechanism(s) of modulation for a given allosteric ligand.  For instance, competition-binding under equilibrium conditions using a fixed low concentration of orthosteric antagonist radioligand in the presence of a CRC of an unlabelled allosteric modulator can

suggest or confirm that a novel compound binds to a site other than the orthosteric site if no inhibition is observed (i.e. allosteric). However, when a test compound causes binding inhibition, further experiments in the form of kinetic binding assays are often required to determine if such inhibition is via direct orthosteric competition with the radioligand or via a negative cooperativity effect (e.g. $1.0 > \alpha > 0$) through an allosteric site. Similar competition binding experiments utilizing CRCs of an orthosteric agonist in the absence and presence of fixed concentration(s) of allosteric modulator can be used to assess the latter's effect on the former's affinity and thereby calculate cooperativity $(\alpha)$[114]. Together, these various functional and binding assays comprise the most common methods for characterizing novel allosteric modulators and have been used throughout many of the mAChR ligand optimization projects described in subsequent Chapters of this thesis (Chapters III-V).

*Discovery of early allosteric modulators of mAChRs*

A number of small molecules emerged early on providing proof of concept for allosteric mAChR modulation (**Fig. 9**). These included gallamine, brucine, thiochromine, and other related compounds, which where found to exhibit positive or negative cooperativity with an orthosteric probe at one or more mAChR in functional and/or binding assays[118,119]. However, most of these ligands possessed poor potency, physiochemical properties, and DMPK liabilities that precluded their widespread use as mAChR research tools. Similarly, one of the first putative mAChR allosteric agonists, AC-42 (**Fig. 9**), exhibited highly $M_1$-selective functional activity but was found to exhibit little to no binding subtype-selectivity and displayed competition with multiple

orthosteric ligands[119,120]. Interestingly, this compound was equipotent and efficacious in assays with the Y381A mutant $M_1$ receptor, which has been used along with evidence from other experiments to argue that AC-42 interacts with either an ectopic site near or overlapping with the orthosteric ACh site or to multiple sites at $M_1$[117,121]. A second example of an early $M_1$ allosteric agonist is *N*-desmethylclozapine (NDMC, **Fig 9**), the primary circulating human metabolite of the commonly used antipsychotic drug clozapine[31]. Although NDMC is not completely selective for $M_1$, strong evidence that it binds to an allosteric site completely distinct from the orthosteric ACh site has been obtained from pharmacological experiments and receptor-mutagenesis studies[31].
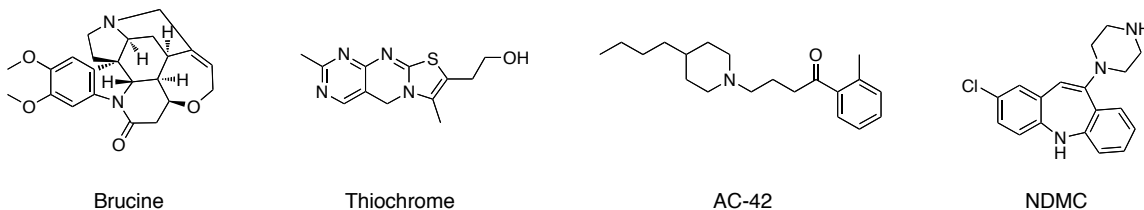


**Figure 9**. Structures of examples of early allosteric mAChR modulators.

Despite these discoveries and until recently, no highly subtype-selective mAChR allosteric ligands possessing the necessary drug-like pharmacological properties for universal and *in vivo* use had been reported. For instance, the AC-42 scaffold (now represented by its newer analog 77-LH-28-1) has been shown to possess a wide range of problematic off-target activities, NDMC exhibits similarly broad polypharmacology, and the early PAMs/NAMs do not represent tractable chemical optimization leads[120,122]. However, contemporary high-throughput screening technology utilizing functional cell-based assays has led to a re-emergence of novel allosteric mAChR modulator discovery

projects.  Subsequent Chapters of this thesis present and discuss our own efforts at identifying, optimizing, and characterizing novel subtype-selective ligands for $M_4$, $M_5$, and $M_1$ with the aim of obtaining useful drug-like tools suitable for the *in vitro* and *in vivo* study of mAChR neurobiology.

## Notes on figure, table, and compound numbering/nomenclature

Throughout the remaining Chapters, all figure and table numbering is specific to each Chapter.  Put differently, each Chapter's figure and table numbering begins with "1".  Similarly, compound numbering formats and nomenclature are specific to each Chapter and in some cases each section of a given Chapter.  Arbitrary numbering and letter codes (e.g. Compound 5 or (**5**), analog 9a or (**9a**)) are used interchangeably throughout each Chapter.  These are often used in place of full VU registration codes (e.g. VU0119498) in order to increase the ease of readability and to reduce space.  Furthermore, for Materials and Methods Chapter II, compound numbering/nomenclature essentially mirrors that found in the associated Chapters III-V.

# Chapter II

## MATERIALS AND METHODS

### Medicinal Chemistry

*General synthetic methods, instrumentation, and workflow*

In general, small scale (20-100 mg) reactions were carried out in parallel to generate compound libraries ranging from approximately 4-100 members in size. A small percentage of the total compounds synthesized across all projects were generated as singletons (i.e. individual isolated syntheses and not part of a library). Standard chemistry techniques were used for all reactions, and traditional thin-layer chromatography (TLC) and liquid chromatography mass spectrometry (LCMS) was used for analytical reaction monitoring. The following general purification methods were utilized depending on the particular chemical/compound and the amount of material: classical normal-phase glass-column silica gel chromatography, mass-directed reverse-phase high-performance liquid chromatography (HPLC), ultraviolet (UV)-directed reverse-phase HPLC, and standard recrystallization/filtration. Only high-quality HPLC grade solvents were used for purifications, and purity was defined as a sample containing approximately >98% of desired product by at least two independent analytical methods (e.g. evaporative light scattering device [ELSD], UV).

Microwave-assisted reactions were conducted using a Biotage Initiator-60. All nuclear magnetic resonance (NMR) spectra were recorded on a 400 megahertz (MHz) Bruker AMX NMR. $^{1}$H chemical shifts are reported in δ values in parts per million

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(ppm) downfield from TMS as the internal standard in dimethyl sulfoxide (DMSO).  Data are reported as follows:  chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), coupling constant (Hz), and integration.  [13]C chemical shifts are reported in δ values in ppm with the DMSO carbon peak set to 39.5 ppm.  Low resolution mass spectra were obtained on an Agilent 1200 LCMS with electrospray ionization.  High resolution mass spectra were recorded on a Waters QToF-API-US plus Acquity system with electrospray ionization.  Analytical thin layer chromatography was performed on 250 μm silica gel 60 $F_{254}$ plates.  Analytical HPLC was performed on an Agilent 1200 analytical LCMS with UV detection at 214 nm and 254 nm along with ELSD detection.  Preparative purification of library compounds was performed on a custom Agilent 1200 preparative LCMS with collection triggered by mass detection.

For purposes of compound tracking and organization, all final compounds were transferred to 4 mL barcoded vials and diluted to a final concentration of 10 (or in some cases 50) mM in DMSO.  Each new compound and each new batch of a previously synthesized compound was registered into a central computer database system, which assigned a unique VU identification number.  A new synthetic batch of a given compound is designated by a corresponding number following a "—" at the end of the VU identification number (e.g. batch one of VU0238429 is registered as VU0238429-1, batch two as VU0238429-2, etc...).  At the present time of writing, the commercially available ChemChart program was used as the compound registration database software.

Following purification, dilution, and registration, compounds were characterized and assayed for pharmacological activity using the methods described in the associated

pharmacology section of this Chapter. In general, optimization projects utilized an iterative parallel library work-flow, wherein a certain region of a chemical scaffold was derivatized while the remainder of the scaffold was held constant for each analog of a given library. After pharmacological evaluation, SAR data from a given present library was used to design/plan the subsequent library such that the optimization proceeds in a cyclic and progressive manner toward improvement of a given property or characteristic of the scaffold (e.g. potency, efficacy, subtype-selectivity, off-target activity, cLogP, etc..). One implementation of this paradigm that was routinely utilized is presented in **Figure 1**. For most projects, an initial HTS using a functional cell-based assay was performed with either the Molecular Libraries Screening Center Network (MLSCN)/Molecular Libraries Probe Center Network (MLPCN) or the Vanderbilt HTS center (VHTSC) compound collections to identify hits. In other projects, lead compounds were identified via cheminformatics or via public disclosures (e.g. conferences, scientific literature, patent literature, etc…).
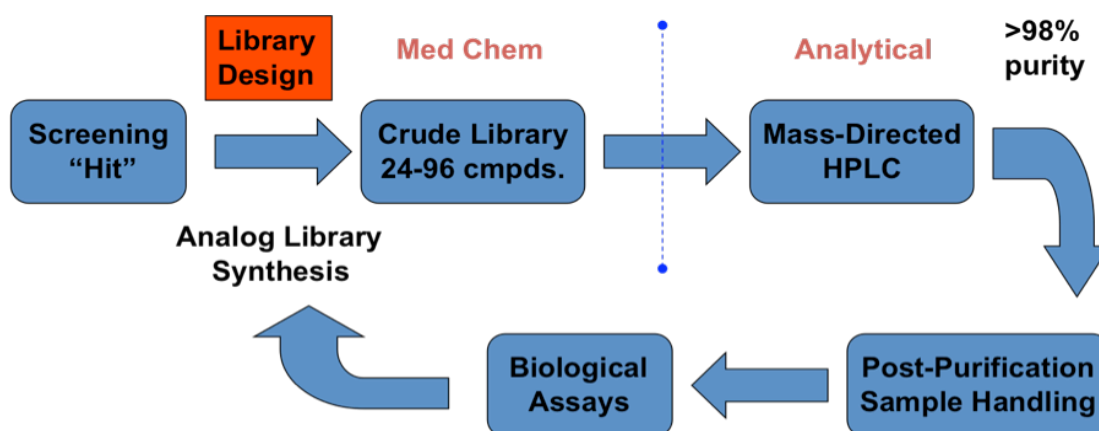


**Figure 1**. Generalized work-flow utilizing iterative synthesis and testing of parallel analog libraries aimed at optimizing a given property or characteristic of the lead structure/scaffold.

The complete details of each compound's full synthetic and purification procedure are too expansive for inclusion in this Chapter, and therefore such are presented only for select key and representative compounds/libraries from the various mAChR small molecule discovery/optimization projects described in this thesis. In general, the procedure for a specific type of reaction (e.g. amide-couplings, *N-* or *O-*alkylations, Suzuki-couplings, etc...) that is described for a given compound/library is applicable to other related compounds/libraries of a project that were made using the same reaction. Therefore, redundant procedures and procedures for non-key compounds/libraries have been excluded for sake of brevity and simplicity. For compounds/libraries whose procedures are not explicitly described, the conditions for their synthesis are presented in abbreviated form in the figures and text of Chapters III-V and in more detail in the associated referenced publications. Furthermore, to aid in clarity and organization, the order in which the syntheses are presented in this Chapter generally reflects the order in which the associated text and figures of Chapters III-V discuss the compounds/libraries. For some compounds whose synthesis is described under an general library procedure, only analytical characterization data is presented. Compound numbering in this Chapter is the same as that which is used in the associated text/figures of Chapters III-V.

*M$_4$ PAM syntheses*

**Initial eastern amide analog library general procedure.** Each of thirty-one glass vials containing 3 mL of CH$_2$Cl$_2$ were loaded with *N,N-*diisopropylethylamine (0.3 mL, 1.70 mmol), 3-amino-4,6-dimethylthioenol[2,3-*b*]-pyridine-2-carboxylic acid (50

mg, 0.225 mmol, SPECS AM-807/25050004), 1-hydroxybenzotriazole hydrate (30.4 mg, 0.225 mmol, 1.0 equivalents), polystyrene-bound *N*-cyclohexylcarbodiimide (317 mg, 0.450 mmol, 1.42 mmol/g, 2.0 equivalents), and one of thirty-one amines (0.225 mmol, 1.0 equivalents). The reactions were stirred for 48 hours at room temperature. Next, macroporous triethylammonium methylpolystyrene carbonate (145 mg, 0.225 mmol, 3.11 mmol/g, 2.0 equivalents) was added, and the reactions were stirred for an additional 3 hours at room temperature. Then, the reactions were filtered and concentrated on a heat-air block to afford 84-99% pure products. Those <99% pure were purified by mass-directed HPLC.

**VU0152099.** To a stirred solution of 3-amino-4,6-dimethylthioenol[2,3-*b*]-pyridine-2-carboxylic acid (3.0 g, 13.51 mmol [Chembridge cat.# 4003557]) in $CH_2Cl_2$ (90 mL) was added *N,N*-diisopropylethylamine (10 mL, 56.66 mmol), 1-hydroxybenzotriazole hydrate (1.83 g, 13.51 mmol, 1.0 equivalents), 4-methoxybenzylamine (2.04 g, 14.86 mmol, 1.1 equivalents), and *N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride (5.18 g, 27.02 mmol, 2.0 equivalents) at 25 °C under room atmosphere. After 48 hours, macroporous triethylammonium methylpolystyrene carbonate (4.4 g, 13.51 mmol, 3.077 mmol/g, 1.0 equivalents) was added to the solution, which was then stirred for 3 hours at 25°C under room atmosphere. Next, the solution was vacuum filtered and the filtrate was separated with citric acid (1.0 M in water) and $CH_2Cl_2$. The organics were dried over $MgSO_4$ and concentrated *in vacuo* to produce a dark yellow solid. The solid was purified by column chromatography (silica gel, fixed 1:2 EtOAc:hexanes) to afford 2.5 g (7.33 mmol, 54%) of the title compound as a bright yellow solid. Analytical LC/MS (J-Sphere80-S4, 3.0 x

50 mm, 4.0 min gradient, 5%[CH$_3$CN]:95%[0.1%TFA/H$_2$O] to 100%[CH$_3$CN]): 2.773

min, >99% (214 nm and ELSD), M+1 peak *m/e* 342.12; ^{1}H NMR (400 MHz, DMSO-d$_6$)

δ 7.27 (d, *J* = 8.8 Hz, 2H), 6.89 (s, 1H), 6.86 (d, *J* = 8.8 Hz, 2H), 6.34 (br s, 2H), 5.80 (s,

1H), 4.53 (d, *J* = 6.0 Hz, 2H), 3.79 (s, 3H), 2.73 (s, 3H), 2.57 (s, 3H); ^{13}C NMR (100

MHz, DMSO-d$_6$) δ 165.9, 159.3, 159.2, 147.7, 143.9, 130.7, 129.3, 123.7, 122.4, 114.4,

98.5, 55.5, 43.3, 24.5, 20.4;    HRMS (Q-ToF): *m/z* calc for C$_{18}$H$_{19}$N$_3$O$_2$S [M + H]:

342.1198; found, 342.1276.

    **VU0152100.**    To a stirred solution of 3-amino-4,6-dimethylthioenol[2,3-*b*]-

pyridine-2-carboxylic acid (2.50 g, 11.26 mmol [Chembridge cat.# 4003557]) in CH$_2$Cl$_2$

(90 mL) was added *N,N*-diisopropylethylamine (10 mL, 56.66 mmol), 1-

hydroxybenzotriazole hydrate (1.52 g, 11.26 mmol, 1.0 equivalents), piperonylamine

(1.87 g, 12.38 mmol, 1.1 equivalents), and *N*-(3-dimethylaminopropyl)-*N'*-ethyl-

carbodiimide hydrochloride (4.32 g, 22.52 mmol, 2 equivalents) at 25 °C under room

atmosphere.  After 48 hours, macroporous triethylammonium methylpolystyrene

carbonate (3.66 g, 11.26 mmol, 3.077 mmol/g, 1.0 equivalents) was added to the solution,

which was then stirred for 3 hours at 25 °C under room atmosphere.  Next, the solution

was vacuum filtered and the filtrate was separated with citric acid (1.0 M in water) and

CH$_2$Cl$_2$.  The organics were dried over MgSO$_4$ and concentrated *in vacuo* to produce a

dark yellow solid.  The solid was purified by column chromatography (silica gel, fixed

1:2 EtOAc:hexanes) to afford 2.0 g (5.63 mmol, 50%) of the title compound as a yellow

solid.    Analytical LC/MS (J-Sphere80-S4, 3.0 x 50 mm, 4.0 min gradient,

5%[CH$_3$CN]:95%[0.1%TFA/H$_2$O] to 100%[CH$_3$CN]): 2.740 min, >99% (214 nm and

ELSD), M+1 peak *m/e* 356.10; ^{1}H NMR (400 MHz, DMSO-d$_6$) δ 8.38 (s, 1H), 7.18 (s,

1H), 6.88 (s, 1H), 6.84 (d, $J$ = 8.0 Hz, 1H), 6.78 (d, $J$ = 8.0 Hz, 1H), 5.98 (br s, 2H), 5.97 (s, 2H), 4.30 (d, $J$ = 5.2 Hz, 2H), 2.77 (s, 3H), 2.57 (s, 3H); $^{13}$CNMR (100 MHz, DMSO-$d_6$) δ 179.9, 164.8, 161.7, 158.0, 153.4, 147.4, 133.8, 122.4, 121.9, 120.5, 108.0, 107.9, 100.8, 92.8, 42.1, 22.4, 20.0;  HRMS (Q-ToF): $m/z$ calc for $C_{18}H_{17}N_3O_3S$ [M + H]: 356.0991; found, 356.1069.

**6-amino-2-mercapto-4-methylnicotinonitrile (6).**  To five 20 mL microwave vials containing 2-cyanothioacetamide (0.01 mol) each was added 3-aminocrotonitrile (0.01 mol) and then EtOH (10 mL).  Once dissolved, pyridine (0.05 mol) was added and the vial was capped.  The reaction mixtures were heated to 160°C for 12 min. consecutively.  The crude reaction mixtures were combined and concentrated to a dark red oil.  To this was added MeOH (150ml) and heated to reflux, upon cooling an orange solid crashed out which was filtered and washed with ether to afford 3.47g of pure **(6)** (42%).  $^1$H NMR (400MHz, DMSO-$d_6$): d= 7.20 (br s, 2H), 5.90 (s, 1H), 2.2 (s, 3H). $^{13}$C (100MHz, DMSO-$d_6$): d= 175.3, 155.1, 153.5, 118.0, 100.2, 98.9, 20.8. HRMS calcd for $C_7H_7N_3S$ [M + H] 166.0435; found 166.0439.

**3,6-diamino-$N$-(4-methoxybenzyl)-4-methylthieno[2,3-$b$]pyridine-2-carboxamide  (8).**  To a 20 mL microwave vial containing **(6)** (3.0 mmol) was added $K_2CO_3$ (6 mmol), and DMF (10 mL).  To this solution was added solid **(7)** then capped, and heated to 160°C for 10 min.  Once complete the reaction was partitioned between $H_2O$ (100 mL) and DCM (100 mL), separated, and the aqueous layer washed 2x with DCM (100 mL).  The organic layer was dried with solid $MgSO_4$, filtered, and concentrated to a light brown solid.  This solid was crystallized in EtOAc and Hexanes and filtered to obtain 0.8g of pure **(8)** as a light yellow solid (75%).  $^1$H NMR (400MHz,

DMSO-$d_6$): d= 8.41 (s, 1H), 7.13 (d, $J$ = 8.8Hz, 2H), 6.98 (br s, 2H), 6.83 (d, $J$ = 8.4 Hz, 2H), 6.12 (s, 1H), 3.89 (s, 2H), 3.72 (s, 3H), 2.22 (s, 3H). $^{13}$C (100MHz, DMSO-$d_6$): d= 167.4, 160.8, 160.1, 158.1, 151.0, 131.1, 128.3, 116.7, 113.7, 103.9, 55.0, 41.9, 32.8, 19.6. HRMS calcd for $C_{17}H_{18}N_4O_2S$ [M + H] 343.1222; found 343.1229.

**Alkylamine library (9a-s) general procedure.** To a 5 mL microwave vial containing **(8)** (0.073 mmol), was added $Cs_2CO_3$ (0.21 mmol), KI (0.073 mmol), $R^1$-Cl (0.080 mmol), and DMF (3 mL). The reaction was stirred 5 min. to eliminate $CO_2$, capped, and heated to 160°C for 30 min. Once complete the reaction was partitioned between $H_2O$ (5 mL), and DCM (5 mL), separated via a 12 mL IST phase separator, concentrated and purified by mass directed HPLC to furnish **9a-s** (15-80%).

**Alkylether library (13a-s) general procedure.** To a 5 mL microwave vial containing **(12)** (0.146 mmol), was added $Cs_2CO_3$ (0.438 mmol), KI (0.291 mmol), $R^1$-Cl (0.219 mmol), and DMF (3 mL). The reaction was stirred 5 min. to eliminate $CO_2$, capped, and heated to 160°C for 30 min. Once complete the reaction was partitioned between $H_2O$ (5 mL), and DCM (5 mL), separated via a 12 mL IST phase separator, concentrated and purified by mass directed HPLC to furnish **13a-s** (15-80%).

**2-mercapto-4-methyl-6-oxo-1,6-dihydropyridine-3-carbonitrile (11).** To twelve 20 mL microwave vials containing 2-cyanothioacetamide (0.01 mol) each was added morpholine (0.01 mol) and then EtOH (10 mL). Once dissolved, ethylacetoacetate (0.01 mol) was added and the vial was capped. The reaction mixtures were heated to 160°C for 10 min. consecutively. The crude reaction mixtures were combined and concentrated to a dark red oil. To this was added DCM (200ml) and heated to reflux. Once cool, the solid product was filtered and washed 2x with DCM (200ml) to give 12.4g

of pale yellow **(11)** (62%). $^1$H NMR (400MHz, DMSO-d$_6$): d= 10.40 (br s, 1H), 8.67 (br s, 1H), 5.38 (s, 1H), 1.99 (s, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 176.3, 162.4, 151.0, 120.7, 106.8, 92.7, 20.5. HRMS calcd for C$_7$H$_6$N$_2$OS [M + H] 167.0277; found 167.0279.

**3-amino-*N*-(4-methoxybenzyl)-4-methyl-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-2-carboxamide (12).** To a flask containing **(11)** (6.0 mmol) was added DMF (30 mL) then TEA (6.6 mmol), and cooled to 0$^o$C with an ice bath. A solution of **(7)** (6.0 mmol) in DMF (30 mL) was slowly added to the reaction via addition funnel over 30 min. and allowed to return to r.t. overnight. The reaction was concentrated then partitioned between DCM (100 mL) and H$_2$O (100 mL), separated, and the aqueous layer washed 2x with DCM (100 mL). The organic layer was dried with solid MgSO$_4$, filtered, and concentrated to a pale yellow solid which was washed with EtOAc (3x 50 mL) to give 1.28g of pure **(12)** as a light yellow solid (62%). $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.52 (br s, 1H), 7.15 (d, *J* = 8.8 Hz, 2H), 6.84 (d, *J* = 8.8 Hz, 2H), 6.40 (s, 1H), 4.22 (d, *J* = 6.0 Hz, 2H), 3.97 (br s, 2H), 3.71 (s, 3H), 2.32 (s, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 167.3, 158.2, 130.9, 128.7, 128.5, 115.5, 113.7, 113.6, 55.0, 42.0, 19.7. HRMS calcd for C$_{17}$H$_{17}$N$_3$O$_3$SNa [M + Na] 366.0888, found 366.0887.

**Ethyl 3-amino-4-methyl-6-oxo-6,7-dihydrothieno[2,3-*b*]pyridine-2-carboxylate (15).** To a flask containing **(11)** (26.5 mmol) was added DMF (250 mL) then TEA (29.2 mmol), and cooled to 0$^o$C with an ice bath. A solution of **(14)** (26.5 mmol) in DMF (60 mL) was slowly added to the reaction via addition funnel over 30 min. and allowed to return to r.t. overnight. The reaction was concentrated then partitioned between DCM (250 mL) and H$_2$O (250 mL), separated, and the aqueous layer

washed 2x with DCM (150 mL). The organic layer was dried with solid $MgSO_4$, filtered, and concentrated to a pale yellow solid which purified via chromatography (1:1.5 EtOAc:Hexanes) to give 4.15g of pure (15) as a light yellow solid (63%).^{1}H NMR (400MHz, DMSO-$d_6$): d= 6.42 (s, 1H), 4.12 (m, 4H), 2.34 (s, 3H), 1.18 (t, $J$ = 7.2 Hz, 3H). [13]C (100MHz, DMSO-$d_6$): d= 168.3, 164.8, 160.0, 154.5, 115.5, 106.8, 97.8, 59.9, 19.7, 13.9. HRMS calcd for $C_{11}H_{13}N_2O_3S$ [M + H] 253.0647, found 253.0644.

**Alkyl pyridone ethyl ester (16a-e) general procedure.** To a 20 mL microwave vial containing (15) (0.04 mol), was added $Cs_2CO_3$ (0.012 mol), KI (0.008 mol), $R^1$-Cl (0.0044 mmol), and DMF (10 mL). The reaction was stirred 5 min. to eliminate $CO_2$, capped, and heated to 160°C for 30 min. Once complete the reaction was partitioned between $H_2O$ (50 mL), and DCM (50 mL), separated, and the aqueous washed 1x with DCM (50 mL). The organic layer was dried with $MgSO_4$, filtered and concentrated. The resulting material was added to a 10g 60CC SCX cartridge and washed with 3 column volumes of MeOH. The product was then eluted with 2 column volumes of 2M $NH_3$ in MeOH to provide pure (16a-e) (75-95%).

**Ethyl 3-amino-6-(3-(dimethylamino)propoxy)-4-methylthieno[2,3-*b*]pyridine-2-carboxylate (16a).** ^{1}H NMR (400MHz, DMSO-$d_6$); d= 6.72 (s, 2H), 6.57 (s, 1H), 4.25 (m, 4H), 2.66 (s, 3H), 2.37 (t, $J$ = 7.2 Hz, 2H), 2.16 (s, 6H), 1.84 (m, 2H), 1.27 (t, $J$ = 6.8 Hz, 3H). [13]C (100MHz, DMSO-$d_6$): d= 164.7, 163.8, 159.3, 150.3, 147.6, 119.1, 109.5, 64.5, 59.7, 55.5, 44.9, 26.3, 19.8, 14.4. HRMS calcd for $C_{16}H_{24}N_3O_3S$ [M + H] 338.1538, found 338.1527.

**Ethyl 3-amino-4-methyl-6-(2-morpholinoethoxy)thieno[2,3-*b*]pyridine-2-carboxylate (16b).** ^{1}H NMR (400MHz, DMSO-$d_6$): d= 6.76 (s, 2H), 6.65 (s, 1H), 4.57

(br s, 2H), 4.24 (q, $J$ = 14.4, 7.2 Hz, 2H), 3.72 (br s, 4H), 3.20 (br s, 6H), 2.70 (s, 3H), 1.27 (t, $J$ = 6.8 Hz, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 164.7, 163.1, 159.0, 150.3, 148.1, 119.6, 118.7, 115.7, 109.6, 64.3, 59.8, 55.5, 52.3, 19.9, 14.4. HRMS calcd for C$_{17}$H$_{24}$N$_3$O$_4$S [M + H] 366.1488, found 366.1476.

**Ethyl 3-amino-4-methyl-6-(pyridin-2-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxylate (16c).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.55 (d, $J$ = 4.4 Hz, 1H), 7.80, (t, $J$ = 8.0 Hz, 1H), 7.46 (d, $J$ = 8.0 Hz, 1H), 7.33 (t, $J$ = 6 Hz, 1H), 6.74 (s, 2H), 6.73 (s, 1H), 5.45 (s, 2H), 4.23 (q, $J$ = 14.0, 7.2 Hz, 2H), 2.70 (s, 3H), 1.26 (t, $J$ = 7.2 Hz, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 164.7, 163.3, 159.0, 156.3, 150.2, 149.1, 148.1, 136.8, 122.9, 121.7, 119.6, 109.6, 68.1, 59.8, 19.9, 14.4. HRMS calcd for C$_{17}$H$_{18}$N$_3$O$_3$S [M + H] 344.1069, found 344.1057.

**Ethyl 3-amino-4-methyl-6-(pyridin-3-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxylate (16d).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.70 (s, 1H), 8.54 (d, $J$ = 4.8 Hz, 1H), 7.89 (d, $J$ = 8.0 Hz, 1H), 7.42 (t, $J$ = 4.8 Hz, 1H), 6.75 (s, 2H), 6.97 (s, 1H), 5.42 (s, 2H), 4.24 (q, $J$ = 14.4, 7.2 Hz, 2H), 2.69 (s, 3H), 1.28 (t, $J$ = 6.8 Hz, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 164.7, 163.3, 159.0, 150.3, 149.4, 149.1, 148.1, 136.1, 132.3, 123.5, 119.6, 109.6, 65.3, 59.8, 19.9, 14.4. HRMS calcd for C$_{17}$H$_{18}$N$_3$O$_3$S [M + H] 344.1069, found 344.1064.

**Ethyl 3-amino-4-methyl-6-(pyridin-4-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxylate (16e).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.56 (d, $J$ = 6.0 Hz, 2H), 7.45 (d, $J$ = 6.0 Hz, 2H), 6.77 (s, 1H), 6.75 (s, 2H), 5.45 (s, 2H), 4.24 (q, $J$ = 14.0, 6.8 Hz, 2H), 2.71 (s, 3H), 1.27 (t, $J$ = 7.2 Hz, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 164.6, 163.1, 159.0,

150.2, 149.6, 148.3, 145.9, 122.0, 109.6, 65.7, 59.8, 19.9, 14.4. HRMS calcd for $C_{17}H_{18}N_3O_3S$ [M + H] 344.1069, found 344.1057.

**3-amino-*N*-(4-methoxybenzyl)-4-methyl-6-(pyridin-4-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxamide dihydrochloride (13k).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.90 (d, *J* = 6.4 Hz, 2H), 8.14 (t, *J* = 5.6 Hz, 1H), 8.05 (d, *J* = 6.4 Hz, 2H), 7.22 (d, *J* = 8.8 Hz, 2H), 6.87 (d, *J* = 8.8 Hz, 2H), 6.84 (s, 1H), 5.73 (s, 2H), 4.32 (d, *J* = 5.6 Hz, 2H), 3.72 (s, 3H), 2.74 (s, 3H). ). $^{13}$C (100MHz, DMSO-d$_6$): d= 165.0, 162.0, 158.1, 156.9, 148.1, 147.9, 142.2, 132.0, 129.3, 128.5, 124.1, 121.0, 115.1, 113.6, 109.4, 65.3, 55.0, 41.6, 19.9. HRMS calcd for $C_{23}H_{23}N_4O_3S$ [M + H] 435.1491, found 435.1490.

**3-amino-*N*-(2,3-difluorobenzyl)-4-methyl-6-(pyridin-4-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxamide dihydrochloride (21n).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.92 (d, *J* = 6.8 Hz, 2H), 8.30 (t, *J* = 5.6 Hz, 1H), 8.09 (d, *J* = 6.4 Hz, 2H), 7.32 (m, 1H), 7.16 (m, 2H), 6.85 (s, 1H), 5.75 (s, 2H), 4.46 (d, *J* = 5.6 Hz, 2H), 2.74 (s, 3H). $^{13}$C (100MHz, DMSO-d$_6$): d= 165.2, 162.1, 157.8, 157.0, 150.8, 148.8, 148.3, 148.1, 146.4, 146.2, 141.4, 129.3 (d, *J* = 11.0 hz), 124.5, 124.3, 120.9, 115.6 (d, *J* = 17.0 Hz), 109.5, 65.3, 35.7, 19.9. HRMS calcd for $C_{22}H_{19}N_4O_2F_2S$ [M + H] 441.1197, found 441.1196.

**3-amino-*N*-(2,5-difluorobenzyl)-4-methyl-6-(pyridin-4-ylmethoxy)thieno[2,3-*b*]pyridine-2-carboxamide dihydrochloride (21o).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 8.57 (d, *J* = 6.0 Hz, 2H), 8.19 (t, *J* = 5.6 Hz, 1H), 7.44 (d, *J* = 6.0 Hz, 2H), 7.24 (m, 1H), 7.10 (m, 2H), 6.77 (br s, 2H), 6.77 (s, 1H), 5.46 (s, 2H), 4.43 (d, *J* = 5.6 Hz, 2H), 2.72 (s, 3H). ). $^{13}$C (100MHz, DMSO-d$_6$): d= 165.3, 162.7, 159.3, 157.2, 156.9, 154.7, 149.6, 148.4, 147.9, 146.0, 128.8 (dd, *J* = 7.0, 17.0 Hz), 121.9, 120.6, 116.6 (dd, *J* = 8.0, 24.0

Hz), 115.2 (dd, $J$ = 5.0, 25.0 Hz), 114.9 (dd, $J$ = 8.0, 24.0 Hz), 109.6, 65.7, 35.9, 19.9.

HRMS calcd for $C_{22}H_{19}N_4O_2F_2S$ [M + H] 441.1197, found 441.1194.

**3-amino-*N*-*tert*-butyl-4-methyl-6-(2-morpholinoethoxy)thieno[2,3-*b*]pyridine-2-carboxamide dihydrochloride (18h).** $^1$H NMR (400MHz, DMSO-d$_6$): d= 6.65 (s, 1H), 6.62 (s, 1H), 6.31 (br s, 3H), 4.71 (t, $J$ = 4.8 Hz, 2H), 3.87 (m, 4H), 3.50 (m, 4H), 3.17 (m, 2H), 2.70 (s, 3H), 1.36 (s, 9H). $^{13}$C (100MHz, DMSO-d$_6$): d= 165.3, 162.2, 162.1, 156.6, 147.6, 147.1, 120.9, 109.5, 62.0, 60.5, 54.7, 51.6, 51.2, 28.8, 19.9. HRMS calcd for $C_{19}H_{29}N_4O_3S$ [M + H] 393.1960, found 393.1958.

*M$_5$ PAM syntheses*

**Initial matrix library of VU0119498 analogs.** Compounds 21-112 were synthesized in parallel according to the following procedure. Isatin starting materials (0.34 mmol) were added to vials containing ACN (3 mL), K$_2$CO$_3$ (0.68 mmol, 2.0 eq), KI (0.03 mmol, 0.1 eq), and respective benzyl halides (0.85 mmol, 2.5 eq). The reactions were microwave irradiated at 160 °C for 10 min. Next, the reactions were partitioned into CHCl$_2$ and H$_2$O and then passed through disposable phase-separator columns (Biotage Isolute). The organics were concentrated on a heated air-drying block and then analyzed by LCMS. Purification by mass-directed Preparative HPLC afforded desired products as colored solids (20-95%) with >98% purity by ELSD and 214 nM UV analysis.

**VU0119498.** This HTS screening hit was resynthesized classically as a singleton prior to the library synthesis. To a vial containing ACN (25 mL) was added isatin (1.00 g, 6.80 mmol), K$_2$CO$_3$ (13.6 mmol, 2.0 eq), KI (0.68 mmol, 0.1 eq), and 4-

bromobenzylbromide (7.48 mmol, 1.1 eq). The reaction was stirred for ~24 hours at room temperature while monitored by TLC. After judging complete, the reaction was partitioned into EtOAc and $H_2O$, and the combined organics were dried over $MgSO_4$, filtered, and then concentrated *in vacuo*. The dried solid was then washed with diethyl ether (3 x 15 mL) to afford the pure 1-(4-bromobenzyl)indoline-2,3-dione title compound as a bright orange solid (1.96 g, 6.18 mmol, 91%). $^1$H-NMR (400MHz, $d_6$ - DMSO) $\delta$ 7.55 (m, 4H), 7.39 (d, $J$ = 8.5, 2H), 7.11 (t, $J$ = 7.8, 1H), 6.94 (d, $J$ = 8.1, 1H), 4.88 (s, 2H). $^{13}$C-NMR (100MHz, $d_6$ - DMSO) $\delta$ 182.91, 158.35, 150.07, 137.87, 135.01, 131.47, 129.64, 124.46, 123.33, 120.65, 117.80, 110.95, 42.27. LCMS (214 nm) 3.25 min (>98%); *m/z* 316.0 [M+H]. HRMS cald for $C_{15}H_{10}BrNO_2$ [M+H] 315.9973 found 315.9974.

**VU0238429.** To a vial containing ACN (15 mL) was added 5-trifluoromethyoxyisatin (1.00 g, 4.33 mmol), $K_2CO_3$ (8.66 mmol, 2.0 eq), KI (0.43 mmol, 0.1 eq), and 4-methoxybenzyl chloride (4.76 mmol, 1.1 eq). The reaction was stirred for ~24 hours at room temperature while monitoring by TLC. After judging complete, the reaction was partitioned into EtOAc and $H_2O$, and the combined organics were dried over $MgSO_4$, filtered, and then concentrated *in vacuo* to afford the pure 1-(4-methoxybenzyl)-5-(trifluoromethoxy)indoline-2,3-dione title compound as an orange solid (1.50 g, 4.26 mmol, 98%). $^1$H-NMR (400MHz, $d_6$ - DMSO) $\delta$ 7.60 (m, 2H), 7.36 (d, $J$ = 8.7, 2H), 7.04 (m, 1H), 6.89 (m, 2H), 4.84 (s, 2H), 3.71 (s, 3H). $^{13}$C-NMR (100MHz, $d_6$ - DMSO) $\delta$ 181.99, 158.73, 158.33, 149.00, 143.83, 130.35, 128.88, 126.94, 121.32, 118.79, 117.66, 114.02, 112.36, 55.05, 42.45. LCMS (214 nm) 3.37 min (>98%); *m/z* 352.1 [M+H]. HRMS calcd for $C_{17}H_{12}F_3NO_4$ [M+H] 352.0797 found 352.0795.

**VU0238441.** To a vial containing ACN (3 mL) was added 7-chloroisatin (150 mg, 0.826 mmol), $K_2CO_3$ (1.652 mmol, 2.0 eq), KI (0.083 mmol, 0.1 eq), and 4-trifluoromethylbenzyl bromide (0.909 mmol, 1.1 eq). The reaction was stirred for ~24 hours at room temperature while monitoring by TLC. After judging complete, the reaction was partitioned into EtOAc and $H_2O$, and the combined organics were dried over $MgSO_4$, filtered, and then concentrated *in vacuo* to afford the pure 7-chloro-1-(4-(trifluoromethyl)benzyl)indoline-2,3-dione title compound as an orange solid (267 mg, 0.786 mmol, 95%). $^1$H-NMR (400MHz, $d_6$ - DMSO) $\delta$ 7.64 (m, 4H), 7.15 (m, 3H), 5.28 (s, 2H). $^{13}$C-NMR (100MHz, $d_6$ - DMSO) $\delta$ 181.56, 159.37, 145.27, 142.25, 139.26, 127.01, 125.30, 124.80, 123.51, 121.40, 115.58, 44.28. LCMS (214 nm) 3.46 min (>98%); *m/z* 340.0 [M+H]. HRMS calcd for $C_{16}H_9ClF_3NO_2$ [M+H] 340.0352 found 340.0353.

**General procedure for Suzuki-coupling libraries**. Glass vials containing 1-2 mL anhydrous THF were charged with starting material bearing a –bromo or –chloro substituted aryl/heteroaryl ring, a substituted boronic acid (1.2 equivalents), aqueous 1M $Cs_2CO_3$ solution (2.0 equivalents), and tetrakis(PPh$_3$)Pd(0) catalyst (0.1-0.2 equivalents). The vials were flushed with argon and then subjected to microwave irradiation for 5-20 min at 120 °C. Once complete, the reactions were passed through disposable celite (Isolute HM-N) columns (rinsing 2-3x column volume with DCM). The filtered organics were then concentrated *in-vacuo* or on a heated-air drying block to provide the crude products, which were analyzed by LCMS and then purified by HPLC.

**VU0400265**. 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 $CHCl_2$ 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%); LCMS (>98%) M+H = 414.09 (1.65 min retention),^{1}H NMR (>95%) (400 MHz, CDCl$_3$) d = 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).

**VU0402735**. A 100 mL round bottom flask containing 5-(trifluoromethoxy)indoline-2-one (250 mg, 1.15 mmol) was vacuum purged 3x with argon, dissolved in DMF (30 mL), and cooled to 0 °C. Solid sodium hydride (82.8 mg, 3.45 mmol, 60% in mineral oil) was then added all at once and the reaction stirred until bubbling ceased. Dibromoethane (259 mg, 1.38 mmol) was added all at once and the reaction allowed to warm to room temperature overnight. Once complete the reaction was quenched with $H_2O$ (4 mL) and concentrated *in situ*. The remaining material was partitioned between $H_2O$ (50 mL) and DCM (50 mL) and filtered through a phase separator, and concentrated. The crude material was purified by HPLC (Gilson, ACN:$H_2O$ w/ 0.1% TFA) obtain the pure intermediate (35 mg, 12.5% yield). LCMS [M+H]: 218.1.

This intermediate (30 mg, 0.138 mmol) was then brought up in ACN (1.5 mL) in a glass vial, to which $K_2CO_3$ (0.274 mmol), 4-methoxybenzyl chloride (0.166 mmol), and KI (0.014 mmol) was added. The vial was sealed and subjected to microwave irradiation

at 160 °C for 10 mins. After LCMS analysis, the reaction was judged complete and the solution was partitioned between DCM and $H_2O$ before passage through a disposable phase separator column (Isolute). The organics were then concentrated on a heated-drying block to afford the crude product. Purification by HPLC (Gilson, ACN:$H_2O$ w/ 0.1% TFA) provided the title compound with 98% purity (LCMS). LCMS [M+H]: 364.33

**VU0402734**. A 50 mL round bottom flask containing VU0238429 as starting material (50 mg, 0.142 mmol) was vacuum purged 3x with argon, dissolved in DCM (2 mL), added EtOH (100 mL) and cooled to 0 °C. Once cool added DAST (68.8 mg, 0.427 mmol) and allowed to warm to room temperature overnight. Once complete the reaction was quenched with saturated sodium bicarbonate (50 mL), $H_2O$ (50 mL) and partitioned between DCM and filtered through a phase separator and concentrated. The crude material was purified by HPLC (Gilson, ACN:$H_2O$ w/ 0.1% TFA) obtain the pure final product (23 mg, 43.4 % yield). LCMS [M+H]: 374.1.

**VU0402733**. A 100 mL round bottom flask containing VU0238429 as starting material (117 mg, 0.333 mmol) was vacuum purged 3x with argon, dissolved in THF (30 mL) and cooled to -78 °C. Once cool added 3.0 M solution of methyl magnesium bromide (0.122 mL, 0.366 mmol) over 5 min. and the reaction allowed to warm to room temperature overnight. Once complete the reaction was quenched with $H_2O$ (50 mL), 1N HCl (50 mL) and partitioned between DCM and filtered through a phase separator and concentrated. The crude material was purified via column chromatography using EtOAc:Hexanes (1:2) to afford the pure final product (45 mg, 37 % yield). LCMS [M+H]: 368.1.

**VU0414747**.   To a glass vial containing 3 mL DMF was added methyl 4-hydroxybenzoate (500 mg, 3.286 mmol), $Cs_2CO_3$ (4.929 mmol), and 2,6-difluoropyridine.   The vial was then sealed and thermally heated to 130 °C for approximately 5 hr before judging complete by TLC/LCMS.   The reaction was then partitioned between EtOAc and $H_2O$, and the organics were washed with brine, dried over $MgSO_4$, filtered, and then concentrated *in-vacuo*.   The crude intermediate product was carried forward without purification as highly pure (>90%) clear oil (738 mg, 2.985 mmol, 91%).

To a vial containing this intermediate product (431 mg, 1.88 mmol) was added 30 mL toluene under air-free argon atmosphere and the solution was cooled to 0 °C by ice bath.   Next, DIBAL (1M solution in toluene, 2.1 equivalents) was added to the vial via argon syringe slowly over approximately 5 min.   The reaction was stirred and allowed to warm to room temperature by removal of the ice bath.   After approximately 2 hr, the reaction was judged complete by LCMS and then quenched with excess Rochelle's salt solution while continuing to stir at room temperature for 1.5 hr.   The solution was then partitioned into EtOAc and $H_2O$, and the organics were dried over $MgSO_4$, filtered, and then concentrated *in-vacuo* to afford the crude product with high (>90%) purity, which was carried forward without further purification (627 mg, 2.86 mmol, 96%).

To a glass vial containing this second intermediate product (100 mg, 0.456 mmol) was added 3 mL anhydrous DCM and then $PBr_3$ (0.547 mmol) under air-free argon atmosphere at 0 °C by ice bath.   The reaction was stirred and allowed to warm to room temperature by removal of the ice bath.   After 2 hr, the reaction was judged complete by LCMS and then quenched with aqueous saturated sodium bicarbonate solution.   The

solution was then partitioned with DCM, the organics were separated and concentrated *in-vacuo* to afford the crude product. Purification by HPLC (Gilson, ACN:H$_2$O w/ 0.1% TFA) provided the pure intermediate (>95% by LCMS) as a clear oily solid (75 mg, 0.266 mmol, 58%).

To a glass vial containing 1.5 mL DMF was added 5-trifluoromethoxyisatin (30 mg, 0.130 mmol), K$_2$CO$_3$ (0.260 mmol), the third intermediate product (0.156 mmol), and KI (0.013 mmol). The vial was then sealed and subjected to microwave irradiation at 120 °C for 30 min. The reaction was judged complete by LCMS and then partitioned between EtOAc and H$_2$O. The organics were then washed with brine, dried over MgSO$_4$, filtered, and concentrated *in-vacuo* to afford the crude final product. Purification by HPLC (Gilson, ACN:H$_2$O w/ 0.1% TFA) provided the title compound with high purity (>98% by LCMS) as an orange solid (8.8 mg, 0.020 mmol, 16%).

**General procedure for *O*-alkylations**. To a glass vial containing 1-2 mL DMF was added a phenol-bearing starting material (25-50 mg), substituted alkyl halide (1.5 equivalents), Cs$_2$CO$_3$ (3.0 equivalents), KI (0.2 equivalents), and HMPA (4.0 equivalents). The vial was then flushed with argon and subjected to microwave irradiation at 160 °C for 15 min. The reaction was then partitioned between EtOAc and saturated aqueous sodium bicarbonate. The organics were washed with brine, dried over MgSO$_4$, filtered, and then concentrated *in-vacuo* or on a heated-air drying block to accord the crude products, which were purified by HPLC.

**General procedure for Mitsunobu libraries**. To glass vials containing 2 mL THF was added PS-PPh$_3$ (2.63 mmol/g, 0.334 mmol) and DIAD (0.243 mmol) under argon. The solutions were stirred for 20 min at room temperature and then a phenol-

bearing starting material (0.152 mmol) and a substituted alcohol (0.197 mmol) was added under argon. The solutions were then stirred overnight at room temperature before being partitioned into DCM and $H_2O$. The solutions were passed through a disposable phase-separator column (Isolute) and the resulting organics were concentrated on a heated-air drying block to afford the crude products. Purification by mass-directed HPLC provided the final products with high purity (>98% by LCMS).

*$M_1$ PAM syntheses*

**VU HTS hit VU0029767.** To a solution of 4-ethoxyaniline (10.0 g, 72.9 mmol) in EtOH (100 mL) was added ethyl 2-bromoacetate (14.61 g, 87.0 mmol) and sodium acetate (8.97 g, 109.0 mmol) and stirred for 48 h at room temperature. Once complete, the reaction was then partitioned into $H_2O$ and EtOAc and the organics washed with brine and dried over $MgSO_4$ before concentration *in vacuo*. The crude product was purified by passage through a silica plug eluting with 5% EtOAc in hexanes to afford the intermediate product ethyl 2-(4-ethoxyphenylamino)acetate (11.7 g, 72%) as a dark brown oil. To a solution of the acetate intermediate (4.0 g, 17.93 mmol) in MeOH (50 mL) was added hydrazine (177.9 mL, 177.9 mmol) and stirred for 16 h at 65 degrees C. Once complete, the reaction was then partitioned into $H_2O$ and EtOAc, and the organics washed with brine and dried over $MgSO_4$ before concentration *in vacuo*. The crude product was purified by column chromatography (silica gel) eluting with 5% MeOH in $CH_2Cl_2$ to afford the intermediate product 2-(4-ethoxyphenylamino)acetohydrazide (2.5 g, 67%) as a white solid. To a solution of the hydrazide intermediate (2.0 g, 9.56 mmol) in DMF (100 mL) was added 2-hydroxy-1-napthaldehyde (1.64 g, 9.56 mmol) and stirred

for 16 h at room temperature. Once complete, the reaction was concentrated *in vacuo* and the crude product was purified by filtration with diethyl ether wash (3x250 mL) to afford title compound (3.41 g, 98%) as a yellow solid; ^{1}H NMR (400 MHz, DMSO-d[6]) δ 9.38 (s, 1H), 8.09 (d, $J$ = 4.2 Hz, 1H), 7.80 (dd, $J$ = 8.8, 4.4 Hz, 2H), 7.50 (t, $J$ = 7.2 Hz, 1H), 7.31 (t, $J$ = 7.2 Hz, 1H), 7.12 (d, $J$ = 8.8 Hz, 1H), 6.67 (d, $J$ = 8.8 Hz, 2H), 6.49 (d, $J$ = 8.8 Hz, 2H), 5.69 (t, $J$ = 6.0 Hz, 1H), 3.80 (q, $J$ = 6.8 Hz, 2H), 3.73 (d, $J$ = 5.6 Hz, 1H), 3.32 (s, 2H), 1.18 (t, $J$ = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d[6]) δ 167.2, 157.8, 150.6, 146.3, 142.7, 132.6, 131.6, 128.9, 127.8, 123.5, 120.7, 118.8, 115.4, 113.5, 113.3, 108.4, 63.3, 47.4, 14.8.; LC-MS (214 nm) 3.09 min (>98%); *m/z* 364.2 [M+H].

**VU HTS hit VU0090157.** To a solution of 6-nitrobenzo[d][1,3]dioxole-5-carbaldehyde (195 mg, 1.0 mmol) in MeOH (100 mL) was added 2-cyanoethyl 3-oxobutanoate (186 mg, 1.2 mmol), 1-methylurea (111 mg, 1.5 mmol), and *p*-TsOH (17 mg, 0.1 mmol) and refluxed for 4 h. Once complete, the reaction was filtered and the crude product was purified by recrystallization with MeOH and $H_2O$ to afford the intermediate product 2-cyanoethyl 1,6-dimethyl-4-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate (283 mg, 78%) as a white crystalline solid. To a solution of the carboxylate intermediate (1.0 g, 2.57 mmol) in acetone/$H_2O$ (6/2 mL) was added 2 M NaOH (4 mL) at 0 degrees C, and the solution was warmed to room temperature and stirred for 2 h. Once complete, the reaction was acidified with 2 N HCl and partitioned into $H_2O$ and $CH_2Cl_2$ and the organics were concentrated *in vacuo*. The crude product was purified by recrystallization with MeOH and $H_2O$ to afford the intermediate product 1,6-dimethyl-4-(6-nitrobenzo[*d*][1,3]dioxol-5-yl)-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid (790 mg, 92%) as a white crystalline solid. To a

solution of the acid intermediate (500mg, 1.49 mmol) in DMF (12 mL) was added cyclopentanol (385 mg, 4.48 mmol), EDC (428 mg, 2.24 mmol), and polystyrene-bound DMAP (1.5 mmol/g, 5.96 mmol) and irradiated at 100 degrees C for 20 m.  Once complete, the reaction was partitioned into 1N HCl in $H_2O$ and $CH_2Cl_2$ and the organics were concentrated *in vacuo*.  The crude product was purified by recrystallization in EtOAc and hexanes to afford the title compound (200 mg, 33%) as a brown crystalline solid. ^{1}H NMR (400 MHz, DMSO-$d_6$) δ 7.60 (s 1H), 7.58 (d, *J* = 1.6 Hz, 1H), 6.95 (s, 1H), 6.23 (s, 1H), 6.20 (s, 1H), 5.81 (d, *J* = 1.4 Hz, 1H), 4.93 (m, 1H), 3.16 (s, 3H), 2.57 (s, 3H), 1.66 (m, 1H), 1.55 (m, 1H), 1.40 (m, 4H), 1.19 (m, 1H), 1.09 (m, 1H).  ^{13}C NMR (100 MHz, DMSO-$d_6$) δ 164.5, 152.6, 152.3, 151.8, 147.0, 141.3, 135.3, 106.9, 104.8, 103.4, 100.4, 75.9, 48.5, 32.0, 29.6, 23.0, 15.9.  ; LC-MS (214 nm) 3.14 min (>98%); m/z 404.2

**General procedure for Suzuki-coupling libraries**.  Glass vials containing 1-2 mL anhydrous THF were charged with starting material bearing a –bromo or –chloro substituted aryl/heteroaryl ring, a substituted boronic acid (1.2 equivalents), aqueous 1M $Cs_2CO_3$ solution (2.0 equivalents), and tetrakis($PPh_3$)Pd(0) catalyst (0.1-0.2 equivalents). The vials were flushed with argon and then subjected to microwave irradiation for 5-20 min at 120 °C.  Once complete, the reactions were passed through disposable celite (Isolute HM-N) columns (rinsing 2-3x column volume with DCM).  The filtered organics were then concentrated *in-vacuo* or on a heated-air drying block to provide the crude products, which were analyzed by LCMS and then purified by HPLC.

**General procedure for BQCA library I.**  Each of seven glass vials containing 2 mL of DMF were loaded with ethyl 8-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate

(25 mg, 0.106 mmol, Maybridge BTB02003EA), $K_2CO_3$ (30 mg, 0.212 mmol, 2.0 equivalents), KI (2 mg, 0.011 mmol, 0.1 equivalents), and one of seven benzyl bromides (0.319 mmol, 3.0 equivalents). The reactions were stirred for 24 hours at room temperature before receiving polystyrene-bound thiophenol (0.159 mmol, 1.5 equivalents) each, and then stirred for an additional 3 hours. The reactions were then judged complete by LCMS, filtered, and separated into $CH_2Cl_2$ and $H_2O$. The organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* yielding seven benzyl-substituted ethyl 8-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylates confirmed by analytical LCMS. Next, crude products (0.1 mmol) and LiOH (8 mg, 0.3 mmol, 3.0 equivalents) were dissolved in 3 mL of THF:$H_2O$ (9:1) in glass vials. The reactions were microwave irradiated at 120 °C for 10 minutes and then separated into EtOAc and $H_2O$, which was acidified to pH 4 drop-wise using 1N HCl. Organics were dried over $MgSO_4$, filtered, and concentrated *in vacuo* yielding seven benzyl-substituted 8-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acids confirmed by LCMS. Purification using mass-directed HPLC afforded the seven compounds (25-85% total yield) as TFA salts with >98% purity.

**General procedure for BQCA library II.** Each of seven glass vials containing 2 mL of DMF were loaded with ethyl 4-oxo-1,4-dihydroquinoline-3-carboxylate (25 mg, 0.115 mmol, Ryan Scientific 6J-050), $K_2CO_3$ (32 mg, 0.230 mmol, 2.0 equivalents), KI (2 mg, 0.012 mmol, 0.1 equivalents), and one of seven benzyl bromides (0.345 mmol, 3.0 equivalents). The reactions were stirred for 24 hours at room temperature before receiving polystyrene-bound thiophenol (0.173 mmol, 1.5 equivalents) each, and then stirred for an additional 3 hours. The reactions were then judged complete by LCMS,

filtered, and separated into $CH_2Cl_2$ and $H_2O$. The organics were washed with brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo* yielding seven benzyl-substituted ethyl 4-oxo-1,4-dihydroquinoline-3-carboxylates confirmed by analytical LCMS. Next, crude products (0.1 mmol) and LiOH (8 mg, 0.3 mmol, 3.0 equivalents) were dissolved in 3 mL of THF:$H_2O$ (9:1) in glass vials. The reactions were microwave irradiated at 120 °C for 10 minutes and then separated into EtOAc and $H_2O$, which was acidified to pH 4 drop-wise using 1N HCl. Organics were dried over $MgSO_4$, filtered, and concentrated *in vacuo* yielding seven benzyl-substituted 4-oxo-1,4-dihydroquinoline-3-carboxylic acids confirmed by LCMS. Purification using mass-directed HPLC afforded the seven compounds (25-85% total yield) as TFA salts with >98% purity.

**General procedure for BQCA library III.** Each of seven glass vials containing 2 mL of DMF were loaded with ethyl 5,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (25 mg, 0.099 mmol, Ryan Scientific 6J-020), $K_2CO_3$ (27 mg, 0.198 mmol, 2.0 equivalents), KI (2 mg, 0.099 mmol, 0.1 equivalents), and one of seven benzyl bromides (0.297 mmol, 3.0 equivalents). The reactions were stirred for 24 hours at room temperature and atmosphere before receiving polystyrene-bound thiophenol (0.149 mmol, 1.5 equivalents) each, and then stirred for an additional 3 hours. The reactions were then judged complete by LCMS, filtered, and separated into $CH_2Cl_2$ and $H_2O$. The organics were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo* yielding seven benzyl-substituted ethyl 5,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylates confirmed by analytical LCMS. Next, crude products (0.1 mmol) and LiOH (8 mg, 0.3 mmol, 3.0 equivalents) were dissolved in 3 mL of THF:$H_2O$ (9:1) in glass vials. The reactions were microwave irradiated at 120 °C for 10 minutes and then separated into

EtOAc and $H_2O$, which was acidified to pH 4 drop-wise using 1N HCl. Organics were dried over $MgSO_4$ and concentrated *in vacuo* yielding seven benzyl-substituted 5,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acids confirmed by LCMS. Purification using mass-directed HPLC afforded the seven compounds (25-85% total yield) as TFA salts with >98% purity.

**BQCA.** To stirred solution of 200 mL DMF in a glass flask was added ethyl 4-oxo-1,4-dihydroquinoline-3-carboxylate (3.40 g, 15.66 mmol, Ryan Scientific 6J-050), $K_2CO_3$ (4.33 g, 31.32 mmol, 2.0 equivalents), KI (260 mg, 1.57 mmol, 0.1 equivalents), and 4-methoxybenzyl bromide (4.70 g, 23.49 mmol, 1.5 equivalents). After 48 hours of stirring at room temperature and atmosphere, the reaction was monitored by LCMS and judged complete. The reaction was then partitioned into $CH_2Cl_2$ and $H_2O$, and the organics were washed with brine, dried over $MgSO_4$, and concentrated *in vacuo*. Purification by diethyl ether washing (6 x 50 mL) afforded the intermediate product ethyl 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (4.99 g, 14.83 mmol, 95%) as an off-white solid at >98% purity by LCMS. To a glass vial containing ethyl 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (4.99 g, 14.83 mmol) in 90 mL THF:$H_2O$ (5:1) was added LiOH (1.07 g, 44.49 mmol, 3.0 equivalents). The reaction was microwave irradiated at 120 °C for 10 minutes and then partitioned into $CH_2Cl_2$ and $H_2O$. The solution was re-acidified to pH 5 drop-wise using 2N HCl. The organics were dried over $MgSO_4$, filtered, concentrated *in vacuo*, and analyzed by LCMS. The crude product was purified by diethyl ether washing (6 x 50 mL) and additional $H_2O$ wash (1 x 100 mL) to afford the intermediate product 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (3.20 g, 10.35 mmol, 70%) as an off-white crystalline

solid at >98% purity by LCMS.  To a stirred solution of 1-(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (1.89 g, 6.11 mmol) in 25 mL DMF in a glass flask at 0 °C was added NaH (143 mg, 5.99 mmol, 0.98 equivalents).  The reaction was allowed to warm to room temperature and stirred for 1 hour before concentration *in vacuo*.  The crude product was washed with diethyl ether (3 x 30 mL) to afford the title compound (1.80 g, 5.44 mmol, 89%) as a white solid at >98% purity by LCMS.  ^{1}H NMR (400 MHz, D$_2$O): d= 9.07 (s, 1H), 8.25 (d, $J$ = 8.0 Hz, 1H), 7.53 (t, $J$ = 8.4 Hz, 1H), 7.45 (d, $J$ = 8.4 Hz, 1H), 7.39 (t, $J$ = 8.0 Hz, 1H), 7.11 (d, $J$ = 8.8 Hz, 2H), 6.79 (d, $J$ = 8.8 Hz, 2H), 5.35 (s, 2H), 3.67 (s, 3H).  ^{13}C NMR (100 MHz, D$_2$O, externally referenced to DMSO-d$_6$): d= 176.3, 172.2, 158.0, 147.4, 138.5, 132.3, 127.5, 127.2, 126.9, 125.5, 124.5, 117.5, 116.9, 113.8, 55.8, 54.7.  HRMS calcd for C$_{18}$H$_{14}$NO$_4$Na$_2$ [M + 2Na] 354.0718, found 354.0716.

**MLPCN M$_1$ PAM probe VU0405652**.
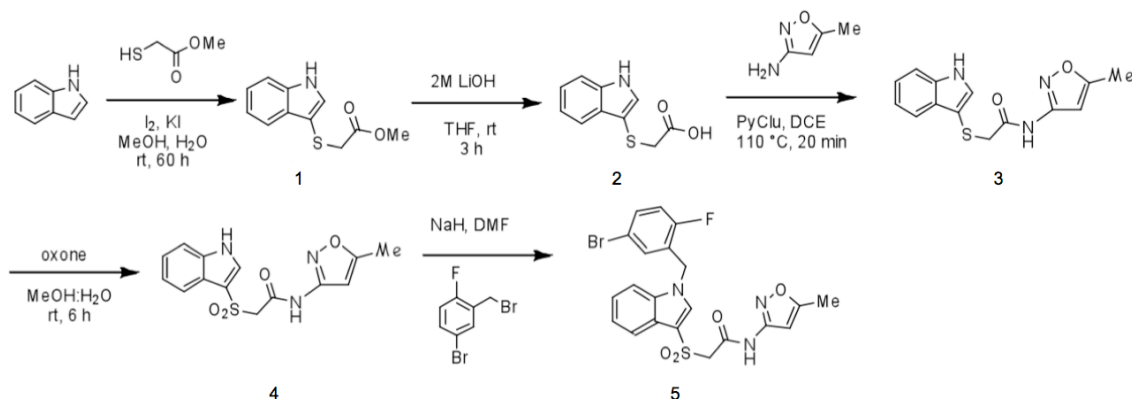


To a solution of indole (3.00 g, 25.6mmol) and methyl thioglycolate (2.40 mL, 25.6 mmol) in methanol:water (80 mL : 20 mL) was added iodine (6.50 g, 25.6 mmol) and potassium iodide (4.25 g, 25.6 mmol).  The reaction mixture was stirred at ambient temperature for 60 hours.  Methanol was removed *in vacuo* and the aqueous layer diluted

with a saturated solution of sodium bicarbonate and extracted with ethyl acetate. The organic layer was dried over magnesium sulfate, evaporated *in vacuo* and the resulting residue was purified on a silica gel column (0-100% ethyl acetate:hexanes over 33 min) to afford compound **1** as an oil (LCMS >98%). Compound **1** was dissolved in a mixture of tetrahydrofuran (20 mL) and 2.0M aqueous LiOH (15 mL), then stirred vigorously at ambient temperature for 30 minutes. Tetrahydrofuran was removed *in vacuo*, the aqueous layer neutralized with 1.2 N HCl and extracted with $CH_2Cl_2$. The organic layer was dried over magnesium sulfate and removed *in vacuo* to produce an oily residue. Upon diluting the residue in dichloromethane a reddish-brown solid formed which was filtered and dried to yield compound **2** (2.00 grams, 9.65 mmol, 38% yield over 2 steps, LCMS >98%).

Compound **2** (650 mg, 3.14 mmol), 3-amino-5-methyl-isoxazole (616 mg, 6.28 mmol), PyClu (2.00 g, 6.28 mmol), and DIEA (1.36 mL, 7.85 mmol) were added to dichloroethane (25 mL) and microwave irradiated at 110 °C for 20 minutes. After cooling, the solvent was removed *in vacuo* and the remaining residue purified on a silica gel column (0-70% ethyl acetate:hexanes over 33 min) to yield compound **3** (642 mg, 2.23 mmol, 71% yield, LCMS >98%). Compound **3** (502 mg, 1.77 mmol) was dissolved in 25 mL (9:1, methanol:water) and oxone (10.0 g, 17.7 mmol) was added. Stirring at ambient temperature continued overnight. Water (20 mL) was added and the mixture extracted with ethyl acetate (3x20 mL). The organics were combined, dried over magnesium sulfate, and concentrated *in vacuo* to give an oily residue which was purified on silica gel (0-50% ethyl acetate:hexanes over 19 min) to yield compound **4** (500 mg, 1.57 mmol, 88% yield, LCMS >98%).

In a 5 mL microwave vial, compound **4** (55.0 mg, 0.174 mmol) was dissolved in DMF (3 mL) and cooled to 0 °C. Sodium hydride (60% by weight, 14.0 mg, 0.348 mmol) was then added in one portion and the reaction mixture vigorously stirred at 0 °C for 15 minutes. 4-bromo-2-bromomethyl-1-flouro-benzene (51.0 mg, 0.191 mmol) was added in one portion and the reaction mixture was stirred while being allowed to warm to ambient temperature over 3 hours. The reaction mixture was quenched with water (2 mL) and the solution was extracted with ethyl acetate (3x4 mL). The combined organics were dried over magnesium sulfate, concentrated *in vacuo* to give an oily residue which was purified on silica gel (0-70% ethyl acetate:hexanes over 19 min) to yield the final product (compound **5**, 45 mg, 0.088 mmol, 51% yield). LCMS >98% 214 nm, $R_T$ = 1.34 min, m/z = 506 ([$^{79}$Br]m+1), 508 ([$^{81}$Br]m+1). $^1$H NMR (400 MHz, DMSO$_{D6}$) 11.27 (s, 1H), 8.23 (s, 1H), 7.82 (d, $J$ = 8.0 Hz, 1H), 7.63 (d, $J$ = 8.0 Hz, 1H), 7.56-7.58 (m, 1H), 7.47 (dd, $J$ = 2.4 Hz, 6.4 Hz, 1H), 7.35-7.23 (m, 3H), 6.54 (s, 1H), 5.62 (s, 2H), 4.43 (s, 2H), 2.37 (s, 3H), HRMS found: 506.0184; calculated for $C_{21}H_{17}BrFN_3O_4S$: 506.0185.

*M$_1$ allosteric agonist syntheses*

**VU0184670.** To a solution of benzoyl chloride (87.7 mg, 0.624 mmol) in dichloromethane (2 mL) was added *N*-boc-ethylenediamine (100.0 mg, 0.624 mmol) and PS-DIEA (2 eq, 356.6 mg). The reaction was agitated overnight at room temperature, after which the reaction mixture was filtered to remove polymer-supported resin. The filtrate was then concentrated and dissolved in 1mL of dichloromethane, to which was added 1mL of trifluoroacetic acid (1:1 DCM:TFA) and stirred at room temperature for 30 min. Following deprotection, the reaction mixture was again concentrated to dryness,

after which the product was dissolved in dimethylformamide (2mL). To this solution was added 1-carbethoxy-4-piperidone (106.8 mg, 0.624 mmol) and MP-BH(OAc)3 (2.5 eq, 645 mg). The reaction mixture was then agitated overnight at room temperature, after which the resin was filtered off.  Aqueous workup of the reaction mixture using brine and ethyl acetate, followed by filtration of the organic layer using an Isolute Phase Separator and sample concentration yielded ethyl 4-(2-(2-methylbenzamido)ethylamino)piperidine-1-carboxylate as a pale yellow oil. The product was then dissolved in 1 mL of DMSO and purified by mass-directed preparative HPLC to afford ethyl 4-(2-(2-methylbenzamido)ethylamino)piperidine-1-carboxylate -2,2,2-trifluoroacetate as a clear oil (64.5 mg, 23.9 %); 1H-NMR (400 MHz, *d6* - DMSO) $\delta$ 7.9-7.8 (m, 1H), 7.52-7.42 (m, 5H), 6.73 (br s, 1H), 4.16-4.11 (q, *J* = 7.07 Hz, 1H), 3.56-3.50 (m, 4H), 2.92-2.90 (m, 6H), 2.29 (s, 1H), 1.38-1.32 (m, 7H); $^{13}$C-NMR (100 MHz, *d6* – CDCl$_3$): $\delta$ 166.95, 154.84, 134.20, 131.82, 128.65, 127.78, 61.24, 54.36, 43.60, 42.03, 36.30, 14.95; HRMS (*m/z*): [M+H] calcd. for C$_{17}$H$_{26}$N$_3$O$_3$, 320.1974; found, 320.1966.

**VU0357017.**  To a solution of *o*-toluoyl chloride (96.5 mg, 0.624 mmol) in dichloromethane (2 mL) was added *N*-boc-ethylenediamine (100.0 mg, 0.624 mmol) and PS-DIEA (2 eq, 356.6 mg). The reaction was agitated overnight at room temperature, after which the reaction mixture was filtered to remove polymer-supported resin. The filtrate was then concentrated and dissolved in 1mL of dichloromethane, to which was added 1mL of trifluoroacetic acid (1:1 DCM:TFA) and stirred at room temperature for 30 min. Following deprotection, the reaction mixture was again concentrated to dryness, after which the product was dissolved in dimethylformamide (2mL). To this solution was added 1-carbethoxy-4-piperidone (106.8 mg, 0.624 mmol) and MP-BH(OAc)3 (2.5 eq,

645 mg). The reaction mixture was then agitated overnight at room temperature, after which the resin was filtered off.  Aqueous workup of the reaction mixture using brine and ethyl acetate, followed by filtration of the organic layer using an Isolute Phase Separator and sample concentration yielded ethyl 4-(2-(2-methylbenzamido)ethylamino)piperidine-1-carboxylate as a pale yellow oil. The product was then dissolved in 1 mL of DMSO and purified by mass-directed preparative HPLC to afford ethyl 4-(2-(2-methylbenzamido)ethylamino)piperidine-1-carboxylate -2,2,2-trifluoroacetate as a clear oil (102.9 mg, 36.9 %); ^{1}H-NMR (400 MHz, $d6$ – CDCl$_3$) $\delta$ 7.37-7.18 (m, 7H), 6.30 (br s, 1H), 4.15-4.10 (q, $J$ = 7.07 Hz, 1H), 3.54-3.50 (q, $J$ = 5.74 Hz, 4H), 2.91-2.86 (m, 6H), 2.35 (s, 1H), 1.27-1.20 (m, 7H); ^{13}C-NMR (100 MHz, $d6$ - DMSO) $\delta$ 130.96, 129.99, 127.76, 125.83, 61.23, 54.32, 43.38, 42.01, 28.07, 20.01, 14.94; HRMS (*m/z*): [M+H] calcd. for C$_{18}$H$_{28}$N$_3$O$_3$, 334.2131; found, 334.2130.

*M$_1$ agonist TBPB analog syntheses*

**Reductive Amination with commercial piperidinones.**  Each of twenty-four glass reaction vials containing 3 mL of CH$_2$Cl$_2$ were loaded with MP-B(OAc)$_3$H (142 mg, 0.345 mmol, 2.42 mmol/g, 3.0 equivalents) and 4-(2-keto-1-benzoimidazolinyl)piperidine (25 mg, 0.115 mmol, 1.0 equivalents).  Then, one of 24 *N*-functionalized piperidinones (0.115 mmol, 1.0 equivalents) with either an alkyl or carbamate moiety was added to each reaction tube.  The reactions were stirred overnight at room temperature.  The next day, the solutions were filtered and washed with CH$_2$Cl$_2$ (3 x 3 mL), then dried before purification by mass-directed preparative HPLC.

**General Procedures for Library Synthesis (Scheme 1): Synthesis of *N*-benzyl piperidinones.** Each of sixty-four glass reaction vials containing 3 mL of $CH_2Cl_2$ and 0.1 mL of MeOH were loaded with piperidone hydrochloride (25 mg, 0.185 mmol, 1.0 equivalents) and $K_2CO_3$ (51 mg, 0.370 mmol, 2.0 equivalents). Then, one of sixty-four functionalized benzyl bromides (0.185 mmol, 1.0 equivalents) was added to each reaction tube. The reactions were stirred overnight at room temperature, partitioned between $CH_2Cl_2$ and $H_2O$, and the organics were concentrated on a heat-air block. Purification by preparative LCMS was performed to afford *N*-benzyl piperidinones products for subsequent reductive amination.

**Reductive Aminations.** Each of sixty-four glass reaction vials containing 3 mL of $CH_2Cl_2$ were loaded with MP-B(OAc)$_3$H (142 mg, 0.345 mmol, 2.42 mmol/g, 3.0 equivalents) and 4-(2-keto-1-benzoimidazolinyl)piperidine (25 mg, 0.115 mmol, 1.0 equivalents) or 5-chloro-1-(4-piperidinyl)-2-benzimidazolone (29 mg, 0.115 mmol, 1.0 equivalents). Then, one of sixty-four tertiary amine hydrates (0.115 mmol, 1.0 equivalents) from the previous alkylation reactions was added to each vial, and the reactions were stirred overnight at room temperature. The next day, the solutions were filtered and washed with $CH_2Cl_2$ (3 x 3 mL), then dried before purification by mass-directed preparative HPLC.

**TBPB.** To a stirred solution of 4-piperidone monohydrate HCl (2.00 grams, 13.02 mmol) in dimethylformamide (100 mL) was added 2-methylbenzyl bromide (2.44 grams, 13.02 mmol) and $K_2CO_3$ (3.60 grams, 26.04 mmol, 2.0 equivalents) at room temperature. The reaction was then monitored by analytical LCMS, and once judged complete, the reaction was separated with water and EtOAc. The organics were next

washed with a saturated solution of NaCl and dried over $MgSO_4$ before being concentrated *in vacuo* to afford 2.59 grams (90%) of the hydrate form of 1-(2-methylbenzyl)piperidin-4-one as a colorless oil. Analytical LCMS (J-Sphere80-C18, 3.0 x 50.0 mm, 4.1 min gradient, 5%[0.05% $TFA/CH_3CN$]:95%[0.05% $TFA/H_2O$]:1.64 min, >99% (214 nm, 254 nm and ELSD) M+1 peak *m/e* 204.1; ^{1}H NMR (400 MHz, DMSO-d-6) d 7.28 (m, 1H), 7.16 (m, 3H), 3.53 (s, 2H), 2.67 (t, $J$ = 6.4 Hz, 4H), 2.36 (s, 3H), 2.32 (t, $J$ = 6.4 Hz, 4H). ^{13}C NMR (100 MHz, DMSO-d6) d 137.0, 136.4, 130.1, 129.3, 127.0, 125.4, 58.7, 52.4, 40.6, 18.8; HRMS calc'd for $C_{13}H_{20}NO_2$[M+H]; 222.1494 found 222.1488.

This intermediate 1-(2-methylbenzyl)piperidin-4-one (2.00 grams, 9.04 mmol) product was then brought up in a stirred solution of $CH_2Cl_2$ (100 mL) to which 4-(2-keto-1-benzimidazolinyl)piperidine (2.16 grams, 9.95 mmol, 1.1 equivalents) was added at room temperature. Then, MP-triacetoxyborohydride (13.57 grams, 27.132 mmol [2.0 mmol/gram loading], 3.0 equivalents) was added and the reaction was monitored by analytical LCMS. Once judged complete, the reaction was filtered and the filtrate was purified by mass-directed preparative HPLC to afford 2.92 grams (80%) of title compound as a pure white solid. Analytical LCMS (J-Sphere80-C18, 3.0 x 50.0 mm, 4.1 min gradient, 5%[0.05% $TFA/CH_3CN$]:95%[0.05% $TFA/H_2O$]:2.34 min, >99% (214 nm, 254 nm and ELSD) M+1 peak *m/e* 405.2; ^{1}H NMR (400 MHz, DMSO-d6) d 7.26 (d, $J$ = 4.0 Hz, 1H), 7.18 (m, 3H), 6.96 (m, 4H), 4.22 (m, 1H), 4.09 (m, 1H), 3.37 (m, 2H), 3.18 (s, 1H), 3.05 (m, 1H), 2.96 (br d, $J$ = 7.6 Hz, 2H), 2.81 (br d, $J$ = 11.2 Hz, 2H), 2.67 (t, $J$ = 6.0 Hz, 1H), 2.57 (br t, $J$ = 11.2 Hz, 1H), 2.28 (m, 6H), 1.93 (br t, $J$ = 10.8 Hz, 2H), 1.64 (m, 2H), 1.41 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d6) d 153.6, 136.9, 130.1,

129.4, 129.2, 129.1, 128.3, 127.0, 125.5, 120.3, 108.8, 61.4, 60.2, 52.4, 49.9, 48.5, 45.8, 40.7, 29.2, 27.9, 18.8; HRMS calc'd for $C_{25}H_{33}N_4O[M+H]$; 405.2654 found 405.2654 .

**TBPB analog 1b (ethyl carbamate derivative).** To a stirred solution of 4-(2-keto-1-benzimidazolinyl)piperidine (50 mg, 0.230 mmol) in $CHCl_2$ (3 mL) was added ethyl 4-oxo-1-piperidinecarboxylate (43.3 mg, 0.253 mmol, 1.1 equivalents) and MP-B(OAc)$_3$H (285 mg, 0.691 mmol, 2.42 mmol/g, 3.0 equivalents). The reaction was then monitored by analytical LCMS, and once judged complete, the reaction was filtered and concentrated. Purification by mass-directed preparative HPLC afforded 32.50 mg (38%) of title compound as a beige-white solid. Analytical LC/MS (J-Sphere80-C18, 3.0 x 50.0 mm, 4.1 min gradient, 5%[0.05% TFA/CH$_3$CN]:95%[0.05% TFA/H$_2$O]:2.05 min, >99% (214 nm, 254 nm and ELSD) M+1 peak *m/e* 373.2; $^1$H NMR (400 MHz, DMSO-d$_6$) d 10.94 (s, 1H), 9.97 (br s, 1H), 7.31 (d. *J* = 4.4 hz, 1H), 7.00 (m, 3H), 4.57 (m, 1H), 4.12 (br d, *J* = 11.6 Hz, 2H), 4.04 (q, *J* = 7.2 Hz, 2H), 3.59 (br d, *J* = 11.2 Hz, 2H), 3.47 (m, 1H), 3.24 (m, 2H), 2.82 (m, 2H), 2.66 (m, 2H), 2.05 (m, 2H), 1.94 (br d, *J* = 12.4 Hz, 2H), 1.59 (m, 2H), 1.19 (t, *J* = 6.8 Hz, 3H). $^{13}$C NMR (100 MHz, DMSO-d$_6$) d 154.4, 153.6, 128.7, 128.4, 120.9, 120.3, 109.1, 108.6, 62.5, 60.9, 48.1, 46.6, 42.1, 25.8, 14.5; HRMS calc'd for $C_{20}H_{29}N_4O_3[M+H]$; 373.2240 found 373.2235.

*M$_1$ antagonist syntheses*

**General parallel analog library synthesis.** To a solution of 3-(benzo[*c*][1,2,5]thiadiazole-4-sulfonamido)-propanoic acid (50 mg, 0.17 mmol) and DIPEA (46 mL, 0.34 mmol) in DCM (2 mL) in a 4-mL vial was added polymer-supported DCC (283 mg, 0.34 mmol, 1.2 mmol/g), HOBt (27 mg, 0.2 mmol) and one of

24 amines (HNR$_1$R$_2$) and rotated at room temperature for 20 h. Then, MP-carbonate (151 mg, 0.5 mmol, 3.3 mmol/g) was added to scavenge excess acid and the HOBt. The reactions were filtered into 13 x 100 mm test tubes, and the resin washed (3 x 5 mL) with DCM. The combined organic layers were dried down on a nitrogen evaporator and the compounds purified to >98% by preparative LCMS on an Agilent 1200 Prep mass-directed LCMS purification system.

**VU0255035 (compound 5b)**. To a solution of 3-(benzo[*c*][1,2,5]thiadiazole-4-sulfonamido)-propanoic acid (2.00 g, 6.94 mmol) and DIPEA (2.43 mL, 13.9 mmol) in DMF (10 mL) was added EDC (1.55 g, 8.33 mmol), HOBt (937 mg, 6.94 mmol) and 1-(4-pyridyl)piperazine (1.36 g, 8.33 mmol) and stirred at room temperature for 20 h. The reaction was diluted with water (80 mL) and extracted with EtOAc (2 x 100 mL). The combined organic extracts were washed with water (2 x 80 mL) and brine (100 mL), dried over MgSO$_4$ and concentrated under vacuum. The residue was triturated with CH$_2$Cl$_2$/hexanes (1:1, 40 mL) to afford amide VU0255035 as a light tan solid (1.45 g, 48 %): mp 159.7-160.2 °C; $^1$H NMR (400 MHz, DMSO-d$_6$) δ 8.37 (d, *J* = 9.0 Hz, 1H), 8.20 (d, *J* = 7.0 Hz, 1H), 8.16 (d, *J* = 6.0 Hz, 2H), 7.85 (dd, *J* = 9.0, 7.0 Hz, 1H), 7.71 (br s, 1H), 6.80 (d, *J* = 6.0 Hz, 2H), 3.51-3.40 (m, 4H), 3.35-3.28 (m, 4H), 3.21-3.12 (m, 2H); $^{13}$C NMR (100 MHz, DMSO-d$_6$) δ 169.1, 155.4, 154.7, 149.5, 149.2, 132.5, 130.9, 129.3, 126.3, 108.6, 45.6, 45.3, 44.2, 41.7, 41.4, 33.0; Analytical LCMS (J-Sphere80-C18, 3.0 x 50.0 mm, 4.1 min gradient, 5%[0.05% TFA/CH$_3$CN]:95%[0.05% TFA/H$_2$O]: 1.95 min, >99%, (214 nM, 254 nm, ELSD), m/z [M+H] = 433.1; HRMS calc'd for C$_{18}$H$_{21}$N$_6$O$_3$S$_2$ [M+H]; 433.1117 found 433.1117.

*Jetmilling/scale-up*

For compounds used in *in vivo* behavioral and/or pharmacokinetic (PK)/plasma-brain levels (PBL) studies, a large-scale synthesis was performed (generally 200 mg to 10 g) to provide the required amount of material. In such cases where compound solubility or other physiochemical properties imposed substantial challenges during vehicle formulation, the material was passed through a jet-milling device (Model 00 Jet-O-Mizer with a High-Yield® Collection Module from Fluid Energy Processing & Equipment Company), which afforded uniform nanoparticles with improved solubility and physical properties.

## *In vitro* Pharmacology

*Cell lines and cell culture*

Rat $M_1$ cDNA, provided by T.I. Bonner (National Institutes of Health, Bethesda, MD), was used to generate the orthosteric-site mutant rat $M_1^{Y381A}$ using the QuikChange site-directed mutagenesis kit. All tissue culture reagents were purchased from Invitrogen Corporation, Carlsbad, CA, unless otherwise noted. Cell lines used for all WT-mAChR studies stably expressed rat $M_1$, human $M_2$ and $G_{qi5}$, human $M_3$, rat $M_4$ and $G_{qi5}$, or human $M_5$ in the Chinese Hamster Ovary (CHO-K1) parental background. The $M_1$, $M_3$, and $M_5$ cell lines were cultured in HAM's F12 medium with 10% FBS, 20 mM HEPES, and 50 μg/mL G418 (Mediatech, Inc., Herndon, VA). $M_2/G_{qi5}$ cells were cultured in the same media with 500 μg/mL hygromycin. $M_4/G_{qi5}$ cells were cultured in the same media 400 μg/mL G418 and 500 μg/mL hygromycin. Cells expressing the rat $M_1^{Y381A}$

orthosteric-site mutant receptor were cultured in the same medium, except without hygromycin. Cell lines used for experiments performed with human $M_4$ were generated and used by Millipore and co-expressed the promiscuous $G_{\alpha 16}$.

*Calcium mobilization assays*

All functional cell-based calcium mobilization assays were performed essentially as previously described (Marlo *et al.*, 2009; Brady *et al.*, 2008) using either an FDSS or Flexstation according to the following protocols. These protocols represent the general methods used for the $M_5$ PAM, $M_4$ PAM, and $M_1$ PAM projects. The $M_1$ allosteric agonist and TBPB projects utilized similar methods with the exception that a single-add format was used wherein test compound is added at 2X alone with no second-add of ACh. For the $M_1$ antagonist project, similar methods were used as described for the $M_5$ PAM, $M_4$ PAM, and $M_1$ PAM projects with the exception that an ACh ~$EC_{80}$ was used in the second-add instead of an ~$EC_{20}$. Likewise for $M_2$-$M_5$ antagonism screening in the $M_1$ allosteric agonist project, an ACh ~$EC_{80}$ was used in the second-add instead of an ~$EC_{20}$ to assess functional antagonism.

For all FDSS screens/assays, cells (10,000 cells/20 µl/well) were plated in black-walled, clear-bottomed, TC treated, 384 well plates (Greiner Bio-One, Monroe, North Carolina) in Ham's F12 medium supplemented with 10% FBS and 20 mM HEPES. The cells were grown overnight at 37 °C in the presence of 5% $CO_2$. The next day, the medium was removed and replaced with 20 µl of 2 µM Fluo-4AM in calcium assay buffer (Hank's Balanced Salt Solution supplemented with 20 mM HEPES and 2.5 mM Probenecid) and the cell plates incubated for 60 minutes at 37 °C. Dye solution was

removed and replaced with 20 µl of fresh assay buffer. Test compounds were transferred from a 384-well source plate (10 mM DMSO, 30 µl/well) to 384-well daughter plates using an ECHO acoustic plate reformatter (Labcyte) and then diluted into assay buffer to 20 µM stock concentration (2X). Acetylcholine (Aldrich) was diluted in a 384-well plate containing submaximal ($\sim EC_{10}$, determined empirically) and maximal (10 µM) stock concentrations (5X). Mobilization of intracellular calcium was measured using the Functional Drug Screening System 6000 (FDSS6000, Hamamatsu). Baseline readings were taken and then test compounds (30 µM final, 20 µl/well) were added using the FDSS's integrated pipettor. After 150 seconds of equilibration, acetylcholine ($EC_{10}$ and maximal concentrations, 10 µl/well) was added using the FDSS's integrated pipettor. Data were obtained as max-min fluorescent ratios and then normalized to percentage of maximum ACh response and represent mean values obtained from three independent determinations (error bars represent +/- SEM) unless otherwise specified.

For Flexstation screens/assays (which were primarily used to determine compound potency, ACh fold-shift efficacy, mAChR subtype-selectivity, etc...) the methods used were essentially the same as previously described (Marlo *et al.*, 2009, Brady *et al.*, 2008) and in a format similar to that described above using the same reagents. CHO cells stably expressing $rM_1$, $hM_3$, $hM_5$, $rM_4/G_{qi5}$, or $rM_2/G_{qi5}$ were plated in 100 mL of growth medium at $5 \times 10^4$ ($rM_1$, $hM_3$, and $hM_5$) or $6 \times 10^4$ ($hM_2$, and $rM_4$) cells per well in Costar 96-well black-walled, TC-treated, clear-bottom plates (Fisher). Cells were incubated overnight at 37 °C under 5% $CO_2$. The next day, medium was removed from the cells, and they were incubated with 50 mL of 2 mM Fluo-4 AM diluted in assay buffer for 1 h at 37 °C. Dye was then removed and replaced with 45 mL of fresh

assay buffer. Test compounds were diluted in assay buffer at 2X concentration and acetylcholine was diluted in assay at a 10X concentration. FLEXstation II (Molecular Devices) automated plate reader was used for assay execution and measurement of calcium flux. After establishing baseline fluorescence, test compounds (45 mL) were added to the cells using the FLEXstation II's integrated pipettor and allowed to equilibrate for 150 seconds before addition of acetylcholine (10 mL). Data were obtained as max-min fluorescent ratios and then normalized to percentage of maximum ACh response. For test compounds exhibiting intrinsic fluorescence (typically found only at 30 μM final), adjustment of analysis time window for max-min values was sometimes performed in order to obtain more accurate baseline readings. Calculation of potentiation $EC_{50}$ and fold-shift of ACh CRC was performed using the curve-fitting software GraphPad Prism (version 4.0c). Data shown represent mean values obtained from at least three independent determinations performed in duplicate or greater (error bars represent +/- SEM) unless otherwise specified.

*Radioligand binding assays*

**M$_4$ PAMs (all) competition binding assays**. M$_4$ cells were washed once with ice-cold phosphate-buffered saline, pH 7.4. Cells were then harvested with a cell scraper, resuspended in ice-cold buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl2, pH 7.4), and homogenized using a glass homogenizer (Dounce). The homogenate was centrifuged at 20,000*g* for 20 min at 4 °C. This final step was repeated twice more with homogenization between centrifugations, and the final pellets were resuspended and homogenized in ice-cold buffer in aliquots at a final protein concentration of 1-3 mg mL−

1 and stored at -80 °C until use. Protein concentrations were measured using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) with serum albumin (Pierce Chemical, Rockford, IL) as the standard. For each binding experiment, membranes were resuspended and homogenized in ice-cold buffer. Non-specific binding, calculated in the presence of 1 µM atropine, was never greater than 10% of total binding; in both saturation and competition binding experiments, bound radioligand did not exceed 10%.

For competition binding experiments, test compounds and atropine were dissolved in 100% DMSO, diluted in assay buffer to make a 3x stock, and then added to the assay plate for a final maximum DMSO concentration of 0.3% for test compounds and 0.01% for atropine. Vehicle for each condition was DMSO-matched control made in assay buffer. For ACh competition experiments, test compound was diluted in assay buffer to make a 5x stock and then added to the assay plate for a maximum final DMSO concentration of 0.1%. Binding reactions were incubated at room temperature for 2 h on a Titer Plate Shaker (Lab-Line Instruments, Melrose, IL), and equilibrium binding was terminated by rapid filtration through Unifilter-96 GF/B filter plates presoaked for at least 2 h with 0.1% polyethylenimine (PEI, Sigma). Filter plates were washed three times with ice-cold harvesting buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) using a 96-well Brandel harvester (Brandel Inc., Gaithersburg, MD). Plates were then dried overnight, and 35 µl MicroScint-20 was added to each well the following day. Radioactivity was counted using a TopCount NXT microplate scintillation and luminescence counter (PerkinElmer Life and Analytical Sciences, Downers Grove, IL).

**$M_5$ PAM VU0238429 and $M_1/M_3/M_5$ PAM VU0119498 competition binding assays.** Membranes were prepared from $M_5$-CHO cells according to the previously

described protocol. Binding reactions contained 0.09 nM [$^3$H]-NMS (obtained commercially from Amersham), 15-20 μg of membrane protein, and test compound or atropine in a total volume of 500 μl assay buffer (100 mM NaCl, 10 mM MgCl$_2$, 20 mM HEPES, pH 7.4). 1 μM (final) atropine was used to determine non-specific binding. The $K_D$ of [$^3$H]-NMS was determined empirically to be 0.264 nM. Binding reactions were incubated for 2 hours at room temperature on a Lab-Line Titer plate shaker at setting 7 (~750 rpm). Reactions were stopped and membranes collected onto 96-well Barex microplates with GF/B filter (1μm pore size) using a Brandel harvester and washed 3X with ice-cold harvesting buffer (50mM Tris-HCl, 0.9% NaCl, pH 7.4). Filter plates were dried overnight and counted in a PerkinElmer TopCount scintillation counter (PerkinElmer Life and Analytical Sciences). True [$^3$H]-NMS concentration was back-calculated after counting aliquots of 5X [$^3$H]-NMS used in the reaction. Atropine $K_i$ determined to be 0.21 by Cheng-Prusoff equation. For all assays, radioligand depletion was kept to approximately 10% or less. Data shown represent mean values obtained from at least three independent determinations performed using three or more replicates (error bars represent +/- SEM).

**ACh +/- M$_5$ PAM VU0238429 competition binding assays.** Membranes were prepared from M$_5$-CHO cells according to a previously described protocol. Binding reactions contained 0.09 nM [$^3$H]-NMS (obtained commercially from Amersham), 20 μg of membrane protein, and ACh plus vehicle or test compound in a total volume of 500 μl assay buffer (100 mM NaCl, 10 mM MgCl$_2$, 20 mM HEPES, pH 7.4). 1 μM (final) atropine was used to determine non-specific binding. The $K_D$ of [$^3$H]-NMS was determined empirically to be 0.264 nM. Binding reactions were incubated for 2 hours at

room temperature on a Lab-Line Titer plate shaker at setting 7 (~750 rpm). Reactions were stopped and membranes collected onto 96-well Barex microplates with GF/B filter (1μm pore size) using a Brandel harvester and washed 3X with ice-cold harvesting buffer (50mM Tris-HCl, 0.9% NaCl, pH 7.4). Filter plates were dried overnight and counted in a PerkinElmer TopCount scintillation counter (PerkinElmer Life and Analytical Sciences). True [$^3$H]-NMS concentration was back-calculated after counting aliquots of 5X [$^3$H]-NMS used in the reaction. Radioligand depletion was kept to approximately 10% or less. Data shown represent mean values obtained from at least three independent determinations performed using three or more replicates (error bars represent +/- SEM).

**M$_1$ PAM VU HTS hits competition binding assays.** Membranes were prepared from M$_1$-expressing CHO cells as described. Binding reactions contained 0.1 nM [$^3$H]-NMS, 20 μg of membranes and compound or vehicle (0.3% DMSO, final, to define total binding) or 1 μM atropine (to define nonspecific binding) in a total volume of 500 μl. The $K_D$ of [$^3$H]-NMS was determined empirically to be 0.21 nM. Compounds were serially diluted in DMSO and then diluted in assay buffer (100 mM NaCl, 10 mM MgCl$_2$, 20 mM HEPES, pH 7.4) to give a final DMSO concentration of 0.3% in the binding reaction. Binding reactions were incubated for 2 hours at room temperature on a Lab-Line Titer plate shaker at setting 7 (~750 rpm). Reactions were stopped and membranes collected onto 96-well Barex microplates with GF/B filter (1μm pore size) using a Brandel harvester and washed 3X with ice-cold harvesting buffer (50mM Tris-HCl, 0.9% NaCl, pH 7.4). Filter plates were dried overnight and counted in a PerkinElmer TopCount scintillation Counter (PerkinElmer Life and Analytical Sciences). For ACh affinity experiments, the $K_I$ of an ACh competition curve was determined in the absence and

presence of fixed concentrations (3 to 30 μM, final) of test compound. Actual [$^3$H]-NMS concentrations were back-calculated after counting aliquots of 5X [$^3$H]-NMS added to the reaction. Radioligand depletion was routinely kept to approximately 5% or less.

**M$_1$ allosteric agonist competition binding assays**. Membranes were prepared from M$_1$, M$_2$, M$_3$, M$_4$, and M$_5$-expressing CHO cells as previously described. Binding reactions contained 0.1 nM [$^3$H]-NMS, 20 μg of membrane and compound or 1 μM atropine (to define nonspecific binding) in a total volume of 500 μl. Compounds were serially diluted in DMSO-matched assay buffer (100 mM NaCl, 10 mM MgCl$_2$, 20 mM HEPES, pH 7.4) to give a final DMSO concentration of 0.3% in the binding reaction. Binding reactions were incubated for 2 hours at room temperature on a Lab-Line Titer plate shaker at setting 7 (~750 rpm). Reactions were stopped and membranes collected onto 96-well Barex microplates with GF/B filter (1μm pore size) using a Brandel harvester and washed 3X with ice-cold harvesting buffer (50mM Tris-HCl, 0.9% NaCl, pH 7.4). Filter plates were dried overnight and counted in a PerkinElmer TopCount scintillation counter (PerkinElmer Life and Analytical Sciences). Actual [$^3$H]-NMS concentrations were back-calculated after counting aliquots of 5X [$^3$H]-NMS added to the reaction. Radioligand depletion was routinely kept to approximately 10% or less.

**Merck M$_1$ PAM BQCA and M$_1$ Antagonist VU0255035 competition binding studies**. Full methods are not described due to redundancy, as details are essentially the same as described previously for M$_4$ PAM and M$_5$ PAM projects. Binding reactions used 15-25 mg of membrane protein prepared from rM$_1$ receptor expressing CHO cells and 0.1 nM [$^3$H]-NMS (GE Healthcare) in a final volume of 1 mL. Non-specific binding was determined in the presence of 1 μM atropine.

**Millipore functional screening.** Prior to conducting *in vivo* experiments with $M_1$ PAM BQCA, $M_4$ PAM VU0152099, and $M_4$ PAM VU0152100, the compounds were submitted to Millipore's GPCR Profiler™ Service where it was evaluated for agonist, antagonist, and allosteric potentiator activity against a panel of 16 GPCRs in a functional screening paradigm ($N$=2). Calcium mobilization assays were used to obtain full compound CRCs, and for PAM screens a standard orthosteric agonist was used for each target.

**MDS Pharma/Ricerca binding screening**. $M_4$ PAM VU0152099, $M_4$ PAM VU0152100 (as reported in Chapter III), numerous $M_5$ PAMs (as reported in Chapter IV), and $M_1$ allosteric agonists (as reported in Chapter V) were submitted to MDS Pharma (now Ricerca) for LeadProfile screening across a panel of 67 different GPCRs, ion channels, enzymes, transporters, and nuclear hormone receptors. Competition binding assays were performed using standard orthosteric radioligands for each target ($N$=2) with test compounds at 10 μM. Results are reported as % inhibition of radioligand binding caused by the test compound, with >50% inhibition signifying substantial activity at a given target.

### *In vivo* Pharmacology

All experiments were conducted in accordance with the National Institutes of Health regulations of animal care covered in Principles of Laboratory Animal Care (National Institutes of Health publication 85-23, revised 1985) and were approved by the

Institutional Animal Care and Use Committee. Methods for studies performed for the $M_5$ PAM and $M_4$ PAM projects are described below, all other behavioral and PK studies associated with $M_1$ PAM, $M_1$ allosteric agonist, TBPB, and $M_1$ antagonist projects are similar to those described below, and in cases where data from a study is discussed in the associated text/figures (i.e. in Chapter V) that is not described below, the full methodology can be found in the associated references.

*Amphetamine-induced hyperlocomotion behavioral rat studies*

All behavioral studies were conducted using male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.; Indianapolis, IN) weighing 270 to 300 grams. Subjects were housed in pairs in a large colony room under a 12-h light/dark cycle (lights on at 6:00 a.m.) with food and water provided *ad libitum*. Test sessions were performed between 6:00 a.m. and 6:00 p.m. Dose groups consisted of 8-16 rats per dose group. All doses of VU0152099 and VU0152100 refer to the salt form and were injected in a 1.0 mL/kg volume. Each compound was dissolved in 10% Tween 80 and double deionized water with the pH adjusted to approximately 7.0 using 1N NaOH.

Studies were conducted using a SmartFrame Open Field System (Kinder Scientific., San Diego, CA) equipped with 32 horizontal (x- and y-axes) infrared photobeams located 1 cm above the floor of the chamber. Changes in ambulation or locomotor activity were measured as the number of total photobeam breaks as expressed in 5 min intervals and were recorded with a Pentium I computer equipped with the Motor monitor system software (Kinder Scientific).

Rats were placed in the open field chambers for a 30 min habituation interval (data not shown), followed by a pretreatment with vehicle or a 56.6 mg/kg dose of either VU0152099 or VU0152100 (I.P.) for an additional 30 min. Next, all rats received an injection of 1 mg/kg (subcutaneous [S.C.]) of amphetamine and locomotor activity was measured for an additional 60 min. Data were analyzed by a one-way ANOVA with comparison with the vehicle+amphetamine control group using Dunnett's test. Calculations were performed using JMP v5.1.2 (SAS Institute Inc., Cary, NC) statistical software.

*Roto-rod behavioral rat studies*

The effects of VU0152100 on motor performance were evaluated using a Rotorod (Columbus Instruments, Columbus, OH). All rats were given an initial training trial of 120 s, followed by 2 additional training trials of 85s approximately 10 min apart, using a rotarod (7.5cm in diameter) rotating at a constant speed of 20 revolutions/minute. After initial training trials, a baseline trial of 85s was conducted, and any rats that did reach the 85s criteria were excluded from the study. Rats were then pretreated for 30 minutes *i.p.* with vehicle or dose of VU0152100, specifically 30, 56.6 or 100 mg/kg, and then the time each animal remained on the rotorod was recorded; animals not falling off the rotorod were given a maximum score of 85s. Data were analyzed by a one-way ANOVA, with comparison to the vehicle control group using Dunnett's test. Calculations were performed using JMP v5.1.2 (SAS Institute Inc., Cary, NC) statistical software.

*Pharmacokinetic/PBL rat studies*

**M₄ PAMs**. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc.; Indianapolis, IN) weighing 225-250g, were fasted overnight prior to dosing. Compounds were dissolved 10% Tween 80 and double deionized water with the pH adjusted to approximately 7.0 using 1N NaOH at a concentration of 56.6 mg/mL and sonicated until a uniform homogenous solution was obtained. The dose was administered ip at 56.6 mg/kg per compound. Three animals were used for each time point. The rat blood and brain were collected at 0.5, 1, 2 and 4h. Animals were euthanized, decapitated and the brains removed and frozen on dry ice. Trunk blood was collected in ethylenediamine tetra-acetic acid (EDTA) Vacutainer tubes and plasma was separated by centrifugation and stored at -80 °C until analysis.

For the brain sample preparation, frozen whole rat brains were weighed (1.5 to 1.8g) and placed in 3 mL of ice-cold solution of acetonitrile and methanol (1:1, volume) with a synthetic internal standard (50 ng/mL) and homogenized using a Sonic Dismembrator Model 100 (Fischer Scientific) at maximal speed for 2 min. Next, a 1ml aliquot of each homogenate was placed into 1.5 mL centrifuge tubes and centrifuged at 16,000 rpm for 5 min. Finally 100 mL of supernatant was injected into LC-MS-MS. Plasma samples (100ml) were combined with 200ml of ice-cold solution of the internal standard (100ng/mL) in acetonitrile with 0.1% formic acid. After vortexing for one minute, the mixture was centrifuged at 16,000 rpm for 5 min in a bench-top Spectrafuge 16M Microcentrifuge (Labnet, Woodbridge, NJ). Again, 100 mL of the supernatant was injected into LC-MS-MS.

For the LCMS Analysis, the LC separation was carried out on a Phenomenex Luna ODS, 5 micron, 2.1 mm x 5 cm column (Torrance, CA) at a flow rate of 0.3 mL/min. The gradient started with 80% solvent A (0.1% Formic acid in water) and 20% solvent B (0.1% Formic acid in CH$_3$CN), held for 1 min and increasing to 100% B in 4 min and held for 1 min. Mass spectrometry was carried out using a ThermoFinnigan TSQ Quantum ultra (Thermo Scientific, Waltham, MA) mass spectrometer in positive ion mode. The software Xcalibur version 2.0 was used to control the instrument and collect data. The ESI source was fitted with a stainless steel capillary (100μm i.d.). Nitrogen was used as both the sheath gas and the auxiliary gas. The ion transfer tube temperature was 300ºC. The spray voltage, tube lens voltage, pressure of sheath gas and auxiliary gas were optimized to achieve maximal response using the test compounds mixing with the mobile phase A (50%) and B (50%) at a flow rat of 0.3 mL/min. Collision-induced dissociation (CID) was performed on the test compounds and internal standards under 1.0 mTorr of argon. Selected reaction monitoring (SRM) was carried out using the transitions from, m/z 356 to 205 @ 30 eV for VU0152099, m/z 342 to 205 @ 27 eV for VU0152100, and m/z 310 to 223 @ 25 eV for our internal standard.

The calibration curves were constructed by spiking known amounts of test compounds in blank brain homogenates and plasma. The samples went through the same extraction steps as described above. A linear response was achieved from 50ng/mL to 100ug/mL in both matrices. Compound exposure following administration was determined by calculating AUC (0-∞) using the trapezoidal method.

**M$_5$ PAMs**. VU0238429 was formulated as a 10% Tween 80 micro-suspension in sterile water at the concentration of 5 mg/mL and administered intraperitoneally to male

Sprague-Dawley rats weighing 225 to 250 g (Harlan Sprague-Dawley, Inc., Indianapolis, IN) at the dose of 10 mg/kg. The rat blood and brain were collected at 0.5, 1, 3, and 6 h. Animals were euthanized and decapitated, and the brains were removed, thoroughly washed in cold phosphate buffered saline and immediately frozen on dry ice. Trunk blood was collected in EDTA Vacutainer tubes, and plasma was separated by centrifugation and stored at -80 °C until analysis. Three animals were used for each time point. On the day of analysis, frozen whole-rat brains were weighed and homogenized in 1:3 (w/w) volumes of ice-cold phosphate buffered saline (pH 7.4). The sample extraction of plasma (100µl) and brain homogenate (250 µl) was performed by a method based on protein precipitation, using three volumes of cold acetonitrile containing 0.1% formic acid and an internal standard (VU-178) having final concentration of 50 ng/mL. Extracts were vortex mixed for 5 min. followed by centrifugation at 14000 rpm for 10 min.

The supernatants of plasma and brain homogenate extracts were analyzed by means of HPLC/MS/MS, using a ThermoFinnigan TSQ Quantum Ultra (Thermo Fisher Scientific, Waltham, MA) mass spectrometer in positive ion mode. The chromatographic separation was achieved on an Acquity UPLC BEH C18 column (1.7um; 2.1x50mm) at a flow rate of 0.8 mL/min. The gradient program was used with the mobile phase, combining solvent A (95: 5: 0.1% formic acid in water: acetonitrile) and solvent B (95: 5: acetonitrile: 0.1% formic acid in water) as follows: 20% B (0.5 min), 20–95% B (0.5 min), 95% B (1 min), 95–20% B (0.2 min), 20% B (2.8 min). The column temperature was set at 50 °C. The software Xcalibur version 2.0 was used to control the instrument and collect data. The electrospray ionization source was fitted with a stainless steel capillary (100 µm i.d.). Nitrogen was used as both the sheath gas and the auxiliary

gas. The ion transfer tube temperature was 300 °C. The spray voltage, tube lens voltage, and pressure of sheath gas and auxiliary gas were optimized to achieve maximal response using the test compounds mixing with the mobile phase A (50%) and B (50%) at a flow rate of 0.8 mL/min. Collision-induced dissociation was performed on compound VU0238429 and internal standard under 1.0 mTorr of argon. Selected reaction monitoring was carried out using the transitions from $m/z$ 352 to 121 for VU0238429, and $m/z$ 310 to 223 for VU-178 (internal standard). The calibration curves were constructed and linear response was obtained in the range of 10- 2000 ng/mL by spiking known amounts of VU0238429 in blank brain homogenates and plasma. Brain concentrations were corrected for dilution in PBS and for residual blood volume using 15μl/g as the vascular space (Brown et al., 1986). The final PK parameters were calculated by non-compartmental analysis using WinNonlin software (version 5.1, Pharsight Inc.).

# DISCOVERY, OPTIMIZATION, AND CHARACTERIZATION OF NOVEL SUBTYPE-SELECTIVE MACHR4 POSITIVE ALLOSTERIC MODULATORS


## Discovery of $M_4$ PAM VU010010


As discussed in the Introduction (Chapter I), a lack of $M_4$-selective small molecule tools has precluded thorough investigation into the role of $M_4$ in basic neurobiology and in target validation for therapeutic discovery. Based on $M_4$ localization data and on results from studies with $M_4$-KO mice, selective activation of $M_4$ may represent an attractive strategy for treatment of various CNS disorders such as schizophrenia[44]. A disclosure by Eli Lilly revealed the first potentially $M_4$-selective small molecule, an $M_4$ PAM termed LY2033298 that was reported to possess high potency ($EC_{50} < 100$ nM) and efficacy at the human receptor with no activity at any other mAChR subtype at high concentrations using cell-based functional assays (**Fig. 1**)[123]. At the time, no data was presented for activity of LY2033298 at rat or mouse $M_4$, and since preclinical research typically relies on rodent studies, the utility of this compound for our own experiments depended largely on its pharmacology in rodents. Thus, we re-synthesized LY2033298 and determined its potency in the presence of an ACh $\sim EC_{20}$ by $Ca^{2+}$ mobilization assay in $rM_4/G_{qi5}$ cells. Importantly, this compound exhibited an approximately two-order of magnitude loss of potency at $rM_4$ ($EC_{50} > 1$ μM, data not shown), rendering it sub-optimal as an $rM_4$ research tool.
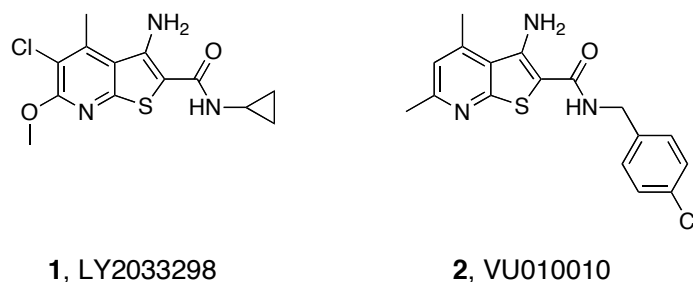
**Figure 1.** Structures of Eli Lilly hM$_4$ PAM LY2033298 (**1**) and Vanderbilt rM$_4$ PAM VU010010 (**2**)

Therefore, we sought to identify novel M$_4$ PAMs with pharmacological profiles suitable for rodent studies[124-126]. Rather than execute a full HTS (as was done for other mAChR PAM projects described in Chapters IV and V), we used a cheminformatic approach wherein a structure similarity search based on LY2033298 was performed on a large commercial drug-like chemical collection. This database screen provided 232 virtual hits, which were then ordered and subsequently screened for rM$_4$ PAM activity in a single concentration-point format using Ca$^{2+}$ mobilization assays. Ultimately, three structurally similar hits were identified that exhibited concentration-dependent M$_4$ PAM activity with sub- to low-micromolar potencies (data not shown). However, these compounds suffered from poor solubility in assay buffer, resulting in incomplete CRC plateaus at higher concentrations. A limited medicinal chemical optimization of the eastern amide side chain in the form of alternative benzyl substitutions identified VU010010 (**Fig. 1**, compound **2**), which possessed relatively greater solubility, an rM$_4$ EC$_{50}$ of approximately 400 nM, and an rM$_4$ ACh CRC fold-shift of 47x at 30 μM.

Characterization of VU010010 in a number of *in vitro* experiments revealed that it was highly selective for M$_4$ and that its potentiation was mediated by enhancement of both ACh affinity and G-protein coupling efficiency (data not shown). Although this early compound represented the first potent and selective rM$_4$ PAM tool useful for *in*

*vitro* cell-based and *ex-vivo* electrophysiology experiments, it still suffered from sub-optimal physiochemical properties that precluded acceptable vehicle formulation for *in vivo* studies and likely contributed to a lack of central brain penetration in a single-dose rat PK/PBL study (data not shown). Therefore, we began a comprehensive chemical optimization project aimed at exploring structure-activity relationships (SAR) around the VU010010 scaffold in order to obtain a compound universally suitable for systemic dosing in a wide range of *in vivo* studies.

### Optimization of M$_4$ PAM VU010010 to obtain VU0152099 and VU0152100

Optimization began with a diversity-oriented synthesis (DOS) approach to explode SAR with a variety of hypothesis-driven, structural changes to the lead compound. The rationale for this approach for the optimization of VU010010 is that SAR for allosteric ligands is often 'flat' or 'shallow', with subtle structural modifications leading to a complete loss of activity, and often only one portion of an allosteric ligand is amenable to change. Therefore, a multi-dimensional, DOS library approach provides the best opportunity to quickly identify productive SAR as opposed to a lead optimization strategy based on classical, single compound synthesis. One explanation for the lack of central activity observed with VU010010 could be the result of the poor physiochemical properties alone, or in combination with efflux through a BBB transporter such as P-glycoprotein (P-gp). The β-aminoamide motif (**Fig. 2a**) present in VU010010 represents a potential P-gp liability, which could be removed by cyclization to analogs such as compounds **4** (**Fig. 2a**). Alternatively, P-gp susceptibility could also be diminished by

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electronically attenuating the basicity of the amine moieties by the incorporation of distal
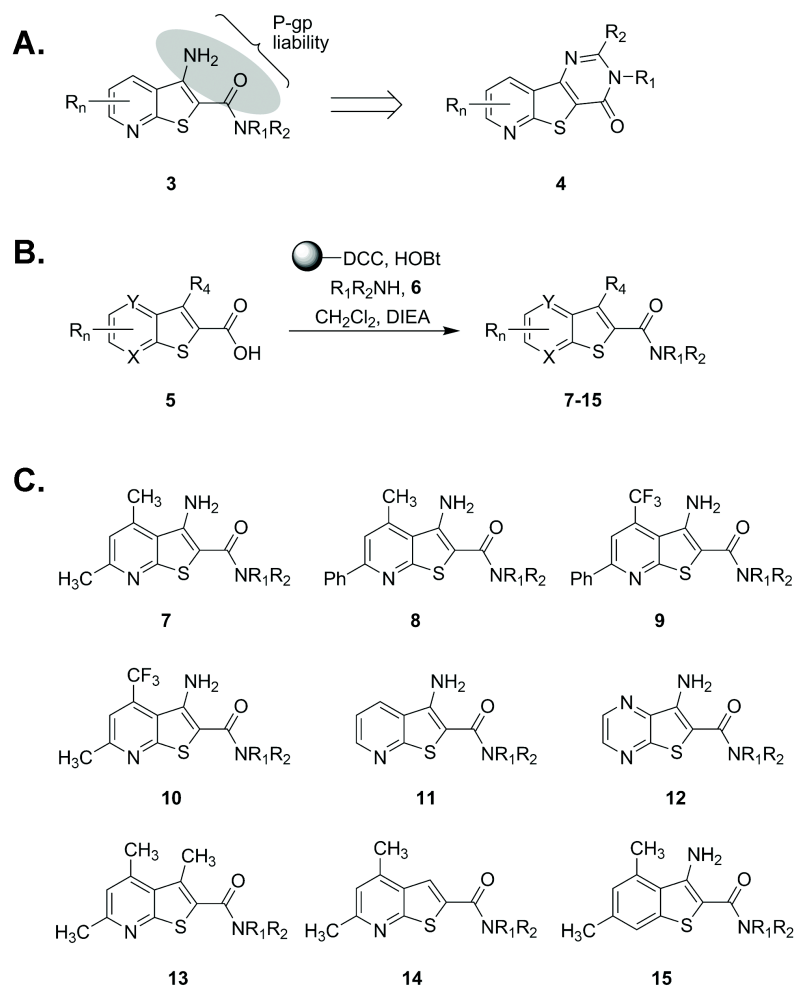
fluorine atoms.



**Figure 2**. Chemical optimization of VU010010. Each of the ten scaffolds (**5**, **7-15**) were derivatized with diverse amide side chains in 12-24 member libraries using standard amide-coupling reaction conditions. Syntheses jointly performed with P. Kennedy.

Utilizing solution phase parallel synthesis, we synthesized small, 12- to 24-member focused libraries around each of the 10 scaffolds **5** and **7-15** (**Fig. 2b-c**), which were then purified by mass-directed preparative HPLC to analytical purity (>98%). This collection of VU010010 analogs incorporated $CF_3$ moieties (scaffolds **9** and **10**) to

electronically attenuate potential P-gp susceptibility, deletion of the β-amino moiety (scaffold **14**), or replacement of the β-amino moiety with an isosteric methyl group (scaffold **13**). Other scaffolds explored the deletion of substituents on the pyridine nucleus (scaffold **11**), incorporation of an additional nitrogen atom to afford a pyrimidine nucleus (scaffold **12**) or removal of the pyridine nitrogen atom in VU010010 (scaffold **15**). Finally, a library around scaffold **7** focused on maintaining the core structure of VU010010, but explored alternative amides, selected to improve physiochemical properties and lower the logP value.

As observed with positive allosteric modulators of Class C GPCRs, SAR around VU010010 was relatively flat, possibly due to a shallow binding pocket. An ACh $EC_{20}$ triage screen, employing a functional, fluorescence-based $Ca^{2+}$ assay in CHO cells stably co-expressing the rat $M_4$ mACh receptor and the chimeric G protein, $G_{qi5}$, quickly eliminated all VU010010 analogs except those in library **7** (**Fig. 3**). Within library **7**, all aliphatic and non-benzyl amides were inactive, and only benzyl and heteroaryl methyl congeners of VU010010, **7a-7p**, retained $M_4$ PAM activity (**Table 1**). The potency of each compound was determined by pre-incubating cells with vehicle or increasing concentrations of test compound followed by the addition of an ACh $EC_{20}$ to yield CRCs. Subtle substitution changes on the arene ring lost activity 5-10-fold in terms of $M_4$ $EC_{50}$ and/or fold-shift of the ACh CRC (**Table 1**). For instance, analog **7d**, in which the 4-Cl moiety of VU0100010 is moved to the 3-position results in a loss in potency of over 9-fold ($EC_{50}$ = 3.7 μM). Similarly, the unsubstituted phenyl congener **7a** retains $M_4$ PAM activity ($EC_{50}$ = 630 nM), but the fold-shift diminishes to 8.6-fold, *versus* the 47-fold shift observed for VU010010. In general, functionalized benzyl amides and pyridyl

methyl congeners (**7f and 7g**) were well tolerated, providing selective $M_4$ PAMs with $EC_{50}$ values ranging from 380 nM to 3.7 μM and with fold-shifts of the ACh CRC from 8.6- to 70-fold.
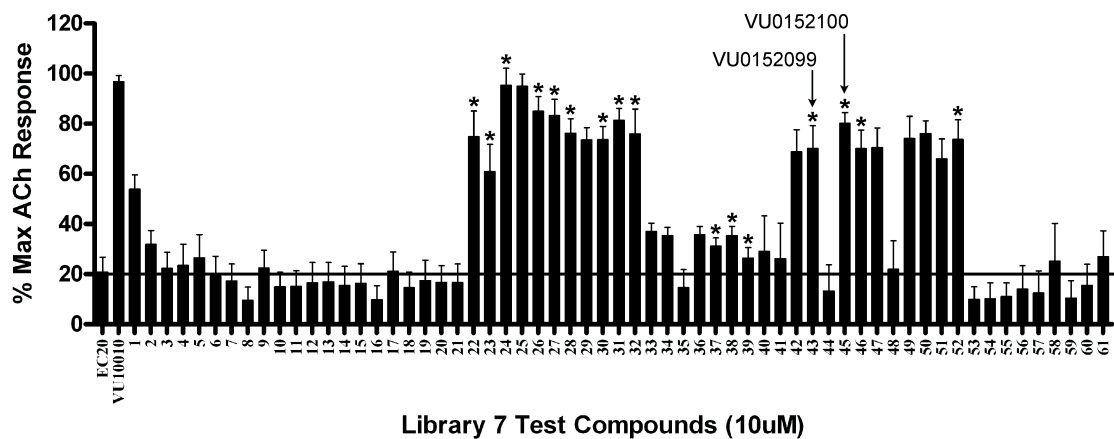


**Figure 3**. Singlepoint screen of $M_4$ PAM VU010010 analogs based on scaffold **7** bearing eastern amide side chain substitutions. $Ca^{2+}$ mobilization was used to obtain singlepoint (10 μM) potentiation responses for each compound in the presence of an ACh ~$EC_{20}$ in $rM_4$ cells. Data represent Mean +/-SEM of at least three independent determinations. Experiments performed by A. Brady.

**Table 1**. M$_4$ PAM potency and ACh CRC fold-shift SAR for VU010010 analogs based on scaffold **7**. Ca$^{2+}$ mobilization was used to obtain CRCs of test compounds in the presence of an ACh ~EC$_{20}$ using rM$_4$ cells. Similar assays determined the fold-shift of an ACh CRC in the presence of 30 μM test compound (N.D. = not determined). Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.
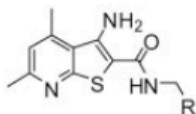


| Compound | R | Rat M$_4$ EC$_{50}$[a] | Rat M$_4$ Ach -Fold Shift |
|---|---|---|---|
| | | μM | |
| 7a | | 0.63 | 8.6 |
| 7b | | 0.83 | 11.8 |
| 7c | | 1.83 | N.D. |
| 7d | | 3.70 | N.D. |
| 7e | | 2.63 | N.D. |
| 7f | | 2.04 | N.D. |
| 7g | | 2.88 | N.D. |
| 7h | | 1.44 | N.D. |
| 7i | | 1.80 | N.D. |
| 7j | | 2.96 | N.D. |
| 7k | | 3.04 | N.D. |
| 7l | | 0.88 | N.D. |
| 7m | | 1.12 | N.D. |
| 7n | | 0.72 | 13.7 |
| 7o | | 0.40 | 29.7 |
| 7p | | 0.38 | 70.1 |

Two compounds were selected for further evaluation based on their ability to potentiate the $M_4$-mediated $Ca^{2+}$ response to ACh. VU0152099 (**7o**), (**Fig. 4a**) and VU0152100 (**7p**), (**Fig. 4d**) retained $M_4$ PAM activity ($EC_{50}$ values of $403 \pm 117$ nM and $380 \pm 93$ nM, respectively) comparable to VU010010, and in the absence of an ACh $EC_{20}$, neither VU0152099 nor VU0152100 elicited a response (**Fig. 4b,e**).
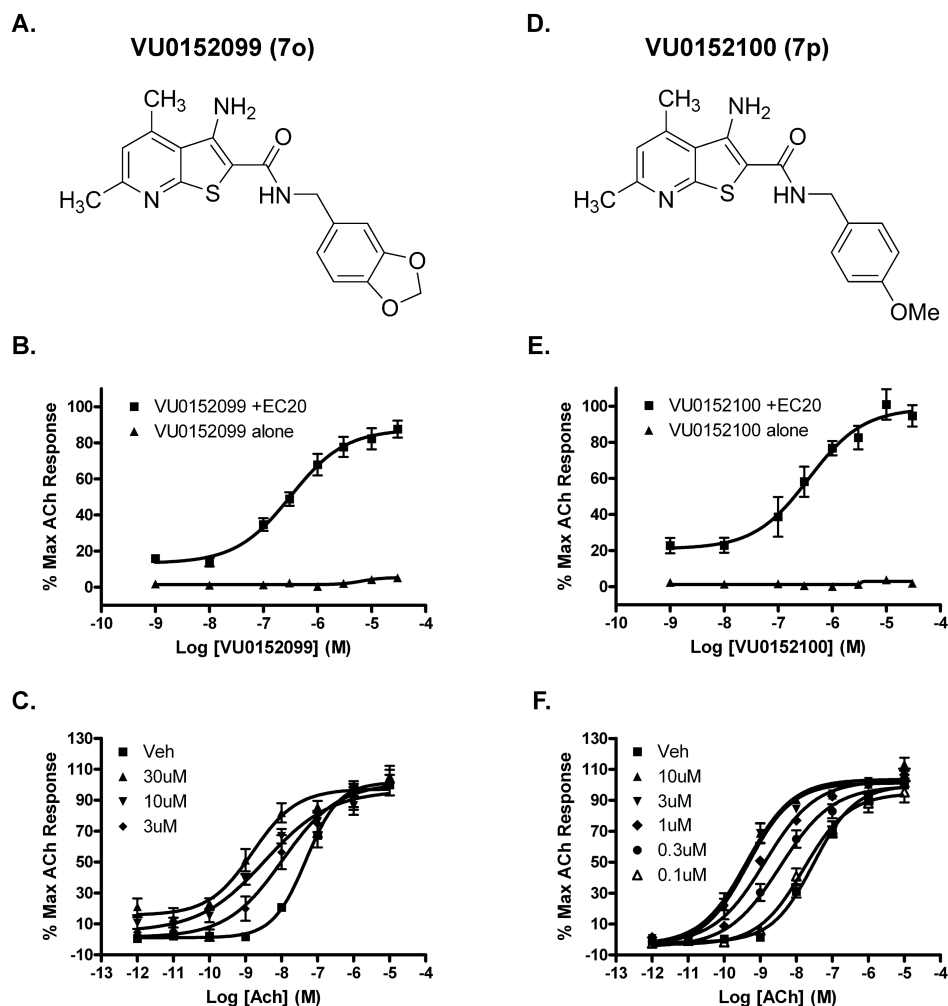


**Figure 4**. Structures, potency CRCs, and ACh CRC fold-shifts for $M_4$ PAM leads A.) VU0152099 (**7o**) and B.) VU0152100 (**7p**). $Ca^{2+}$ mobilization was used to obtain: B., E.) CRCs of test compound in the absence and presence of an ACh $\sim EC_{20}$, ($EC_{50}$ values: VU0152099 = 403 nM, VU0152100 = 380 nM) C., F.) CRCs of ACh in the absence or presence and of increasing fixed concentrations of test compound (fold-shift values: VU0152099 = 30x, VU0152100 = 70x). Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.

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We next determined the effects of maximal concentrations of each compound on the CRC of ACh. Cells were pre-incubated with a fixed concentration (0.1-30 μM) of test compound and subsequently stimulated with increasing concentrations of ACh. Both VU0152099 and VU0152100 induced a concentration-dependent left-shift of the ACh CRC with maximal shifts of 30-fold observed with 30 μM VU0152099 (**Fig. 4c)** and 70-fold observed with 10 μM VU0152100 (**Fig. 4f**).

### *In vitro* characterization of M$_4$ PAMs VU0152099 and VU0152100

Using Ca$^{2+}$ mobilization to assess the functional activity of VU0152099 and VU0152100 at the M$_4$ receptor requires co-expression of the chimeric G protein, G$_{qi5}$, in order to link the G$_{i/o}$-coupled M$_4$ receptor to the PLCβ/Ca$^{2+}$ pathway. As an alternative approach to measure M$_4$ PAM activity, we chose to take advantage of a novel functional assay recently developed in our lab that takes advantage of the ability of endogenous G$_{βγ}$ subunits of G$_{i/o}$-coupled GPCRs to alter the kinetics of GIRK channels to conduct the ion, thallium. For these studies, HEK293 cells stably co-expressing heteromeric GIRK1/2 channels and the human M$_4$ muscarinic receptor were pre-incubated with test compound and then stimulated with agonist (ACh) in the presence of thallium ion. Both VU0152099 and VU0152100 concentration-dependently potentiated the response to an EC$_{20}$ concentration of ACh with EC$_{50}$ values of 1.2 ± 0.3 μM and 1.9 ± 0.2 μM, respectively, and increased the maximal response to ACh to approximately 130% (**Fig. 5a**). As observed in the Ca$^{2+}$ mobilization assays described above, both VU0152099 and VU0152100 (10 μM) also enhanced the potency of ACh to induce GIRK-mediated

thallium flux, as manifest by a robust ($\approx$30-fold) left-shift of the ACh CRC from $77 \pm 1.2$ nM to $2.09 \pm 0.3$ nM and $2.35 \pm 0.5$ nM, respectively (**Fig. 5a**). Taken together, these *in vitro* studies suggest that VU0152099 and VU0152100 are potent positive allosteric modulators that enhance the response of the $M_4$ receptor to the endogenous agonist, ACh.
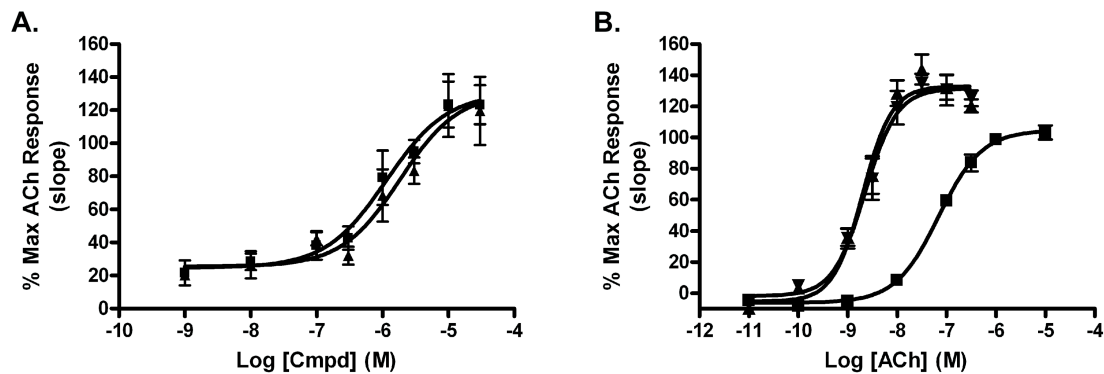


**Figure 5**. GIRK-mediated thallium flux $M_4$ PAM assays with lead compounds VU0152099 and VU0152100. A.) CRCs of test compounds in the presence of an ACh $\sim EC_{20}$ ($EC_{50}$ values: VU0152099 = 1.2 $\mu$M, VU0152100 = 1.9 $\mu$M) and B.) CRCs of ACh in the absence and presence of a fixed 10 $\mu$M concentration of test compound (fold-shift values: VU0152099 and VU0152100 both = $\sim$30x). Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.

To further confirm an allosteric mechanism of action by the novel $M_4$ PAMs, we evaluated the effect of VU0152099 and VU0152100 on equilibrium radioligand binding studies using membranes prepared from cells expressing the rM4 receptor. We first assessed the ability of increasing concentrations of the two $M_4$ PAMs to displace the orthosteric radioligand, $[^3H]$-*N*-methylscopolamine ($[^3H]$-NMS) (0.1 nM). Unlike the orthosteric antagonist, atropine, which potently inhibited $[^3H]$-NMS binding ($K_i$= 0.54 $\pm$ 0.1 nM), neither $M_4$ PAM displaced $[^3H]$-NMS at concentrations up to 30 $\mu$M (**Fig. 6a**), strongly suggesting that VU0152099 and VU0152100 act at a site on the $M_4$ receptor that is distinct from the orthosteric binding site.

In addition, we evaluated the effect of VU0152099 and VU0152100 on the affinity of ACh for the $M_4$ receptor by assessing the ability of an increasing concentration of ACh to displace [³H]-NMS (0.1 nM) binding in the absence or presence of the $M_4$ potentiators. VU0152099 and VU0152100 were found to induce a 20-25x leftward shift in the potency of ACh to displace [³H]-NMS binding to $M_4$ as manifest by a reduction in the ACh $K_i$ from 252 ± 17.9 nM (veh) to 10.4 ± 0.91 nM (VU0152099) and 12.2 ± 0.49 nM (VU0152100) (**Fig. 6a**). These data present a possible mechanism whereby these compounds could enhance receptor activation by increasing the affinity of $M_4$ for acetylcholine, and are in agreement with data previously determined for VU010010, where a 14 fold decrease in the ACh $K_i$ was reported.
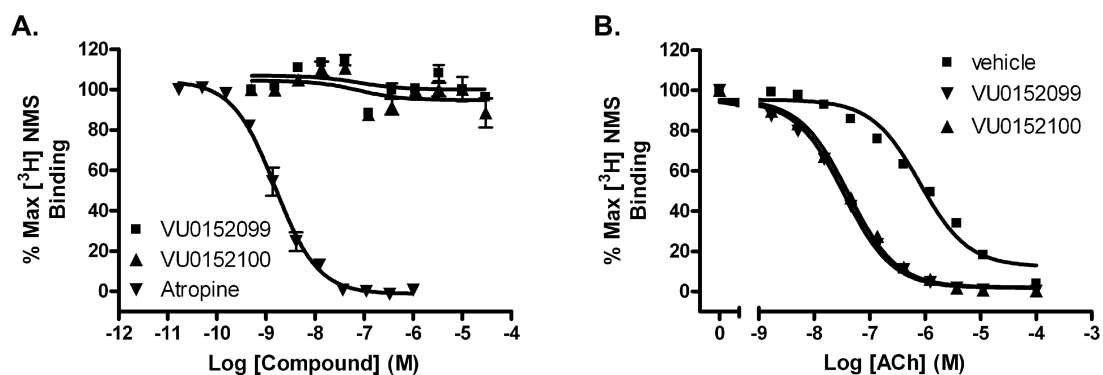


**Figure 6**. [³H]-NMS competition binding experiments with $M_4$ PAMs VU0152099 and VU0152100 at $rM_4$ cell membranes. A) CRCs of test compound and atropine positive control (atropine $K_i$ = 0.54 nM). B.) CRCs of ACh in the absence (vehicle) and presence of (10 μM) test compounds (ACh $K_i$ values: vehicle = 252 nM, VU0152099 = 10.4 nM [25x left-shift], VU0152100 = 12.2 nM [21x left-shift]). Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.

We next evaluated VU0152099 and VU0152100 in $Ca^{2+}$ mobilization assays for effects at all mAChR subtypes to determine whether these compounds are selective for $M_4$. Both VU0152099 and VU0152100 were selective for $M_4$ relative to $M_1$, $M_2$, $M_3$ and

$M_5$. Neither VU0152099 (**Fig. 7a**) nor VU152100 (**Fig. 7b**) had any effect on the ACh CRCs at these other mAChR subtypes at concentrations up to 30 μM. To further assess selectivity of these compounds for $M_4$ relative to other potential targets, the activity of VU0152099 and VU0152100 also were evaluated in radioligand binding assays against a large panel of 67 discrete GPCRs, ion channels, transporters, nuclear hormone receptors, and enzymes (**Table 2**). These compounds were largely inactive at each of the targets in this panel screen. At concentrations of 10 μM, both compounds were completely inactive at most targets and induced less than 50% displacement of binding for all targets tested, with the single exception of $GABA_A$ receptors (assessed by flunitrazepam binding), where VU0152099 displayed 51% displacement. This suggested that VU0152099 may interact with the flunitrazepam binding site with an $IC_{50}$ value of approximately 10 μM, which still provides high selectivity for $M_4$ relative to $GABA_A$ receptors.
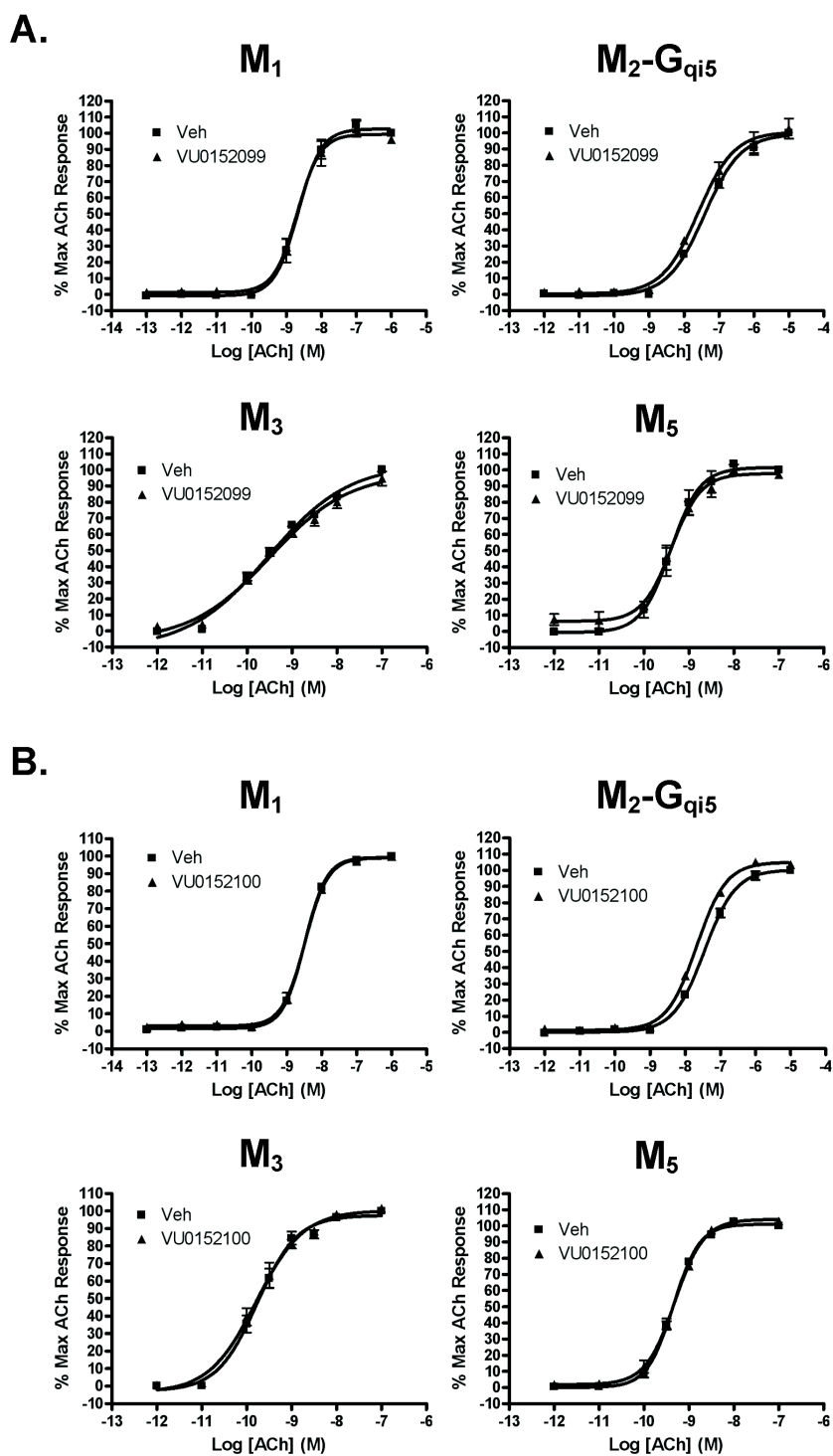
**Figure 7**. mAChR subtype-selectivity profiles for $M_4$ PAMs A.) VU0152099 and B.) VU0152100 determined by ACh CRC fold-shift. $Ca^{2+}$ mobilization was used to obtain CRCs of ACh in the presence and absence of 30 μM test compound using $M_1$, $M_2$, $M_3$, and $M_5$ cells. Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.

**Table 2**. Ancillary pharmacological activity of $M_4$ PAMs VU0152099 and VU0152100 in singlepoint (10 μM) competition binding assays across a large panel of off-targets. Experiments performed by MDS Pharma/Ricerca.

| Target | Species | % Inhibition VU0152099 | VU0152100 | Target | Species | % Inhibition VU0152099 | VU0152100 |
|---|---|---|---|---|---|---|---|
| Adenosine A1 | human | 28 | -1 | Histamine $H_3$ | human | 10 | 9 |
| Adenosine A2 | human | 46 | 43 | Imidazoline $I_2$, Central | rat | 7 | 8 |
| Adenosine A3 | human | 28 | 40 | Interleukin IL-1 | mouse | -19 | -4 |
| Adrenergic $\alpha_{1A}$ | rat | 10 | 9 | Leukotriene, Cysteinyl $CysLT_1$ | human | -4 | -6 |
| Adrenergic $\alpha_{1B}$ | rat | -7 | -7 | Melatonin $MT_1$ | human | 15 | 20 |
| Adrenergic $\alpha_{1D}$ | human | 1 | 1 | Muscarinic $M_1$ | human | -3 | 0 |
| Adrenergic $\alpha_{2A}$ | human | 7 | 17 | Muscarinic $M_2$ | human | -1 | -4 |
| Adrenergic $\beta_1$ | human | 17 | 17 | Muscarinic $M_3$ | human | -1 | -2 |
| Adrenergic $\beta_2$ | human | 30 | 35 | Neuropeptide Y $Y_1$ | human | 2 | 9 |
| Androgen (testosterone)AR | rat | 6 | 6 | Neuropeptide Y $Y_2$ | human | -2 | 2 |
| Bradykinin $B_1$ | human | 13 | 1 | Nicotinic Acetylcholine | human | 6 | -6 |
| Bradykinin $B_2$ | human | -5 | -9 | Nicotinic Acetylcholine $\alpha 1$, Bungarotoxin | human | -10 | -4 |
| Calcium channel L-type, benzothiazepine | rat | 13 | 7 | Opiate $\delta$ (OP1, DOP) | human | 2 | -13 |
| Calcium channel L-type, dihydropyridine | rat | 21 | 17 | Opiate $\kappa$ (OP2, KOP) | human | 5 | 2 |
| Calcium channel N-type | rat | -8 | -4 | Opiate $\mu$ (OP3, MOP) | human | 2 | -2 |
| Dopamine $D_1$ | human | 6 | 7 | Phorbol Ester | mouse | 2 | 4 |
| Dopamine $D_{2S}$ | human | 2 | 0 | Platelet Activating Factor (PAF) | human | 13 | 22 |
| Dopamine $D_3$ | human | -1 | 5 | Potassium Channel [$K_{ATP}$] | hamster | 3 | 6 |
| Dopamine $D_{4.2}$ | human | -13 | -11 | Potassium Channel hERG | human | 5 | 5 |
| Endothelin $ET_A$ | human | -8 | -8 | Prostanoid $EP_4$ | human | 15 | 7 |
| Endothelin $ET_B$ | human | 2 | -4 | Purinergic $P_{2X}$ | rabbit | 6 | -4 |
| Epidermal Growth Factor (EGF) | human | 9 | -5 | Purinergic $P_{2Y}$ | rat | 15 | 14 |
| Estrogen $ER^\alpha$ | human | -1 | -1 | Rolipram | rat | 36 | 29 |
| G protein-coupled receptor GPR103 | human | -3 | -4 | Serotonin (5-Hydroxytryptamine) $5-HT_{1A}$ | human | 2 | 2 |
| $GABA_A$ Flunitrazepam, central | rat | 51 | 43 | Serotonin (5-Hydroxytryptamine) $5-HT_3$ | human | -2 | 14 |
| $GABA_A$ Muscimol, central | rat | 6 | 9 | Sigma $\sigma_1$ | human | 2 | 6 |
| $GABA_{B1A}$ | human | -7 | 12 | Sigma $\sigma_2$ | rat | -8 | -3 |
| Glucocorticoid | human | -3 | 8 | Sodium Channel, Site 2 | rat | 11 | 20 |
| Glutamate, Kainate | rat | 3 | -15 | Tachykinin $NK_1$ | human | -15 | -18 |
| Glutamate, NMDA, Agonism | rat | 17 | 22 | Thyroid Hormone | rat | -1 | 0 |
| Glutamate, NMDA, Glycine | rat | 3 | 2 | Transporter, Dopamine (DAT) | human | 27 | 46 |
| Glutamate, NMDA, Phencyclidine | rat | 2 | 3 | Transporter, GABA | rat | 26 | 13 |
| Histamine $H_1$ | human | 14 | 7 | Transporter, Norepinephrine (NET) | human | 34 | 22 |
| Histamine $H_2$ | human | 7 | 10 | Transporter, Serotonin (5-Hydroxytryptamine) (SERT) | human | 0 | 4 |

Since VU0152099 and VU0152100 are allosteric modulators of $M_4$, it is possible that they have activity at similar allosteric sites on other GPCRs. If so, this would not be apparent in the radioligand binding assays of **Table 2**. The finding that these compounds are completely inactive at other mAChR subtypes makes this less likely since $M_4$ is more closely related to the other mAChR subtypes than to other GPCRs. However, to further evaluate the selectivity of VU0152099 for $M_4$ relative to other family A GPCRs, we contracted with Millipore to determine the effects of these compounds on functional responses of a panel of 15 other GPCRs to activation by their respective agonists (**Fig. 8**). For these studies, we chose family A GPCR subtypes that are among the closest relatives of mAChRs.
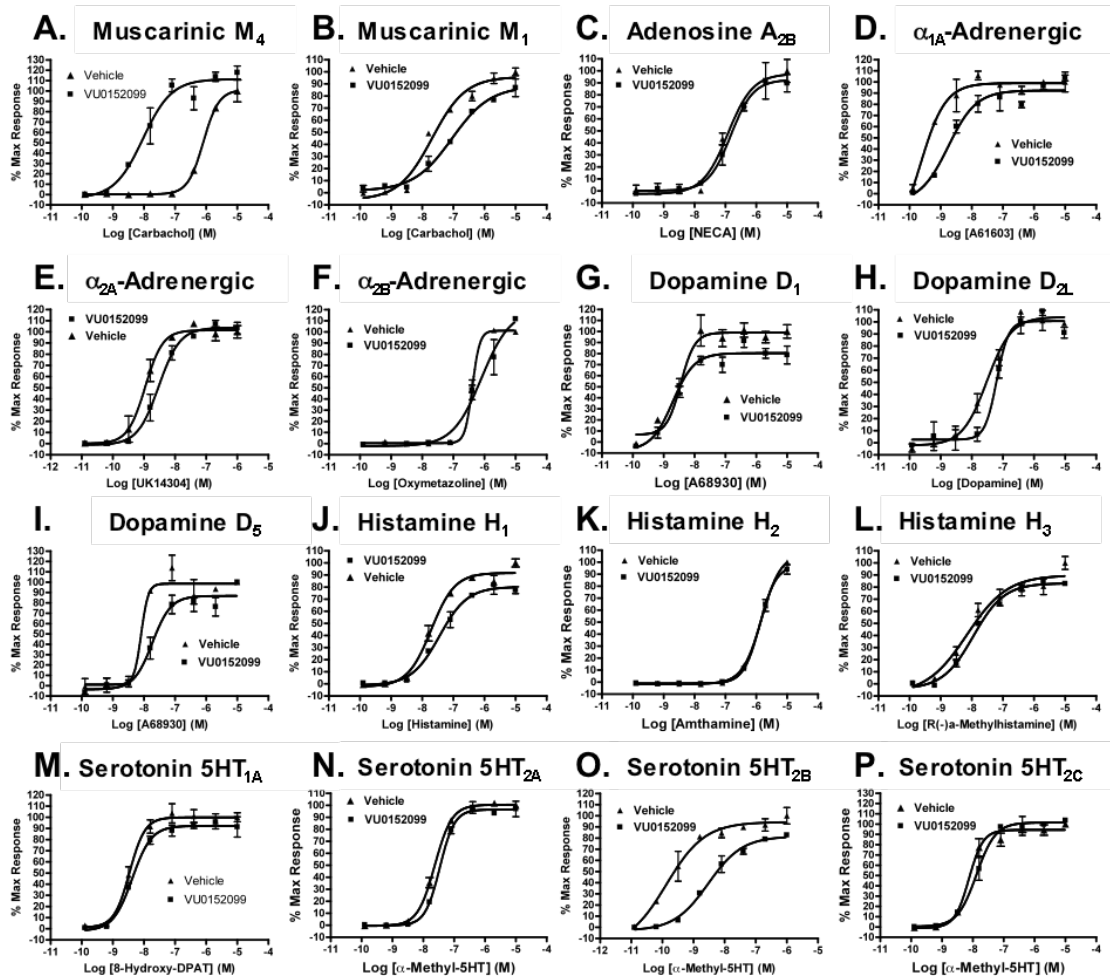
**Figure 8**. Functional GPCR off-target activity screening for $M_4$ PAMs VU0152099 and VU0152100. $Ca^{2+}$ mobilization assays using multiple cell lines expressing each of the GPCR targets and promiscuous G protein, $G_{\alpha 15/16}$ were used to obtain CRCs of orthosteric agonists in the absence and presence of test compound at 10 μM. VU0152099 exhibited no agonist activity at any of the targets tested, and was found to possess antagonist activity only at the $5HT_{2B}$ receptor (as indicated by an ≈16 fold right-shift in the agonist CRC in the presence of VU0152099). At the human $M_4$ mAChR, VU0152099 elicited a robust ≈80 fold left-shift in the carbachol CRC ($EC_{50s}$: CCh alone = 800 nM, CCh +VU0152099= 10 nM). Similar CRC curves were generated for additional GPCRs: Muscarinic $M_1$, Adenosine $A_{2B}$, $\alpha_{1A^-,\ 2A^-,\ 2B^-}$ Adrenergic, Dopamine $D_1$ ,$D_{2L}$, $D_5$, Histamine $H_1$, $H_2$, $H_3$, and Serotonin $5HT_{1A,\ 2A,\ 2B,\ 2C}$, with no comparable left-shift observed at any other target tested. All data represent Mean of two independent determinations. Experiments performed by Millipore GPCR Profiler Service™ service.

We first determined the effects of VU0152099 alone on each receptor and found that these compounds had no agonist activity at any receptor studied (data not shown). We then determined the effects of VU0152099 on full CRCs of agonists of each of these

receptors (**Fig. 8**). This allows unambiguous evaluation of whether the compounds possess antagonist activity (either allosteric or orthosteric) or allosteric potentiator activity at these other GPCRs. Consistent with our internal studies, VU0152099 induced a robust potentiation of ACh-induced activation of $M_4$, but had no potentiator activity at $M_1$ (**Fig. 8**). In addition, VU0152099 had no allosteric potentiator activity at any of the other GPCR subtypes tested (**Fig. 8**). The only significant activity detected for VU0152099 in this functional panel screen was weak antagonist activity at the serotonin $5HT_{2B}$ receptor (**Fig. 8**). Together, these data suggested that VU0152099 and VU0152100 possess clean ancillary pharmacology profiles, which would allow us to pursue the behavioral effects of selective $M_4$ activation *in vivo*.


### *In vivo* **pharmacokinetics of M$_4$ PAMs VU0152099 and VU0152100**


Prior to conducting *in vivo* studies with VU0152099 and VU0152100, pharmacokinetic studies were undertaken to assess brain/plasma ratios following systemic dosing of these compounds. In contrast, to the high log partition coefficient (logP) of VU010010 (4.5), both VU0152099 and VU0152100 possessed logP values of 3.7 and 3.6, respectively, a full order of magnitude less lipophilic than VU010010. As a consequence, both VU0152099 and VU0152100 displayed improved physiochemical properties and afforded homogeneous dosing solutions in multiple vehicles acceptable for *in vivo* studies. Further, we conducted *in vivo* PK/PBL studies in rats at the dose of 56.6 mg/kg (intraperitoneal [I.P.]). Both compounds exhibited substantial systemic absorption and brain penetration (**Fig. 9**). After administration, peak brain concentrations for both

compounds were in the range of 3-5 µg/ml (**Table 3**). VU0152100 (**Fig. 9b, Table 3**) was far superior to VU0152099 (**Fig. 9a, Table 3**) in terms of brain penetration, as evident from area under the curve (AUC) (0-∞) values. The AUC brain/AUC plasma ratio for VU0152099 was calculated to be 0.39 ± 0.01, while the ratio for VU0152100 was determined to be 0.86 ± 0.08 (**Table 3**). The half-life of the compounds in brain was 1.25 ± 0.02 h (VU0152099) and 1.12 ± 0.01 h (VU0152100) (**Table 3**). Therefore, our earlier concern of P-gp susceptibility within this series was likely unwarranted, and the lack of central activity for VU010010 was most likely due solely to physiochemical properties.
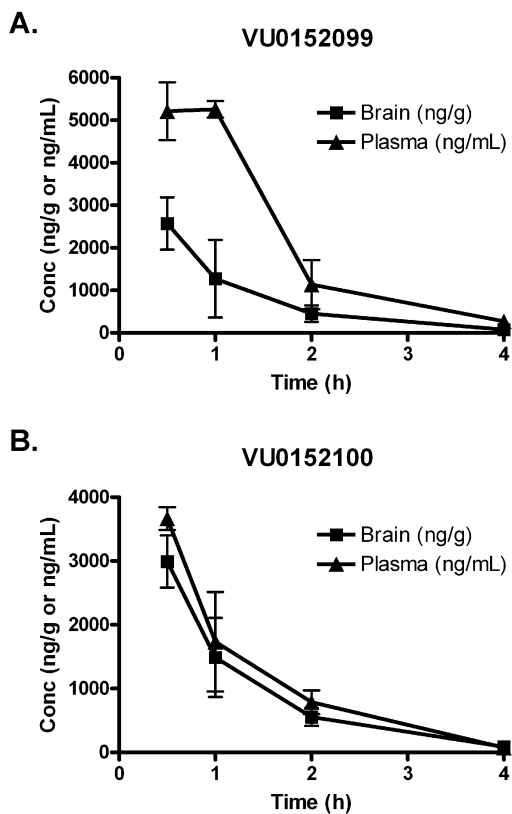


**Figure 9**. *In vivo* rat pharmacokinetic/PBL studies with $M_4$ PAMs VU0152099 and VU0152100. Concentrations were measured over time in brain and plasma following a single 56.6 mg/kg (IP) dose using male Sprague-Dawley rats (*N*=3 per compound). Experiments performed by S. Jadhav.

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**Table 3.** Pharmacokinetic parameters calculated for $M_4$ PAMs VU0152099 and VU0152100 from a rat PK/PBL study using a single 56.6 mg/kg (IP) dose each compound.

| PK Parameter | VU0152099 | VU0152100 |
|---|---|---|
| Mean AUC $(0-\infty)$ brain $(ng \cdot h/g)$ | $4751.80 \pm 666.17$ | $5726.35 \pm 694.68$ |
| Mean AUC$(0-\infty)$ plasma $(ng \cdot h/ml)$ | $11928.00 \pm 1472.36$ | $6570.35 \pm 235.87$ |
| AUC brain / AUC plasma | $0.39 \pm 0.01$ | $0.86 \pm 0.08$ |
| $t_{1/2}$ plasma (h) | $1.66 \pm 0.39$ | $1.62 \pm 0.69$ |
| $t_{1/2}$ brain (h) | $1.25 \pm 0.023$ | $1.12 \pm 0.01$ |

### *In vivo* behavioral studies with VU0152099 and VU0152100

Previous studies using the $M_1/M_4$-preferring muscarinic agonist xanomeline produced robust effects in several preclinical models predictive of antipsychotic-like activity, including reversal of amphetamine-induced hyperlocomotion in rats, as discussed in the Introduction (Chapter I). Based on our initial PK studies suggesting that systemic administration of VU0152099 and VU0152100 provides robust brain levels of these compounds, the effects of VU0152099 and VU0152100 were evaluated in reversing amphetamine-induced hyperlocomotion using a dose of 56.6 mg/kg (I.P.) for each compound with a 30 min pretreatment interval. As shown in **Fig. 10a**, both VU0152099 and VU0152100 produced robust decreases in amphetamine-induced hyperlocomotion over the time course tested. In addition, to provide further confirmation that the $M_4$ PAMs have no effect on baseline levels of motor performance, which could complicate the interpretation of the amphetamine-induced hyperlocomotion data, we evaluated the effects of one of the $M_4$ PAMs, specifically VU0152100, after administration alone on performance in the rotorod test (**Fig. 10b**). As shown, VU0152100 had no effect on performance in the rotorod test, even when tested at a dose of 100 mg/kg, which was higher than that required to observe reversal of amphetamine-induced hyperlocomotion.

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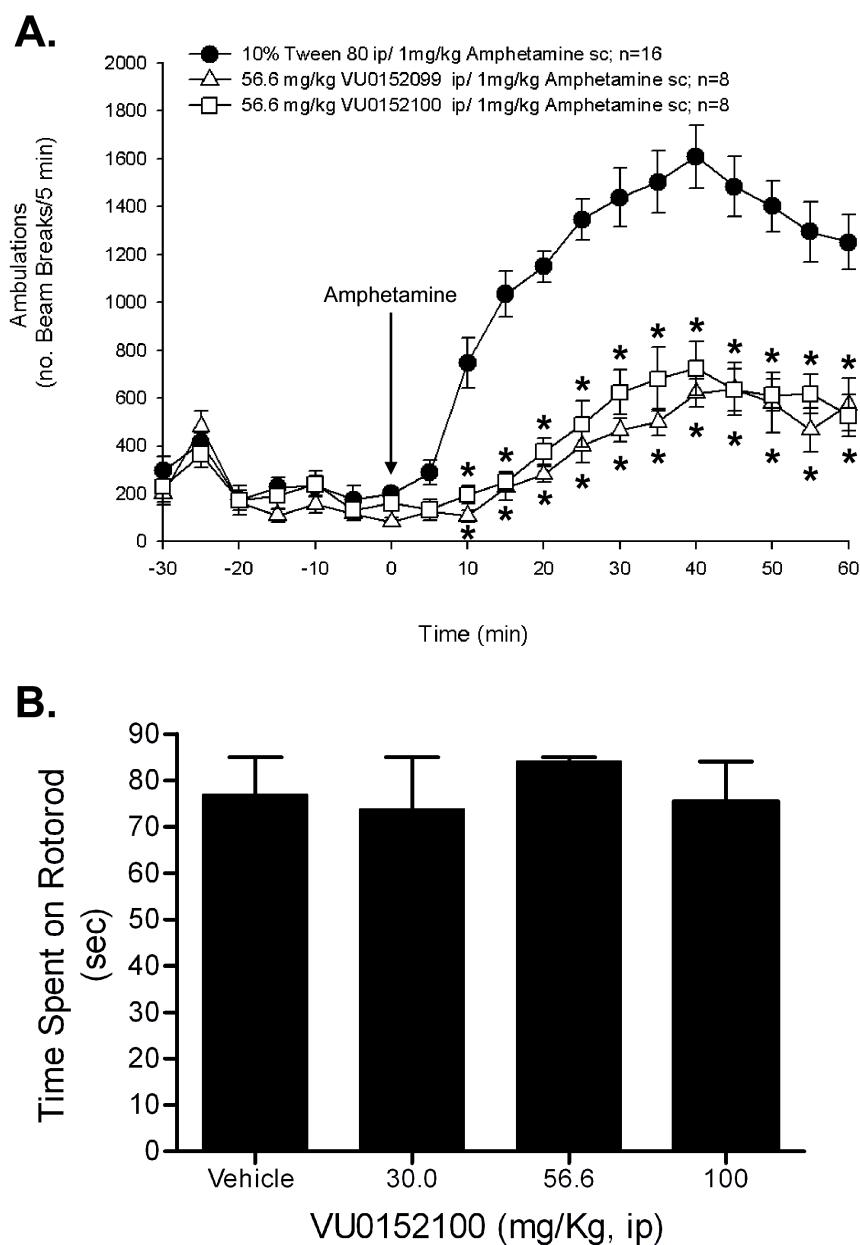
**Figure 10**. Reversal of amphetamine-induced hyperlocomotion and absence of sedation *in vivo* by $M_4$ PAMs VU0152099 and VU0152100. A.) Rats were pretreated for 30 min with vehicle or a 56.6 mg/kg dose of either VU0152099 or VU0152100 (IP). Next, all rats received an injection of 1 mg/kg (SC) of amphetamine and locomotor activity was measured for an additional 60 min. Each point represents the Mean +/-SEM of eight-sixteen rats. *, $P < 0.05$ versus veh+amphetamine control group, Dunnett's test. B.) Lack of effect of VU0152100 on motor performance on the Rotorod. After initial training, rats were pretreated for 30 minutes (IP) with vehicle or VU0152100 (30, 56.6 or 100 mg/kg), and time each animal remained on rotorod was recorded; Each bar graph represents the Mean +/-SEM of eight-ten rats. Experiments performed by C. Jones.

These behavioral findings represented the first published direct *in vivo* pharmacological evidence that selective $M_4$-modulation may be efficacious in the treatment of psychosis. Although much is known about the relationship between $M_4$ and regulation of central DA signaling based on $M_4$ localization and KO-mouse studies, which suggest that activation or positive allosteric modulation of $M_4$ is likely to inhibit DA-mediated motor activity, no potent, selective, and CNS penetrant $M_4$ activators were available to test this hypothesis in rodents, prior to our discovery of VU0152099 and VU0152100. Furthermore, these results shed important light on questions raised from clinical trials with xanomeline, the $M_1/M_4$-preferring orthosteric agonist that demonstrated robust anti-psychotic efficacy in multiple human trials (Chapter I)[100,110]. Specifically, our data support the hypothesis that the mechanism(s) of action underlying xanomeline's effects were mediated at least in-part by activation of $M_4$. However, it is possible, perhaps even likely, that xanomeline-induced $M_1$ activation also contributed to the drug candidate's broad clinical efficacy across multiple symptom clusters. Nonetheless, further investigation into the neurochemistry and neurocircuitry involved in mediating the observed *in vivo* effects (**Fig. 10**) of $M_4$ PAMs VU0152099 and VU0152100 is required.

## Optimization of $M_4$ PAMs VU0152099 and VU0152100

Although the novel $M_4$ PAMs VU0152099 and VU0152100 represented important advances over both LY2033298 and our first generation compound VU010010 and served as highly useful *in vivo* probes of selective $M_4$ modulation, we opted to

continue our optimization campaign in order to further improve upon these leads (**Fig. 11**). Both VU0152099 and VU0152100 possessed high potency, efficacy, and subtype-selectivity, with acceptable *in vivo* PK profiles, and were devoid of ancillary pharmacological activity; however, *in vitro* metabolic stability experiments revealed that these compounds readily underwent phase-I oxidative metabolism with <10% of parent compound remaining after 90 min incubation with rat liver microsomes (data not shown). Subsequent metabolite identification experiments indicated that hydroxylation of the pyridine ring was the major oxidative metabolite. In order to prevent this metabolic liability and further explore SAR around the lead scaffold, we employed an iterative parallel library synthesis approach for new analog generation.
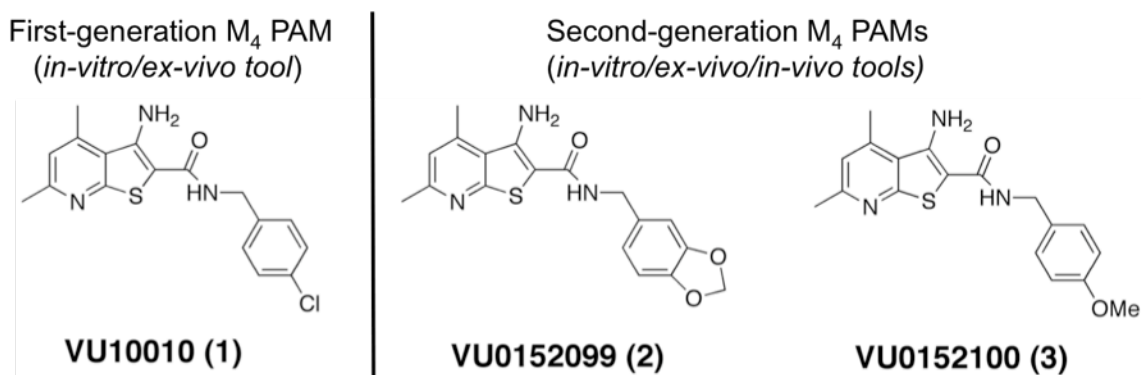


**Figure 11**. Structures of first- and second-generation $M_4$ PAMs based on optimization of the dimethylamino-thienopyridine amide scaffold. All three compounds possess approximately ~400 nM $rM_4$ potency with 30-70x ACh CRC fold-shift, yet suffer from poor *in vitro* metabolic stability.

Having tentatively designated the *p*-methoxybenzamide moiety of lead compound **3** as the most-favored substituent (of those screened in the initial amide side chain scan described previously) based on its functional activity at rat $M_4$, we first held this side chain constant while exploring alternative substituents at the 6-position to prevent formation of the hydroxy metabolite. Synthesis of initial 20-member alkylamine and 20-

member ether libraries, as shown in **Figure 12**, began with condensation between 3-aminocrotonitrile **4** and 2-cyanothioacetamide **5** to furnish the aminopyridyl core **6**. This was cyclized with a-chloro-p-methoxybenzamide **7** to give the diaminothieno[2,3-b]pyridine scaffold with the p-methoxybenzamide **8**. To produce the 20-member amine library, **8** was reacted with various alkyl chlorides to generate **9a-s**. The ether library was synthesized in a similar fashion by reacting ethylacetoacetate **10** with 2-cyanothioacetamide **5** to provide the core pyridone **11**, which was then cyclized with **7** at 0 °C then allowed to warm to r.t. overnight. Once pure, **12** was alkylated to obtain analogs **13a-s**.
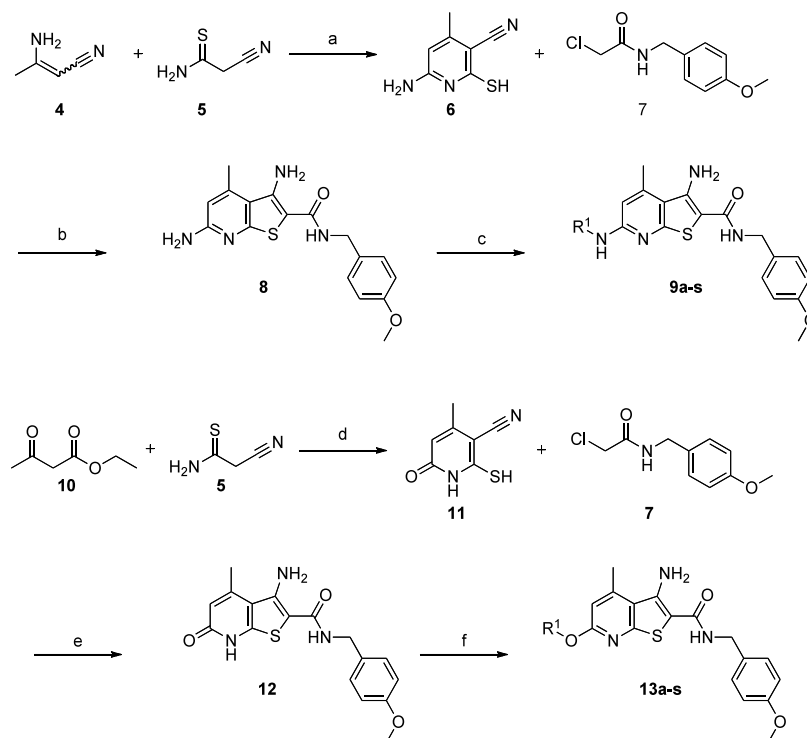


**Figure 12**. Synthesis of M$_4$ PAM analog libraries **9a-s** and **13a-s**. Reagents and conditions: a) Piperidine, EtOH, MW 160 °C, 12 min. 42%; b) K$_2$CO$_3$, MW 160 °C, 10 min., 75%; c) R$_1$-Cl, KI, Cs$_2$CO$_3$, DMF, MW 160 °C, 30 min. 15-80%; d) Morpholine, EtOH, MW 160 °C, 10 min. 62%; e) K$_2$CO$_3$, DMF, 0 °C->r.t., 24h 65%; f) R$_1$-Cl, KI, Cs$_2$CO$_3$, DMF, MW 160 °C, 30 min. 15-80%.

These libraries were first screened in a singlepoint $Ca^{2+}$ mobilization assay using a fixed 10 μM concentration of test compound added to rat $M_4/G_{qi5}$-cells prior to addition of a submaximal concentration ($\sim EC_{20}$) of ACh. This allowed efficient triage of analogs for further characterization. In general, alkylamine library **9a-s** showed weak efficacy with elevation of the ACh response ranging from none (inactive) to modest (<50% ACh max). However, ether library **13a-s** contained a number of robust potentiators ($\sim$60-90% ACh max). The $EC_{50}$ values for compounds selected from both libraries based on their potentiation efficacy and structural characteristics were then obtained from full CRCs in similar $Ca^{2+}$ assays testing for potentiation of an ACh $\sim EC_{20}$ (**Table 4**).

**Table 4**. M$_4$ PAM SAR for select analogs from alkylamine library **9a-s** and ether library **13a-s** chosen based on an initial singlepoint triage screen. Ca$^{2+}$ mobilization was used to obtain CRCs of each compound in the presence of an ACh ~EC$_{20}$ using rM$_4$ cells. Similar assays were used to obtain CRCs of ACh in the presence and absence of 30 μM of each test compound to determine fold-shift values (- denotes not determined). Data represent Means from at least three independent determinations.

| Compound | R$_1$ | EC$_{50}$ (μM) | Fold-shift |
|---|---|---|---|
| 9a | (structure) | >10 | - |
| 9b | (structure) | >10 | - |
| 9c | (structure) | 9.1 | 1x |
| 9e | (structure) | >10 | - |
| 9l | (structure) | >10 | - |
| 9n | (structure) | >10 | - |
| 9o | (structure) | >10 | - |
| 9p | (structure) | >10 | 1x |
| 13a | (structure) | >10 | 1x |
| 13b | (structure) | >10 | - |
| 13c | (structure) | 6.5 | 37x |
| 13d | (structure) | 8.2 | 25x |
| 13e | (structure) | >10 | - |
| 13f | (structure) | >10 | - |
| 13g | (structure) | >10 | - |
| 13k | (structure) | 2.0 | 50x |
| 13l | (structure) | 2.2 | 9x |
| 13m | (structure) | 1.8 | 5x |
| 13p | (structure) | >10 | - |

More than half of these compounds possessed $EC_{50}$ values over 10 μM with potentiation effects emerging only at the 10 μM and 30 μM concentrations. Among alkylamines **9a-s**, only the ethyl morpholine congener **9c** had an $EC_{50}$ just below 10 μM, reflecting relatively weak activity of this library. In contrast, compounds from the ether library **13a-s** possessed improved potencies. Particularly, picolyl analogs **13k**, **13l**, and **13m** each exhibited an $EC_{50}$ of approximately 2 μM. A concentration-response curve for **13k** in the presence of fixed ACh $EC_{20}$ is presented in **Figure 13a**. Analog **13k** elicited a robust potentiation of $M_4$ activation, elevating the submaximal ACh response to over 130% of the maximum response induced by a high concentration of ACh alone. Looking ahead to *in vivo* studies, the structure of **13k** was particularly attractive, as the presence of a basic amine would allow for an HCl salt to confer greater aqueous solubility for vehicle formulation. Based on these potency data, compounds having $EC_{50}$ values below 10 μM were examined for their ability to shift a full ACh CRC to the left when applied at a fixed 30 μM concentration in a similar functional $Ca^{2+}$ assay with $rM_4/G_{qi5}$-cells (**Table 4**). The ACh CRC fold-shift data for analog **13k** is shown in **Figure 13b**. In the case of other allosteric modulators of GPCRs, compound potency often fails to correlate tightly with fold-shift magnitude. For example, a PAM with high potency but low efficacy can exhibit next to no fold-shift effect, and conversely one with low potency but high efficacy may induce a substantial fold-shift. Hence, evaluation of fold-shift for novel potentiators having upper single-digit micromolar potencies can sometimes uncover helpful SAR that would have otherwise been missed.
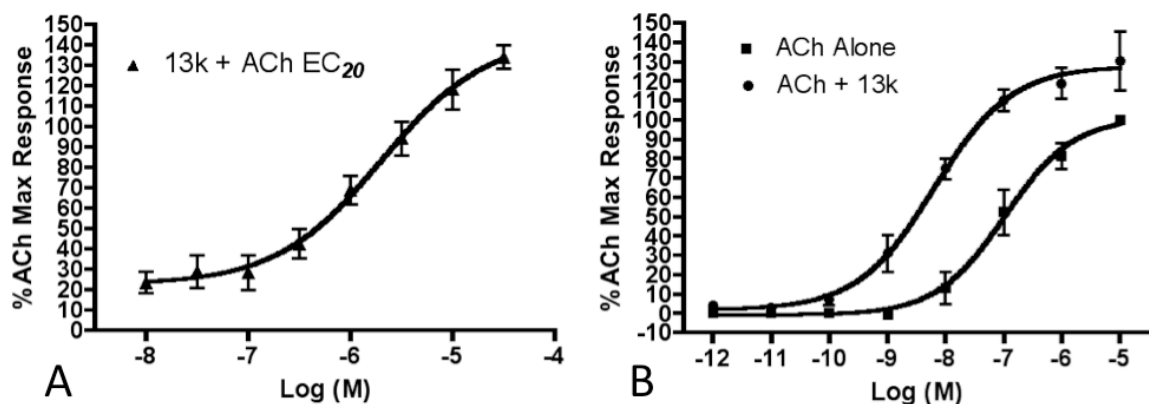
**Figure 13**. CRCs and ACh fold-shift for analog **13k** in rat $M_4$ cells by $Ca^{2+}$ mobilization assay. A.) CRC for **13k** in the presence of an ACh ~$EC_{20}$ ($EC_{50}$ = 2.0 μM). B.) CRC for ACh in the presence and absence of a 30 μM concentration of **13k** (fold-shift = 50x). Data represent Mean +/-SEM from at least three independent determinations.

As shown in **Table 4**, neither morpholino compound **9c** nor ethyl compound **9p** caused a left-shift in the ACh CRC, demonstrating the compromised activity found with alkylamine modification at the 6-position of the scaffold. The same lack of effect was seen with the tertiary amine analog **13a** from the ether library. However, the ether-linked morpholino **13c** and pyrrolidine **13d** analogs demonstrated strong ACh left-shifts of 37x and 25x, respectively. Interestingly, movement of the nitrogen from the 2-position or 3-position of the picolyl ethers **13m** and **13l** to the 4-position of **13k** progressively increased the fold-shift from 5x to 9x and ultimately 50x, respectively (**Fig. 13b**).

Taken together, these data suggested ether-linked modifications to the 6-position of the scaffold were more tolerated and optimal than alkylamine-linked changes. However, despite retention of robust PAM properties in terms of ACh CRC fold-shift for **13c**, **13d**, and particularly **13k**, the potency of these analogs was moderately diminished relative to the parent compound **3** ($EC_{50}$ = 380 nM) (**Fig 13**). Furthermore, the SAR for these two libraries underlines the importance of considering both fold-shift and potency

when optimizing allosteric modulators. Although each of the three picolyl ether analogs had ~2 µM $EC_{50}$ values, their left-shift effects on the ACh CRC revealed dramatic differences in maximal PAM efficacy.

For the next library iteration, we postulated that with the picolyl or ethyl morpholine ether moieties on the left-hand side of the molecule, the *p*-methoxybenzyl of the right-hand side might no longer be optimal for $M_4$ potency. Therefore, we opted to re-scan the amide with 18 side chain groups while holding constant each of the three picolyl ether modifications, the morpholino ether, and the dimethylpropylamine ether (**Fig. 14**). The morpholino and 4-picolyl were clear choices based on their degree of ACh CRC fold-shift, but the 2-picolyl and 3-picolyl were also included to be comprehensive. The dimethylpropylamine ether was chosen to provide for the possibility that a different amide side chain may rescue the activity of **13a** (i.e. a matrix-like approach to broaden SAR). These libraries began with the cyclization between pyridone **11** and ethyl chloroacetate **14** to produce thienopyridone ethyl ester **15**. To obtain the five alkyl ethers **16a-e**, material **15** was reacted with the five selected side chains from our previously library. These five scaffolds were saponified and immediately coupled with 18 amines to produce five alkyl ether libraries with different amide side chains **17a-p**, **18a-r**, **19a-o**, **20a-p**, **21a-o** (**Fig. 14**).
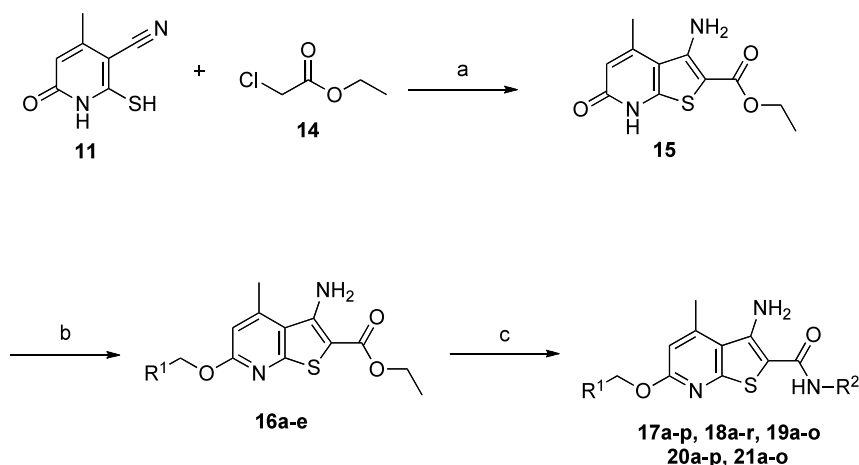
**Figure 14**. Synthesis of M$_4$ PAM analog libraries **17-21**. Reagents and conditions: a) TEA, DMF, 0 °C->r.t. 62%; b) R$_1$-Cl, KI, Cs$_2$CO$_3$, DMF, MW 160 °C, 30 min. 75-95%; c) 1) 3:1 1M NaOH:EtOH, MW 120 °C, 30 min. 2) R$_2$-NH$_2$, DIC, HOBt, 9:1 DMF:DIEA 20-80%.

As before, these libraries were screened first in a singlepoint 10 μM PAM assay that tested their ability to enhance the response of a submaximal (~EC$_{20}$) concentration of ACh in r$_{M4}$/G$_{qi5}$-cells (**Fig. 15**). PAM activity ranged from absent to robust within each of these libraries, revealing generally consistent SAR across all of the five ether-linked modifications that were held constant on the left-hand side of the structure.
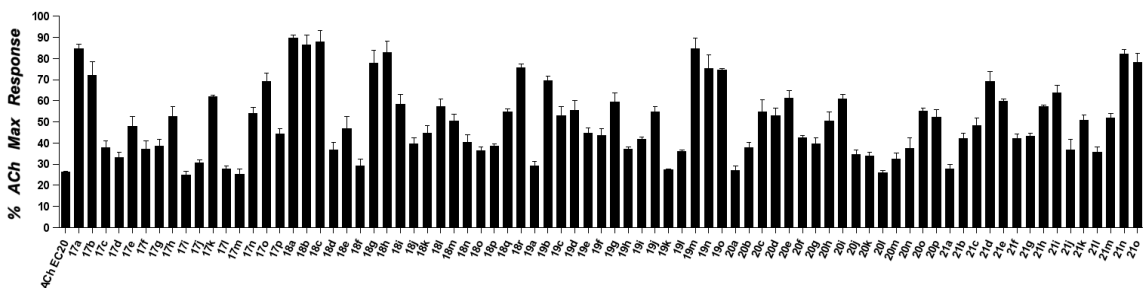


**Figure 15**. Singlepoint (10 μM) M$_4$ PAM screen of analog sets **17-21**. Ca$^{2+}$ mobilization was used to obtain % ACh max values for each compound in the presence of an ACh ~EC$_{20}$. Data represent Mean +/- SEM from at least three independent determinations.

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From this screen, eleven compounds were selected for CRCs and ACh fold-shift assays based on their degree of potentiation. The associated SAR data for the chosen compounds from libraries **17-21** obtained from these assays are shown in **Table 5**. All selected compounds possessed an $EC_{50}$ below 10 μM, except for **19n**, the di-flurobenzyl substituted 2-picolyl analog. Similar to earlier libraries, ACh CRC fold-shift magnitude did not track closely with potency, as shown for example in the case of compound **18h**. This t-butyl substituted moropholine analog had near 9 μM potency but caused a robust 62x foldshift of the ACh CRC. Further, the two dimethylpropyl analogs **17a** and **17o** displayed approximately 3 μM potency yet induced only a moderate ACh fold-shift. Interestingly, this dimethylpropylamine motiety at $R_1$ conferred poor potency ($EC_{50} > 10$ μM) in its parent compound **9b** that bore a *p*-methoxybenzyl group at $R_2$, but the amide scan library **17a-p** discovered side chains that rescued activity for this western side modification.

**Table 5**.  M$_4$ PAM SAR for select compounds from second library iteration analogs **17-21**.  Ca$^{2+}$ mobilization was used to obtain CRCs of each compound in the presence of an ACh ~EC$_{20}$ using rM$_4$ cells. Similar assays were used to obtain CRCs of ACh in the presence and absence of 30 μM of each test compound to determine fold-shift values (- denotes not determined).  Data represent Means from at least three independent determinations.

| Compound | R$_1$ | R$_2$ | EC50 (μM) | Foldshift |
|---|---|---|---|---|
| 17a | | | 2.58 | 7x |
| 17o | | | 2.75 | 14x |
| 18a | | | 7.42 | 40x |
| 18b | | | 6.25 | 67x |
| 18c | | | 5.81 | 51x |
| 18g | | | 7.15 | 36x |
| 18h | | | 8.73 | 62x |
| 19m | | | 5.40 | 28x |
| 19n | | | >10 | 41x |
| 21n | | | 2.44 | 44x |
| 21o | | | 3.78 | 64x |

In general, difluorinated benzylic substitutions at R$_2$ were favored, providing analogs with EC$_{50}$s in the 2-5 μM range at rat M$_4$ and with large ACh CRC fold-shift values (**Table 5**).  The 4-picolyl moieties of R$_1$ with the 2,3-difluoro and 2,5-difluoro substitutions at R$_2$ of **21n** and **21o** proved most desired when seeking a balance of both potency and potentiation efficacy, consistent with previous SAR.  However, the moropholines at R$_1$ of library **18a-r** with bare alkyl and mono-oxygenated side chains at

$R_2$ possessed strong fold-shift effects despite moderately weaker potency compared to

**21n** and **21o**. **Figure 16** presents the CRC for elevation of an ACh ~ $EC_{20}$ and fold-shift

effect on an ACh CRC for analog **21n**. Interestingly, this 2,3-difluorobenzyl substituted

analog did not elevate the maximal response of ACh at the top of the CRC (**Fig. 16b**),

which constrasts with 4-methoxybenzyl analog 13k (**Fig. 13b**).
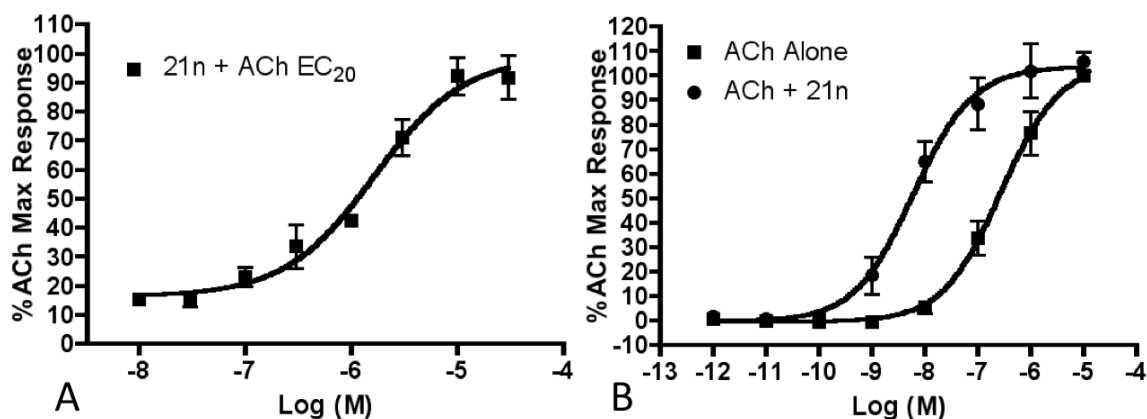


**Figure 16**. $M_4$ PAM characterization of analog **21n** in rat $M_4/G_{qi5}$-CHO cells by functional $Ca^{2+}$ mobilization assay. A.) CRC for compound **21n** in the presence of an ACh ~$EC_{20}$ ($EC_{50}$ = 2.4 μM). B.) ACh CRC in the presence and absence of a fixed 30 μM concentration of **21n** (foldshift = 44x). Data represent Mean +/-SEM from at least three independent determinations.

Despite generation of a multi-dimensional library of analogs varying both sides of

the lead molecule's scaffold, the approximately 400 nM rM4 potency of the first- and

second- generation compounds **1-3** could not be maintained despite retention of robust

PAM efficacy in terms of ACh CRC fold-shift (e.g. >50x). Indeed, compounds **13k**, **18b**,

and **21o** each caused large left-shifts of ACh CRCs when applied at 30 μM, but were

approximately an order of magnitude less potent than the earlier lead compounds. These

SAR suggest the presence of a possible ~ 2 μM potency floor for this chemotype with 6-

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position ether or amine modifications, as variation of the amide side chain failed to provide congers with $EC_{50}$ values below this level.

We next evaluated the microsomal stability of novel analogs **13k**, **21n**, **21o**, and **18h** in both rat and human fortified liver microsomes. Replacement of the metabolically labile 6-Me group with the ether linkage did indeed improve metabolic stability for all four analogs **13k**, **21n**, **21o**, and **18h** (>90% parent remaining after 90 minutes) as compared to **1-3** (<10% parent remaining after 90 minutes). Moreover, incorporation of the basic amine moieties in these four compounds also improved solubility providing either homogeneous solutions or uniform micro-suspensions, as the HCl salts, across a panel of pharmaceutically acceptable vehicles (β-cyclodextrin, PEG400/$H_2O$, etc...) relative to initial lead compounds **2** (VU0152099) and **3** (VU0152100), which were only soluble in 10% Tween-80. In particular, **21n** afforded a homogeneous solution at 15 mg/mL in pH 3 saline. Thus, our optimization efforts revealed interesting and useful SAR that led to improved metabolic stability and physiochemical properties for this series of $M_4$ PAMs.

### *In vivo* behavioral studies with 3rd-generation optimized $M_4$ PAMs

Despite low micromolar potency at rat $M_4$, we evaluated analogs **13k**, **21n**, **21o**, and **18h** in our standard amphetamine-induced hyperlocomotion *in vivo* model. Recall, both **2** (VU0152099) and **3** (VU0152100) ($EC_{50}$s ~400 nM, ACh CRC fold-shifts of 30x and 70x, respectively) were efficacious in this behavioral model predictive of antipsychotic efficacy. In the event, **13k**, **21n**, **21o**, and **18h** all failed to reverse

amphetamine-induced hyperlocomotion following dosing at 56.6 mg/kg (I.P.), suggesting perhaps that the new compounds were too weak at rat $M_4$ (data not shown). This possibility is underlined by the fact that LY2033298 possesses similarly modest $rM_4$ potency and failed to achieve efficacy when dosed alone in recently disclosed *in vivo* rat behavioral studies, thereby requiring co-dosing of a muscarinic agonist (Oxotremorine) to achieve significant effects[127].

## Species-specific differences in 3rd-generation $M_4$ PAM potencies

Primarily, our efforts were aimed at exploring SAR at rat $M_4$ and optimizing this series for beneficial DMPK and vehicle formulation properties suitable for *in vivo* rodent behavioral studies. While stability and physiochemical properties were improved, potency at rM4 was diminished to a point where, most likely, *in vivo* efficacy was lost. However, rat and human mAChRs do diverge in structure (~95% amino acid sequence identity) and species-specific differences in on-target pharmacology have been noted for other mAChR PAMs. Therefore, we opted to evaluate representative compounds **13k**, **21n**, **21o**, and **18h** in analogous functional cell-based $Ca^{2+}$ assays using cells expressing the human $M_4$ receptor (and promiscuous $Gq_{15/16}$ for $Ca^{2+}$ mobilization readout). To this end, all four compounds were submitted to Millipore and assayed in their GPCR Profiler Service™, which provided potency and ACh CRC fold-shift values with the human $M_4$ receptor. Remarkably, each compound possessed $EC_{50}$ values approximately in the 100-200 nM range at human $M_4$ (**Fig. 17a**), more than an order of magnitude greater potency than at the rat $M_4$ receptor. Each compound also elicited large left-shifts of the control

ACh CRC in human $M_4$ cells (**Fig. 17b**) at 30 μM, similar to their fold-shifts at rat $M_4$. In contrast, the prototypical $M_4$ PAMs **1-3**, displayed near equivalent $EC_{50}$s on rat and human $M_4$, suggesting the possibility that the basic residues in these newer analogs contact divergent residues in human $M_4$.
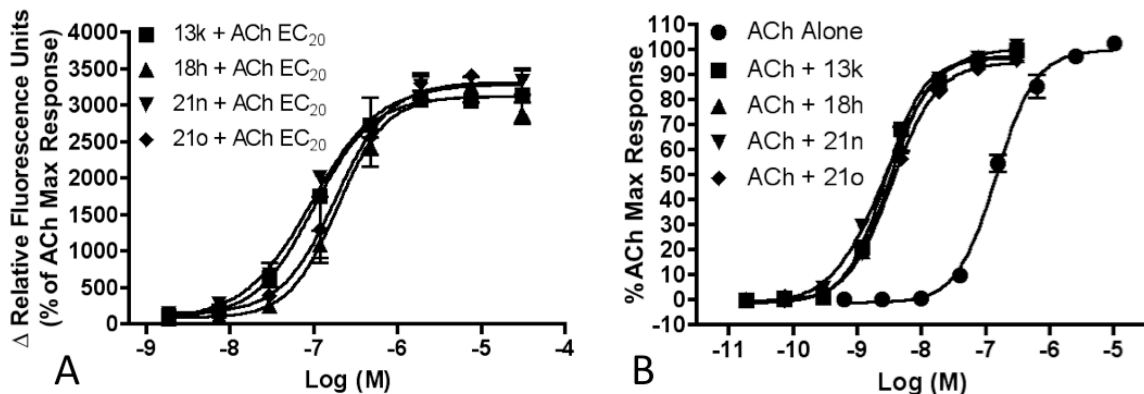


**Figure 17**. Effects of compounds **13k**, **18h**, **21n**, and **21o** in human $M_4/G_{q15/16}$-expressing cells by functional $Ca^{2+}$ mobilization assay. A.) CRCs for potentiators in the presence of an ACh ~$EC_{20}$ (**13k** $EC_{50}$ = 100 nM, **18h** $EC_{50}$ = 192 nM, **21n** $EC_{50}$ = 95 nM, **21o** $EC_{50}$ = 183 nM). B.) for ACh in the presence and absence of a fixed 30 μM concentration of potentiator (**13k** foldshift = 63x, **18h** foldshift = 44x, **21n** foldshift = 60x, **21o** foldshift = 49x).

## Potential for dual $M_4$ PAM / $H_3$ antagonist anti-psychotic activity

While $M_1$ PAMs and allosteric agonists have the potential to affect multiple symptom clusters of schizophrenia, $M_4$ PAMs may only prove efficacious in the treatment of positive symptoms. If so, combination therapy or possibly a monotherapy possessing poly-pharmacology may be more clinically effective for comprehensive symptom treatment in psychotic patients. For the cognitive symptom cluster specifically,

antagonism of the central histamine $H_3$ receptor ($H_3$) has been proposed as a potentially viable strategy[128-130].

We observed that $M_4$ PAMs **13k**, **21n**, **21o**, and **18h** possessed the general features of the refined $H_3$ receptor antagonist pharmacophore (**Fig. 18**), and we therefore evaluated these compounds for their ability to function as $H_3$ antagonists (using an *in vitro* binding assay), which may provide pro-cognitive attributes *in vivo*[128]. Compounds **13k**, **21o** and **18h** were found to inhibit human $H_3$ with $IC_{50}$ values of ~10 μM, while **21n** afforded an $IC_{50}$ value of 6.3 μM. Although weak, this result suggest that it may be possible to 'dial in' $H_3$ antagonist activity to this new series of $M_4$ PAMs via further optimization. Testing of this hypothesis via additional syntheses and dual $M_4/H_3$ screening will be required and has not yet been performed.
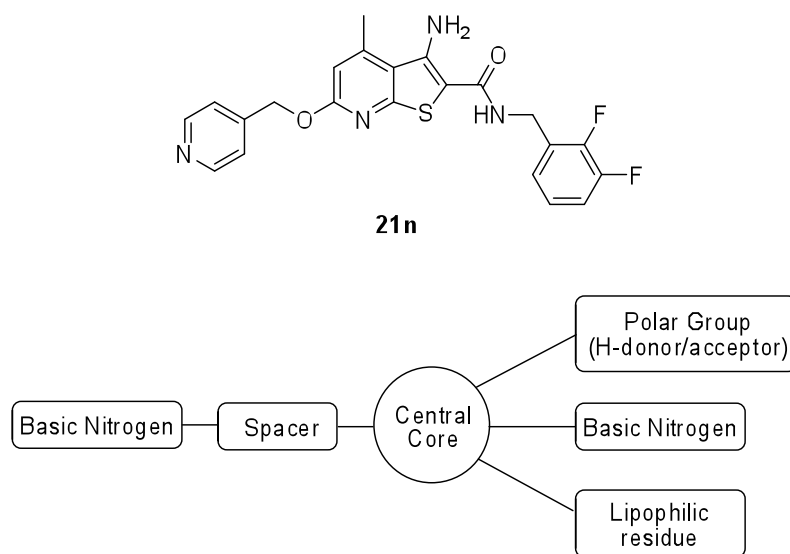


**21n**



**Figure 18**. Alignment of $M_4$ PAM lead compound **21n** with the refined histamine $H_3$ receptor antagonist pharmacophore.

**Summary**


Ultimately, a lead optimization campaign around $M_4$ PAMs **1-3** provided novel analogs with substantially improved metabolic stability and physiochemical properties, but diminished potency at rat $M_4$ ($EC_{50}s \sim 2$ μM) while retaining comparable ACh CRC fold-shift (14x-67x) relative to early leads **1-3**. Surprisingly, we noted significant species differences within this series of $M_4$ PAMs, where analogs such as **21n** displayed an order of magnitude greater potency at human $M_4$ ($EC_{50}$ = 95 nM) than at the rat $M_4$ receptor ($EC_{50}$ = 2.4 μM) but with comparable ACh fold-shifts (human, 60x; rat, 44x).

To further expand the therapeutic relevance of $M_4$ positive allosteric modulation for the treatment of schizophrenia beyond the positive symptom cluster, we evaluated analogs against the $H_3$ receptor, as they align well with the refined $H_3$ antagonist pharmacophore model. $M_4$ PAM **21n** was found to provide modest inhibition of $H_3$ radioligand binding with an $IC_{50}$ of 6.3 μM, suggesting that it might be possible to discover optimized analogs with dual $M_4$ PAM and $H_3$ antagonist activity. Such compounds could be used to test the hypothesis that dual $M_4/H_3$ pharmacology would possess broad anti-psychotic potential across a range of preclinical behavioral studies aimed at predicting efficacy in treating different/multiple symptom clusters of schizophrenia.

**Chapter IV**

**DISCOVERY, OPTIMIZATION, AND CHARACTERIZATION OF NOVEL SUBTYPE-SELECTIVE MACHR5 POSITIVE ALLOSTERIC MODULATORS**

**Discovery of $M_1$/$M_3$/$M_5$ PAM VU0119498**

In order to identify novel $M_1$ PAMs, an HTS of the VHTSC compound collection (~160,000 compounds total) was performed using a fluorescence-based $Ca^{2+}$ mobilization assay[27]. Test compounds were added to CHO-cells stably expressing the $M_1$ receptor and allowed to equilibrate for approximately three minutes before addition of a submaximal (~$EC_{20}$) concentration of the orthosteric agonist carbachol (CCh). Compounds that potentiated the CCh response at least three standard deviations over the vehicle-control response were marked as primary hits (1,634 compounds). Secondary screening (singlepoint or CRC assays) at $M_1$ and counter-screening (CRC assays) at $mGluR_4$ identified a subset of hits (77 compounds), which exhibited concentration-dependent PAM activity at $M_1$ with no activity at $mGluR_4$.
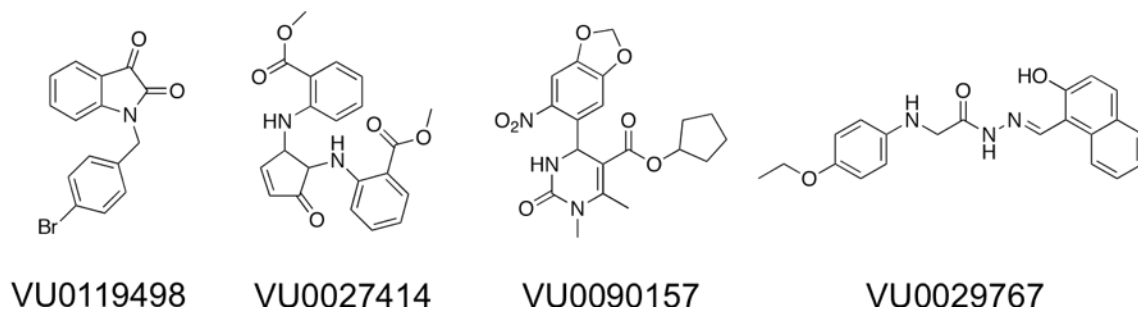


VU0119498          VU0027414          VU0090157          VU0029767

**Figure 1.** Compounds identified by $M_1$ PAM HTS chosen for *in vitro* characterization.

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Four of these hits (**Fig. 1**) were chosen for further characterization, including *in vitro* mAChR subtype-selectivity assays, which identified VU0119498 as a mAChR PAM with similar potencies (~3-6 μM) and efficacies (~45-65% CCh max) in $M_1$, $M_3$, and $M_5$ CHO cell lines and with no PAM activity in $M_2/G_{qi5}$ or $M_4/G_{qi5}$ CHO cell lines, as shown in **Fig. 2**. Subsequent functional and radioligand-binding assays demonstrated that VU0119498 (30 μM) induces a 14x left-shift of an ACh CRC at $M_1$ by $Ca^{2+}$ mobilization assay and does not compete with $[^3H]$-NMS for binding to $M_1$ cell membranes (**Fig. 2**), similar to the other three novel mAChR PAMs evaluated from the HTS and consistent with an allosteric mode of modulation.
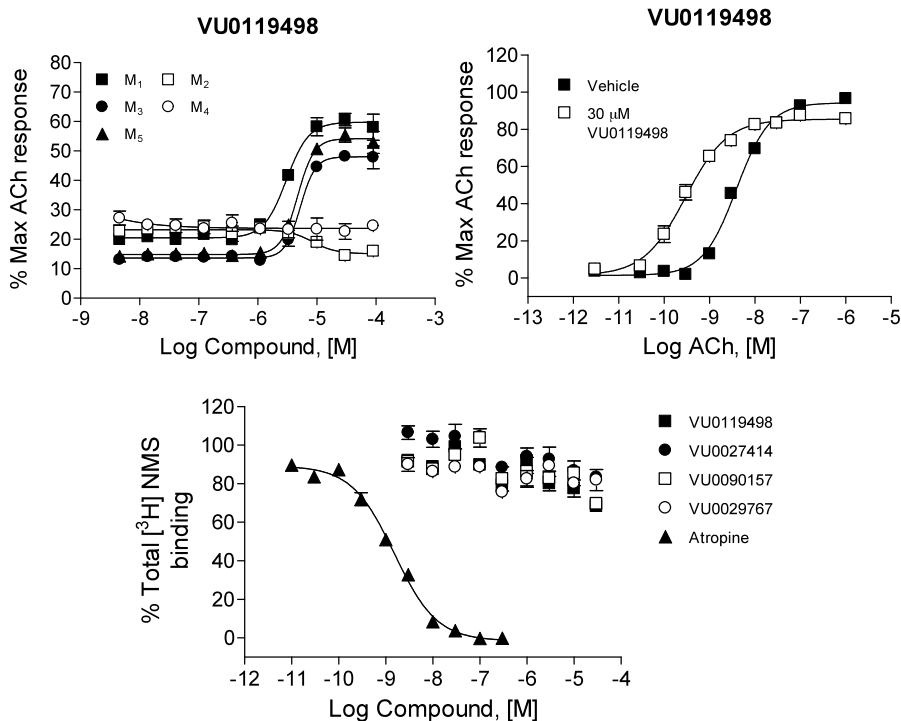


**Figure 2**. *In vitro* characterization of VU0119498. mAChR subtype-selectivity curves (top left panel) were obtained by $Ca^{2+}$ mobilization assays in the presence of an ~$EC_{20}$ of ACh at all five mAChR subtype cell lines. ACh curves in the absence and presence of VU011948 (top right panel) were obtained by $Ca^{2+}$ mobilization assays using $M_1$ cells. Competition curves (bottom panel) were obtained in the presence of 0.1 nM $[^3H]$-NMS using membranes from $M_1$-expressing CHO cells. Atropine $K_i = 1.08$ +/- 0.09 nM. Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results. Experiments performed by J. Marlo.

In light of the unprecedented mAChR PAM subtype-selectivity profile of VU0119498, we hypothesized that structural modification via chemical optimization of this novel compound would lead to the discovery of PAMs selective (either functionally or via binding specificity) for $M_5$[131,132]. As discussed in the introduction (Chapter I), the current and longstanding lack of available small molecules with selectivity for $M_5$ made this an attractive endeavor with the possibility of providing a substantial contribution to the mAChR research community and thereby facilitating subsequent *ex-vivo* and/or *in vivo* $M_5$ neurobiology studies. To this end we executed an exploratory optimization project utilizing (Chapter I) medicinal chemistry methods. However, prior to synthesizing VU019498 analogs, the parent compound itself was resynthesized and retested in order to confirm the validity of the structure and associated pharmacological characteristics (**Fig. 3**). *N*-alkylation of commercially available isatin with 4-bromobenzyl bromide under standard conditions provided the desired compound with high yield (**Fig. 3**), which was then tested in mAChR subtype-selectivity CRC assays by $Ca^{2+}$ mobilization.
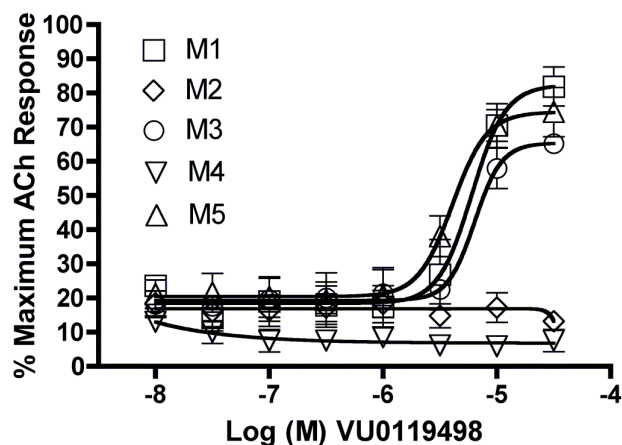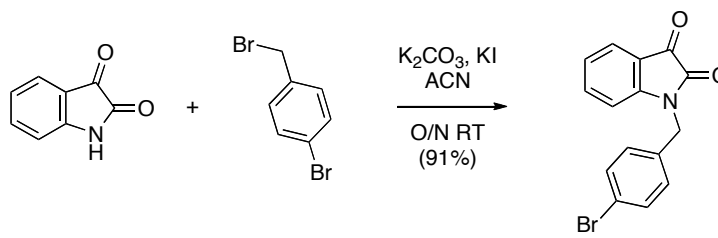
**Figure 3.** Resynthesis and retesting of HTS hit VU0119498. Isatin was reacted with 4-bromobenzyl bromide in the presence of $K_2CO_3$, and KI, in ACN and stirred overnight at room temperature to provide desired compound (top panel). $Ca^{2+}$ mobilization assays with $M_1$-$M_5$ cells ($M_2$ and $M_4$ co-expressed $G_{qi5}$) were used to obtain CRCs (bottom panel) of resynthesized VU0119498 ($EC_{50}$ values: $M_1$ = 6.0 μM 82% ACh max, $M_3$ = 6.4 μM 66% ACh max, $M_5$ = 4.1 μM 74% ACh max). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

This was an important step because hits from HTS campaigns can sometimes fail to retest following resynthesis due to reasons such as incorrect structure assignment from the compound vendor or in-house management system, sample or assay plate/well contamination, etc... Gratifyingly, the resynthesized batch of VU0119498 exhibited a virtually identical mAChR subtype-selectivity, potency, and efficacy profile as that of the parent batch obtained from the HTS (**Fig. 3**), which increased confidence that a subsequent optimization effort on this chemical scaffold would be worthwhile.

**Optimization of VU0119498 to obtain M$_5$ PAM VU0238429**

Given SAR findings from other medicinal chemistry projects centered on GPCR allosteric modulators that demonstrated often pronounced effects of subtle structural modifications on subtype-selectivity and other pharmacological characteristics, we chose to initially and rapidly explore SAR around VU0119498 via synthesis of a 92-member analog library in matrix format wherein each of 8 commercially available isatins was reacted with approximately 12 benzyl halides under standard microwave alkylation conditions (**Fig. 4**). Resulting analogs (compounds 21-112) were then screened in a singlepoint format at 30 µM in Ca$^{2+}$ mobilization assays using M$_5$ and M$_1$ cells receiving a fixed submaximal (~EC$_{20}$) concentration of ACh (**Fig. 4**). This method was utilized to allow for efficient triage of analogs displaying high M$_1$ vs. M$_5$ or M$_5$ vs. M$_1$ preference. Interestingly, some analogs displayed robust potentiation effects at M$_5$ (i.e. elevation of ACh ~EC$_{20}$ to >50-60% of maximum ACh response) with absent or weak potentiation at M$_1$, thus exhibiting strong preference for M$_5$ versus M$_1$ activity (**Fig. 4**).
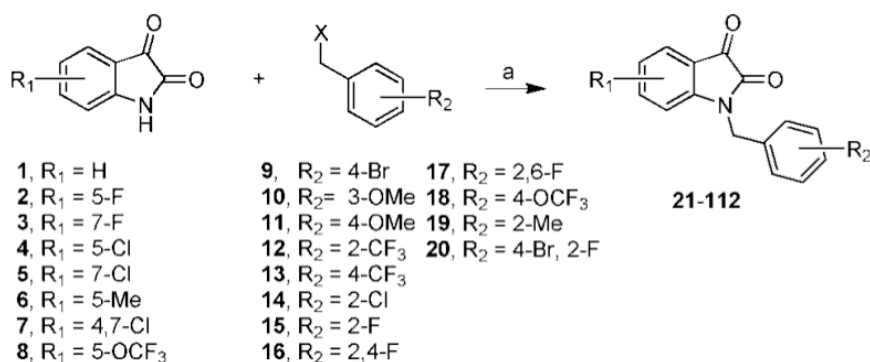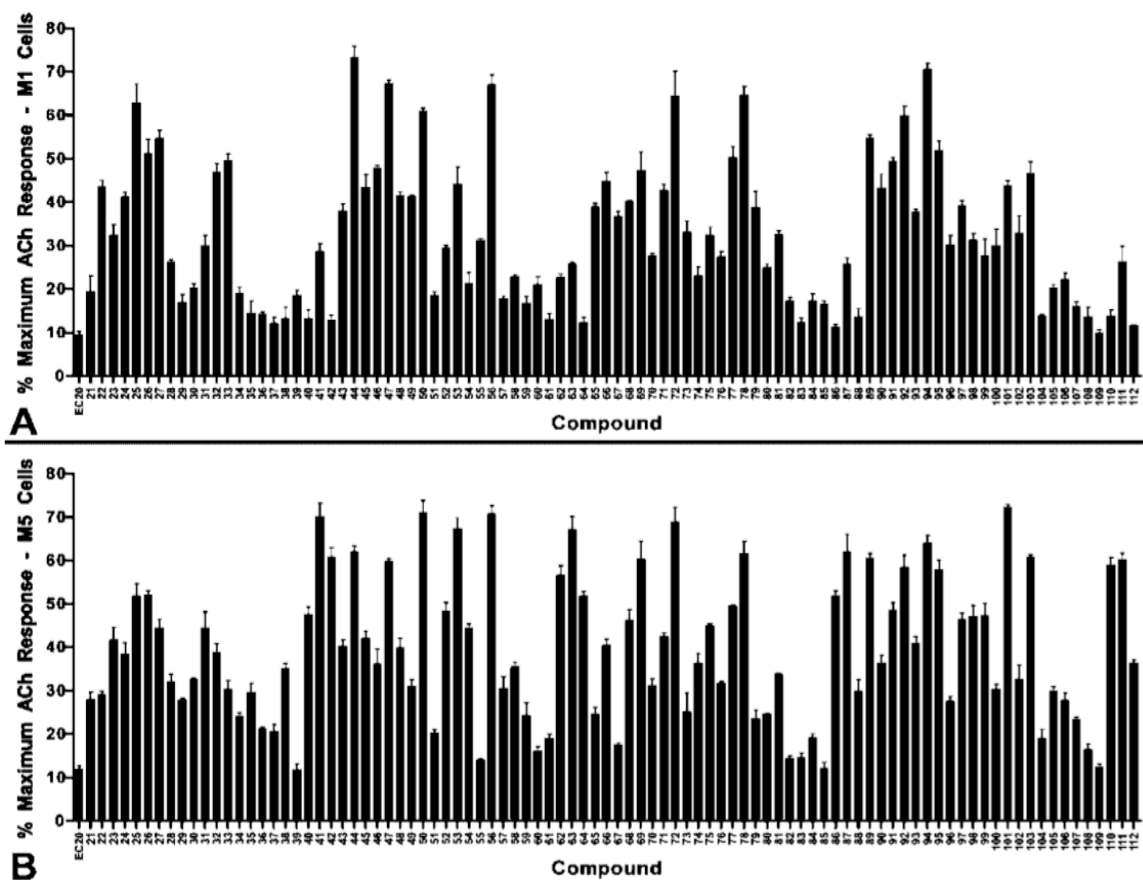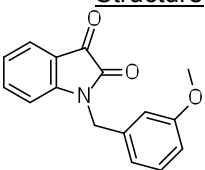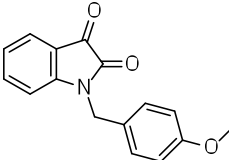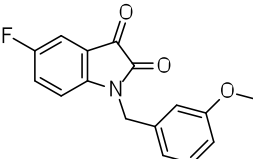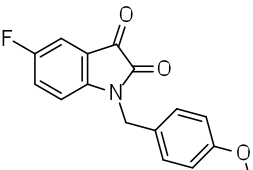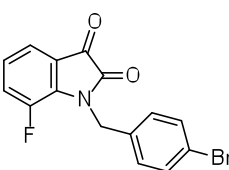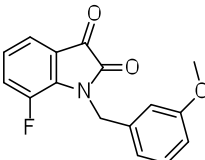
**Figure 4.** Synthesis and singlepoint screening of initial matrix library of VU0119498 analogs. Different substituted isatins were each *N*-alkylated with various benzyl halides under microwave conditions in the presence of $K_2CO_3$ and KI in ACN to generate a 92-member library (top panel). $Ca^{2+}$ mobilization assays with $M_1$ and $M_5$ cells were used to obtain singlepoint (30 μM) potentiation responses for each analog in the presence of a submaximal ($\sim EC_{20}$) concentration of ACh (middle and bottom panels). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

**Table 1.** Structures for compounds **21-112** and associated PAM activity data from the initial single concentration (30 μM) potentiator screen against $M_1$ and $M_5$. Values shown for each compound are $Ca^{2+}$ mobilization responses as percentages of maximum ACh response. The synthetic method used to generate each compound is indicated by the following abbreviations: M, microwave; C, classical. The molecular formula for each compound is also presented. Data represent the Mean of at least 3 independent experiments with similar results.

| Structure | Cmpd # | Synthesis | Formula | $M_1$ %Max | $M_5$ %Max |
|---|---|---|---|---|---|
| | 21 | M | C16H13NO3 | 19.40 | 28.04 |
| | 22 | M | C16H13NO3 | 43.58 | 29.19 |
| | 23 | M | C16H12FNO3 | 32.32 | 41.61 |
| | 24 | M | C16H12FNO3 | 41.14 | 38.28 |
| | 25 | M | C15H9BrFNO2 | 62.67 | 51.69 |
| | 26 | M | C16H12FNO3 | 51.07 | 51.97 |

| | | | | | |
|---|---|---|---|---|---|
| 27 | M | C16H12FNO3 | 54.62 | 44.40 |
| 28 | M | C15H9BrClNO2 | 26.13 | 32.03 |
| 29 | M | C16H12ClNO3 | 16.94 | 27.87 |
| 30 | M | C16H12ClNO3 | 20.31 | 32.61 |
| 31 | M | C15H9BrClNO2 | 29.87 | 44.34 |
| 32 | M | C16H12ClNO3 | 46.88 | 38.74 |
| 33 | M | C16H12ClNO3 | 49.44 | 30.32 |
| 34 | M | C16H12BrNO2 | 18.98 | 24.06 |

| | | | | | |
|---|---|---|---|---|---|
| | 35 | M | C17H15NO3 | 14.49 | 29.57 |
| | 36 | M | C17H15NO3 | 14.09 | 21.18 |
| | 37 | M | C15H8BrCl2NO2 | 12.08 | 20.67 |
| | 38 | M | C16H11Cl2NO3 | 13.26 | 35.11 |
| | 39 | M | C16H11Cl2NO3 | 18.60 | 11.73 |
| | 40 | M | C16H9BrF3NO3 | 13.21 | 47.56 |
| | 41 | M | C17H12F3NO4 | 28.55 | 70.08 |
| | 42 | M, C | C17H12F3NO4 | 12.69 | 60.73 |

| | | | | | |
|---|---|---|---|---|---|
| | 43 | M | C16H10F3NO2 | 37.89 | 40.23 |
| | 44 | M | C16H10F3NO2 | 73.26 | 61.99 |
| | 45 | M | C15H10ClNO2 | 43.42 | 41.96 |
| | 46 | M | C16H9F4NO2 | 47.78 | 36.15 |
| | 47 | M | C16H9F4NO2 | 67.30 | 59.76 |
| | 48 | M | C15H9ClFNO2 | 41.35 | 39.90 |
| | 49 | M | C16H9F4NO2 | 41.26 | 30.92 |

| | 50 | M | C16H9F4NO2 | 60.86 | 71.10 |



| | 51 | M | C15H9ClFNO2 | 18.53 | 20.27 |



| | 52 | M | C16H9ClF3NO2 | 29.56 | 48.35 |



| | 53 | M | C16H9ClF3NO2 | 44.14 | 67.39 |



| | 54 | M | C15H9Cl2NO2 | 21.27 | 44.35 |



| | 55 | M | C16H9ClF3NO2 | 31.14 | 13.99 |



| | 56 | M,C | C16H9ClF3NO2 | 67.04 | 70.76 |

| | 57 | M | C17H12F3NO2 | 17.81 | 30.57 |
|---|---|---|---|---|---|
| | 58 | M | C17H12F3NO2 | 22.89 | 35.45 |
| | 59 | M | C16H12ClNO2 | 16.80 | 24.30 |
| | 60 | M | C16H8Cl2F3NO2 | 21.09 | 16.02 |
| | 61 | M | C15H8Cl3NO2 | 13.06 | 18.94 |
| | 62 | M | C17H9F6NO3 | 22.63 | 56.54 |
| | 63 | M | C17H9F6NO3 | 25.69 | 67.22 |

| | | | | |
|---|---|---|---|---|
| 64 | M | C16H9ClF3NO3 | 12.29 | 51.88 |
| 65 | M | C15H10FNO2 | 38.87 | 24.49 |
| 66 | M | C15H9F2NO2 | 44.84 | 40.38 |
| 67 | M | C15H9F2NO2 | 36.65 | 17.44 |
| 68 | M | C15H9F2NO2 | 40.11 | 46.18 |
| 69 | M | C15H8F3NO2 | 47.31 | 60.32 |
| 70 | M | C15H8F3NO2 | 27.59 | 31.11 |
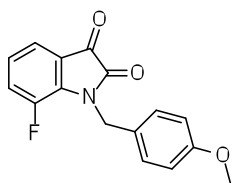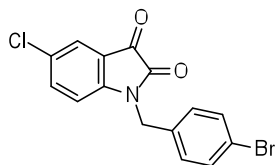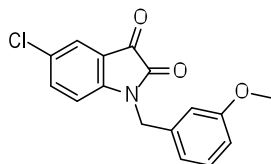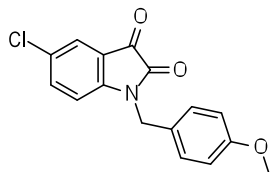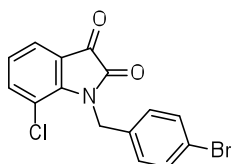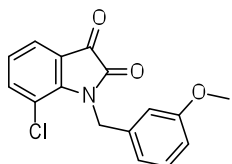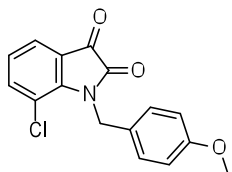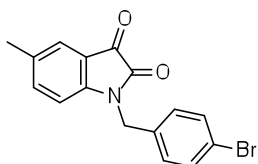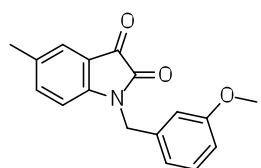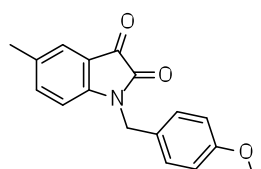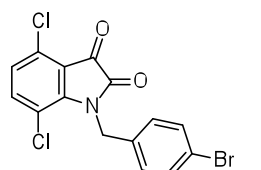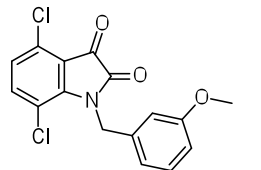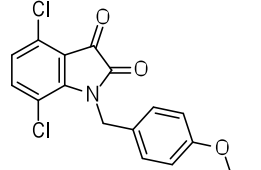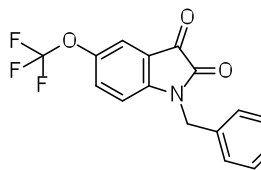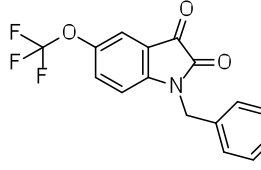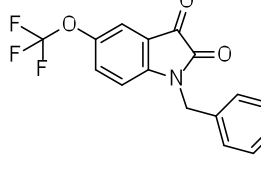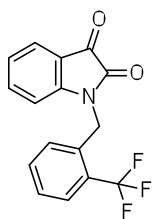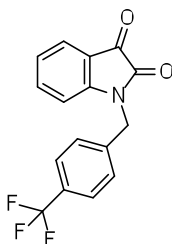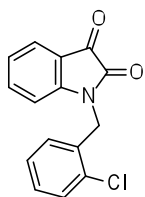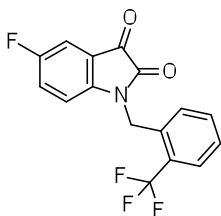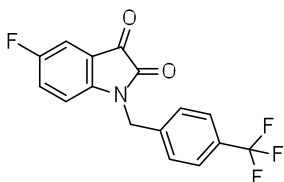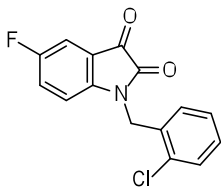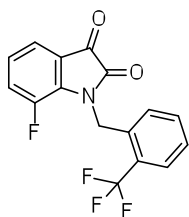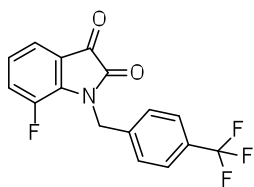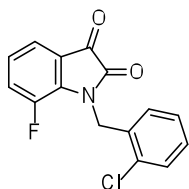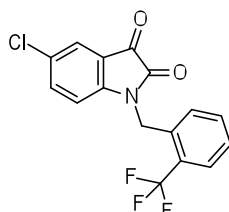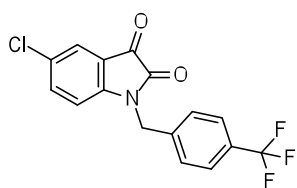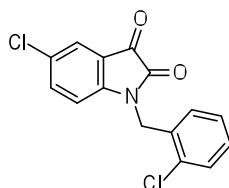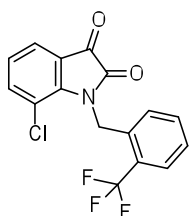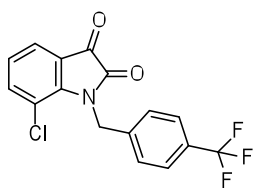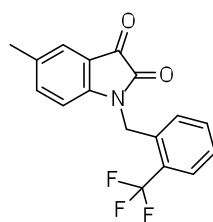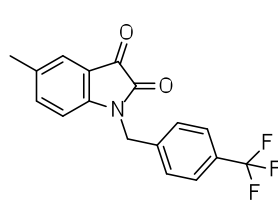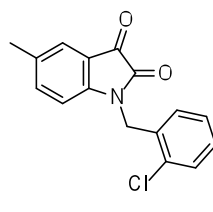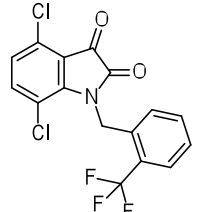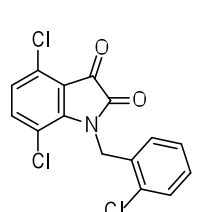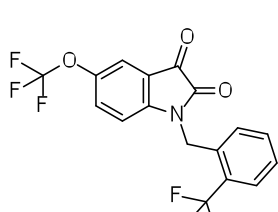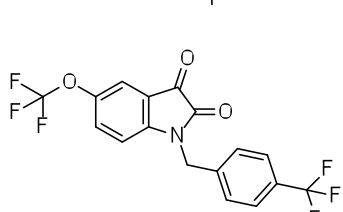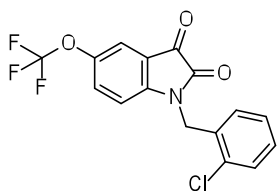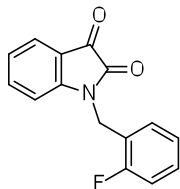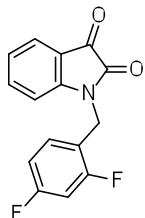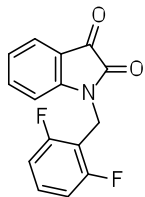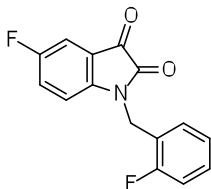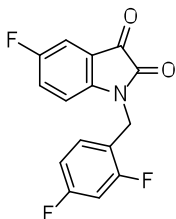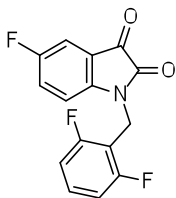
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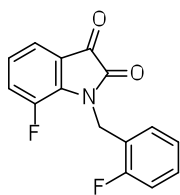
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|---|---|---|---|---|---|
| | 72 | M | C15H8F3NO2 | 64.42 | 68.93 |
| | 73 | M | C15H8F3NO2 | 33.10 | 25.10 |
| | 74 | M | C15H9ClFNO2 | 22.98 | 36.33 |
| | 75 | M | C15H8ClF2NO2 | 32.39 | 44.90 |
| | 76 | M | C15H8ClF2NO2 | 27.34 | 31.82 |
| | 77 | M | C15H9ClFNO2 | 50.42 | 49.54 |

| | 78 | M | C15H8ClF2NO2 | 64.64 | 61.59 |
| --- | --- | --- | --- | --- | --- |
| | 79 | M | C15H8ClF2NO2 | 38.83 | 23.59 |
| | 80 | M | C16H12FNO2 | 24.85 | 24.60 |
| | 81 | M | C16H11F2NO2 | 32.56 | 33.81 |
| | 82 | M | C16H11F2NO2 | 17.28 | 14.40 |
| | 83 | M | C15H8Cl2FNO2 | 12.36 | 14.70 |
| | 84 | M | C15H7Cl2F2NO2 | 17.23 | 19.10 |

|   | 85 | M | C15H7Cl2F2NO2 | 16.40 | 12.19 |



|   | 86 | M | C16H9F4NO3 | 11.30 | 51.77 |



|   | 87 | M | C16H8F5NO3 | 25.85 | 61.90 |



|   | 88 | M | C16H8F5NO3 | 13.57 | 29.82 |



|   | 89 | M | C16H10F3NO3 | 54.72 | 60.47 |



|   | 90 | M | C16H13NO2 | 43.16 | 36.34 |



|   | 91 | M | C15H9BrFNO2 | 49.20 | 48.52 |

| | 92 | M | C16H9F4NO3 | 59.81 | 58.39 |
| | 93 | M | C16H12FNO2 | 37.72 | 40.76 |
| | 94 | M | C15H8BrF2NO2 | 70.54 | 63.96 |
| | 95 | M | C16H9F4NO3 | 51.78 | 57.70 |
| | 96 | M | C16H12FNO2 | 30.17 | 27.52 |
| | 97 | M | C15H8BrF2NO2 | 39.19 | 46.34 |

| | | | | | |
|---|---|---|---|---|---|
| | 98 | M | C16H9ClF3NO3 | 31.28 | 47.07 |
| | 99 | M | C16H12ClNO2 | 27.65 | 47.26 |
| | 100 | M | C15H8BrClFNO2 | 29.89 | 30.39 |
| | 101 | M | C16H9ClF3NO3 | 43.65 | 72.32 |
| | 102 | M | C16H12ClNO2 | 32.83 | 32.56 |
| | 103 | M | C15H8BrClFNO2 | 46.52 | 60.75 |

| | | | | | |
|---|---|---|---|---|---|
| | 104 | M | C17H12F3NO3 | 13.83 | 18.92 |
| | 105 | M | C17H15NO2 | 20.19 | 29.85 |
| | 106 | M | C16H11BrFNO2 | 22.32 | 27.77 |
| | 107 | M | C16H8Cl2F3NO3 | 16.03 | 23.24 |
| | 108 | M | C16H11Cl2NO2 | 13.68 | 16.42 |
| | 109 | M | C15H7BrCl2FNO2 | 9.93 | 12.38 |

| | 110 | M | C17H9F6NO4 | 13.82 | 58.87 |



| | 111 | M | C17H12F3NO3 | 26.16 | 59.97 |



| | 112 | M | C16H8BrF4NO3 | 11.56 | 36.36 |
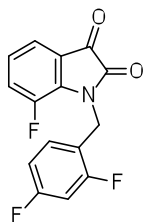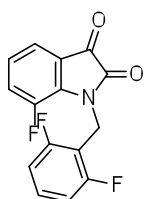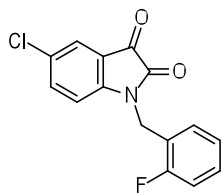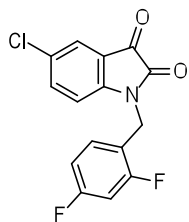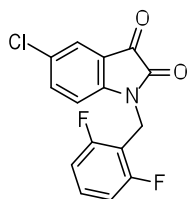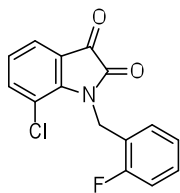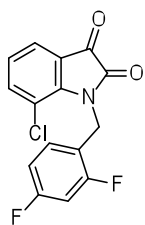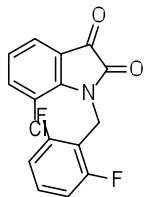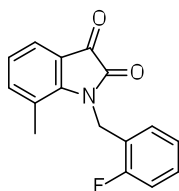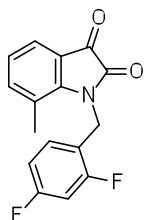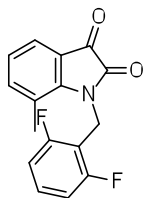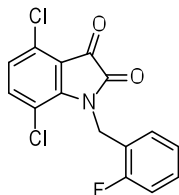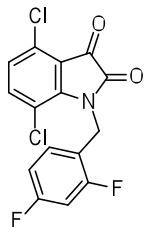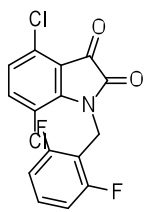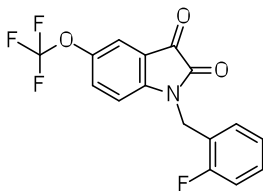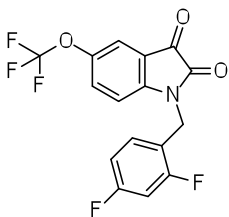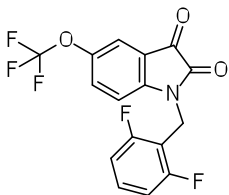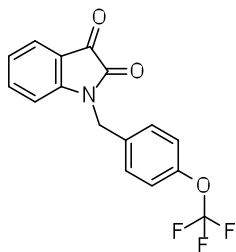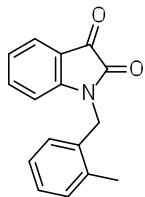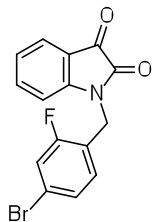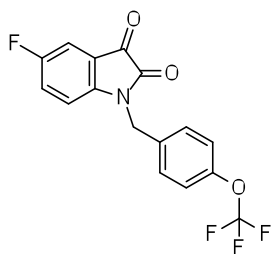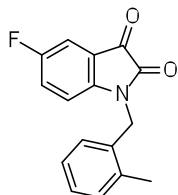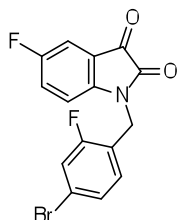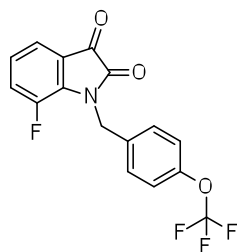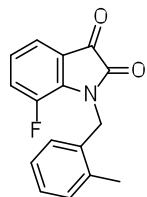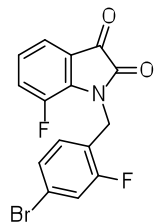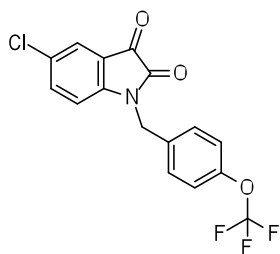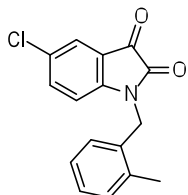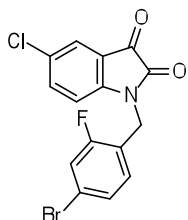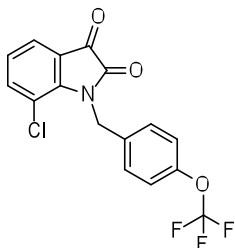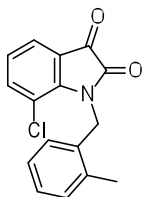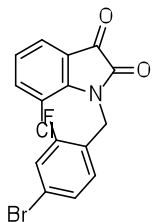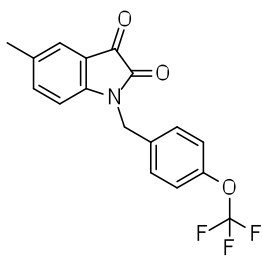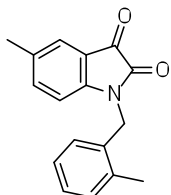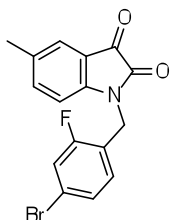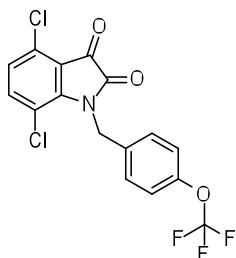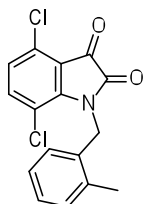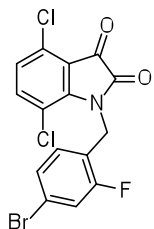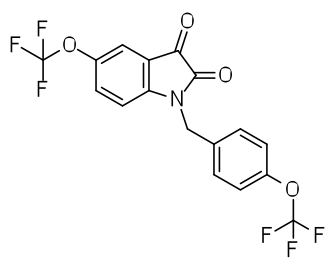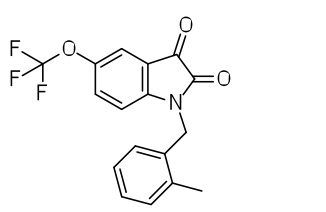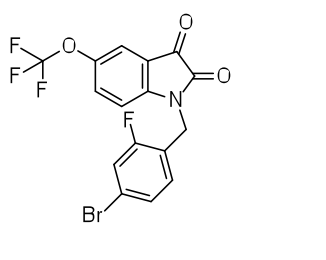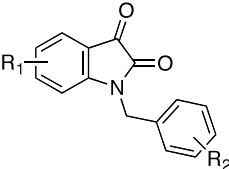
In terms of maximal potentiation efficacy, SAR from the initial 30 μM screen of the entire library suggested that 5-OCF$_3$ substitution of the isatin core was generally favored for greater M$_5$ versus M$_1$ activity, while halogen substitutions at the 7- position conferred a more dual M$_1$/M$_5$ activity. The % ACh max values for each compound at both M$_1$ and M$_5$ from the full singlepoint screen are show in **Table 1**. Based on the results of this screen, 18 compounds were selected for follow-up CRCs at M$_1$ and M$_5$ to determine potencies (**Table 2**). All 5-OCF$_3$ substituted compounds chosen from the initial screen possessed 1-5 μM potencies at M$_5$ and >30 μM potencies at M$_1$ in CRC assays, with the exception of compounds **63** and **112**, which nonetheless possessed greater M$_5$ versus M$_1$ activities (**Table 2**). Numerous benzylic substitutions were tolerated for M$_1$ and M$_5$ activity (depending also on the isatin core), methoxybenzyl was

generally favored for $M_5$ versus $M_1$ activity, and trifluoromethylbenzyl was generally favored for dual $M_1$/$M_5$ PAM activity. 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 (**Table 2**).

**Table 2.** Potency SAR at $M_1$ and $M_5$ for 18 compounds selected from an initial singlepoint screen. $Ca^{2+}$ mobilization assays with $M_1$ and $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh. Data represent the Mean of at least 3 independent experiments with similar results.



| Cmpd | $R_1$ | $R_2$ | $M_1$ $EC_{50}$ (µM) | $M_5$ $EC_{50}$ (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 |

Based on these findings, specifically that subtle structural changes to VU0119498 confer dramatic effects on mAChR subtype-selectivity, we also hypothesized that some of the compounds (of this 92-member library) that were inactive at $M_1$ and $M_5$ in the singlepoint screen may represent $M_3$ PAMs. Although identification of PAMs for $M_3$

was not a priority of this project, we nonetheless tested this hypothesis by evaluating 13 $M_1/M_5$ inactive compounds in a similar singlepoint (30 μM) screen at $M_3$ by $Ca^{2+}$ mobilization assay (**Fig. 5**). Surprisingly, all were devoid of robust PAM activity at $M_3$ (**Fig. 5**). With the exception of only two compounds (VU0238437 and VU0238469, which bore 7-F substitution at $R_1$) all of these analogs contained either 5-Me or 4,7-Cl substitution at $R_1$, suggesting that these modifications dial-out functional mAChR PAM activity from the VU0119498 scaffold or prevent mAChR binding altogether. The structures and VU numbers for these 13 compounds are shown in **Table 3**.
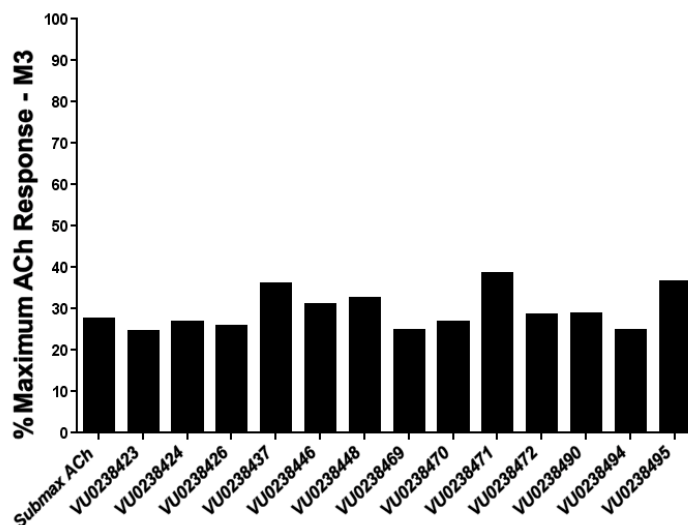


**Figure 5.** Singlepoint screen at $M_3$ of a subset of VU0119498 analogs chosen based on inactivity at $M_1$ and $M_5$. $Ca^{2+}$ mobilization assays with $M_3$ cells were used to obtain singlepoint (30 μM) potentiation responses for each analog in the presence of a submaximal (~$EC_{20}$) concentration of ACh. Data represent results from a single experiment.

**Table 3**. Structures and VU numbers for 13 VU0119498 analogs chosen for a M$_3$ PAM singlepoint screen based on their inactivity at M$_1$ and M$_5$ in the initial singlepoint screen of 92-member matrix library.

| R$_1$ | R$_2$ | Compound |
|-------|-------|----------|
| 5-Me | 4-OMe | VU0238423 |
| 4,7-Cl | 4-Br | VU0238424 |
| 4,7-Cl | 4-OMe | VU0238426 |
| 7-Fl | 2-Cl | VU0238437 |
| 4,7-Cl | 2-CF$_3$ | VU0238446 |
| 4,7-Cl | 2-Cl | VU0238448 |
| 7-Fl | 2-Fl | VU0238469 |
| 4,7-Cl | 2-Fl | VU0238470 |
| 4,7-Cl | 2,4-Fl | VU0238471 |
| 4,7-Cl | 2,6-Fl | VU0238472 |
| 5-Me | 4-OCF$_3$ | VU0238490 |
| 4,7-Cl | 2-Me | VU0238494 |
| 4,7-Cl | 2-Fl,4-Br | VU0238495 |

### *In vitro* characterization of M$_5$ PAM VU0238429

In light of the high M$_5$ versus M$_1$ potentiation preference displayed by analog **42** (1.16 μM M$_5$ EC$_{50}$ and >30 μM M$_1$ EC$_{50}$) in these assays, the full subtype-selectivity profile of this compound was obtained in similar Ca$^{2+}$ assays using M$_2$, M$_3$, and M$_4$ cells (**Fig. 6**). Likewise, full subtype-selectivity determination was also performed with compound **56**, which was the most potent dual M$_1$/M$_5$ potentiator in the initial evaluation at both receptors (2.11 μM M$_5$ EC$_{50}$ and 3.20 μM M$_1$ EC$_{50}$), as shown in **Figure 7**.
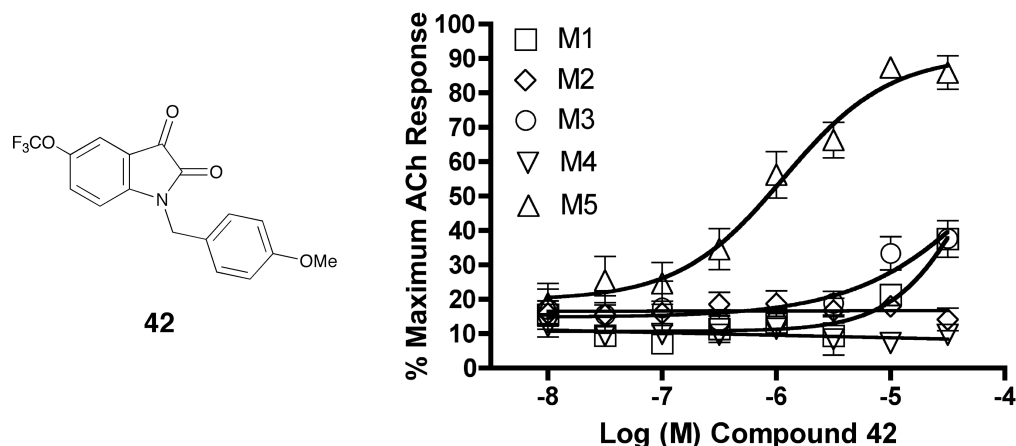
**Figure 6**. mAChR subtype-selectivity of compound **42** (VU0238429) displaying high $M_5$ PAM selectivity. $Ca^{2+}$ mobilization assays with $M_1$-$M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: $M_1$ = >30 μM, $M_3$ = >30 μM, $M_5$ = 1.1 μM 91% ACh max). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.
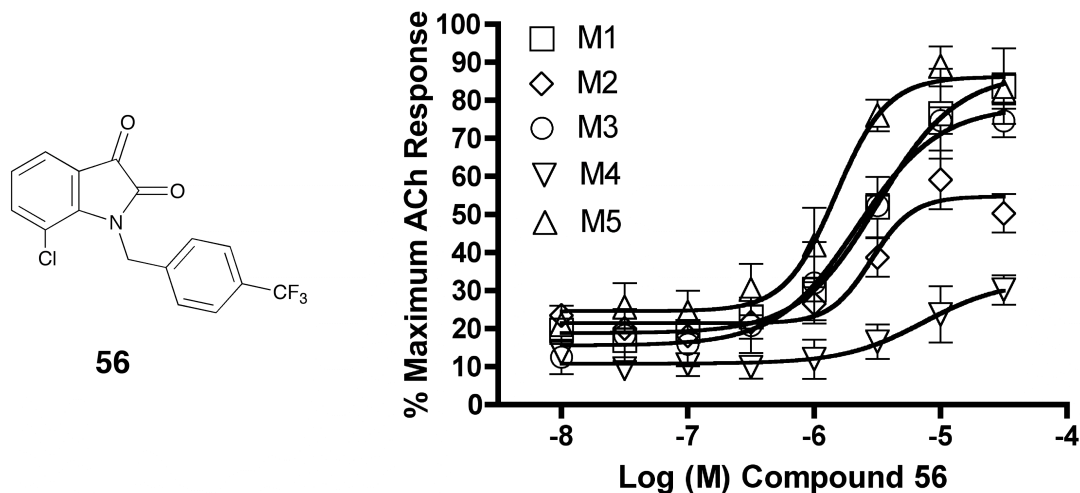


**Figure 7**. mAChR subtype-selectivity of compound **56** displaying pan-mAChR PAM activity. $Ca^{2+}$ mobilization assays with $M_1$-$M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: $M_1$ = 3.2 μM 87% ACh max, $M_3$ = 2.2 μM 78% ACh max, $M_5$ = 2.1 μM 86% ACh max, $M_2$ = 2.8 μM 55% ACh max, $M_4$ = >10 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Remarkably, compound **42** (VU0238429) had similarly poor potency at $M_3$ as at $M_1$, with potentiation of the ACh ~$EC_{20}$ beginning to emerge only in the double-digit

micromolar range (**Fig. 6**). Gratifyingly, this compound maintained its lack of potentiation activity at $M_2$ and $M_4$ (in cells co-transfected with $G_{qi5}$) at up to 30 μM (**Fig. 6**). Given these data, the approximately 1.16 μM $M_5$ potency of compound **42** provides >30x PAM selectivity for $M_5$ versus the other four subtypes, thus representing the first highly $M_5$-preferring $M_5$ ligand ever reported. Conversely, compound **56** (VU0238441) was found to retain the $M_1$/$M_3$/$M_5$ potentiation profile of the parent HTS hit **113** and also interestingly possessed modest potentiation at $M_2$ and $M_4$ at higher concentrations (**Fig. 7**). Despite the high $M_5$-preference of VU0238429 observed in these functional PAM assays, it is unknown if this compound (or any of the other analogs) possesses high $M_5$-binding specificity or if its selectivity is primarily functional with similar binding affinities for multiple subtypes. Binding experiments aimed at answering this question are hindered by an absence of radioligand(s) for the putative allosteric site at which these PAMs bind; however, functional studies may be pursued, which are discussed later in this thesis (Chapter VI).

To further evaluate the ability of compound **42** (VU0238429) to potentiate ACh-induced activation of $M_5$, we obtained full CRCs for ACh in $M_5$ cells in the presence of either vehicle or a fixed (30 μM) concentration of compound **42** (**Fig. 8**). In these assays, compound **42** induced a robust 14x left-shift of the ACh CRC, which was virtually identical to that found with the parent HTS hit VU0119498 when evaluated in the similar assay at $M_1$. Moreover, compound **42** lacked intrinsic agonist activity at $M_5$ when tested up to 30 μM in the absence of ACh (**Fig. 8**).
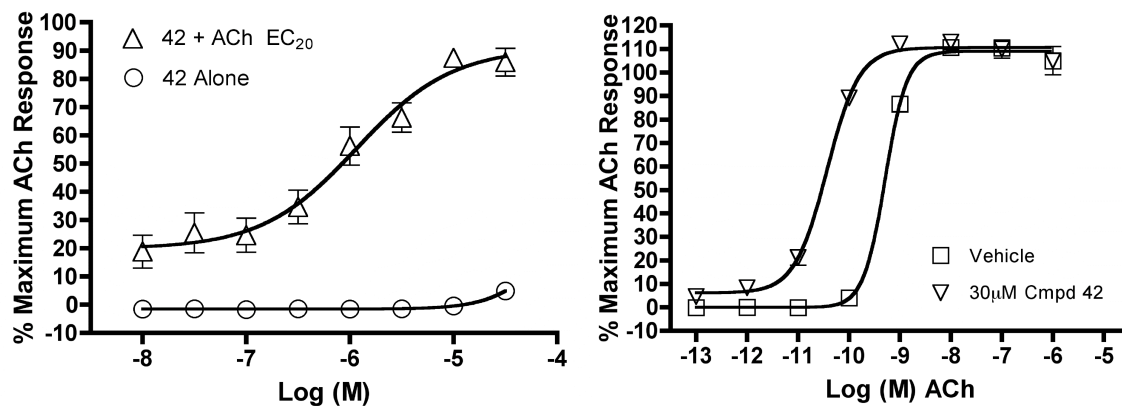
**Figure 8.** Compound **42** (VU0238429) requires ACh to activate $M_5$ and causes a robust increase in ACh potency at $M_5$. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence and absence of a submaximal ($\sim EC_{20}$) concentration of ACh (left panel) and ACh CRCs in the presence and absence of compound (30 μM, right panel). ACh $EC_{50}$ was left-shifted 14x by compound 42 (VU0238429). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

We also evaluated HTS hit VU0119498 and the highly $M_5$-preferring analog **42** (VU0238429) in competition-binding experiments with $M_5$-CHO membrane preparations using the orthosteric radioligand [$^3$H]-NMS, a potent pan-mAChR antagonist. At up to 30 μM, compound **42** lacked competition with [$^3$H]-NMS, consistent with an allosteric mode of binding to $M_5$ (**Fig. 9**). By contrast, VU0119498 exhibited approximately 50% inhibition of radioligand binding at 30 μM (**Fig. 9**). This inhibition by VU0119498 could be due either to direct competitive orthosteric binding or to negative cooperativity via an allosteric site; however, further experiments to investigate these possibilities were not performed due to a greater focus on VU0238429. When competition curves of ACh were obtained (**Fig. 9**) in the presence and absence of VU0238429 (30 μM) at $M_5$ cell membranes, this PAM caused a pronounced increase in the $K_i$ of the low affinity binding state of the receptor ($\sim$15x) with only a modest effect on the $K_i$ of the high affinity binding state ($\sim$1.5x), which suggests that the primary mechanism of potentiation by

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VU0238429 at $M_5$ is by enhancement of ACh binding, possibly via stabilization of the high affinity receptor conformation (**Fig. 9**).
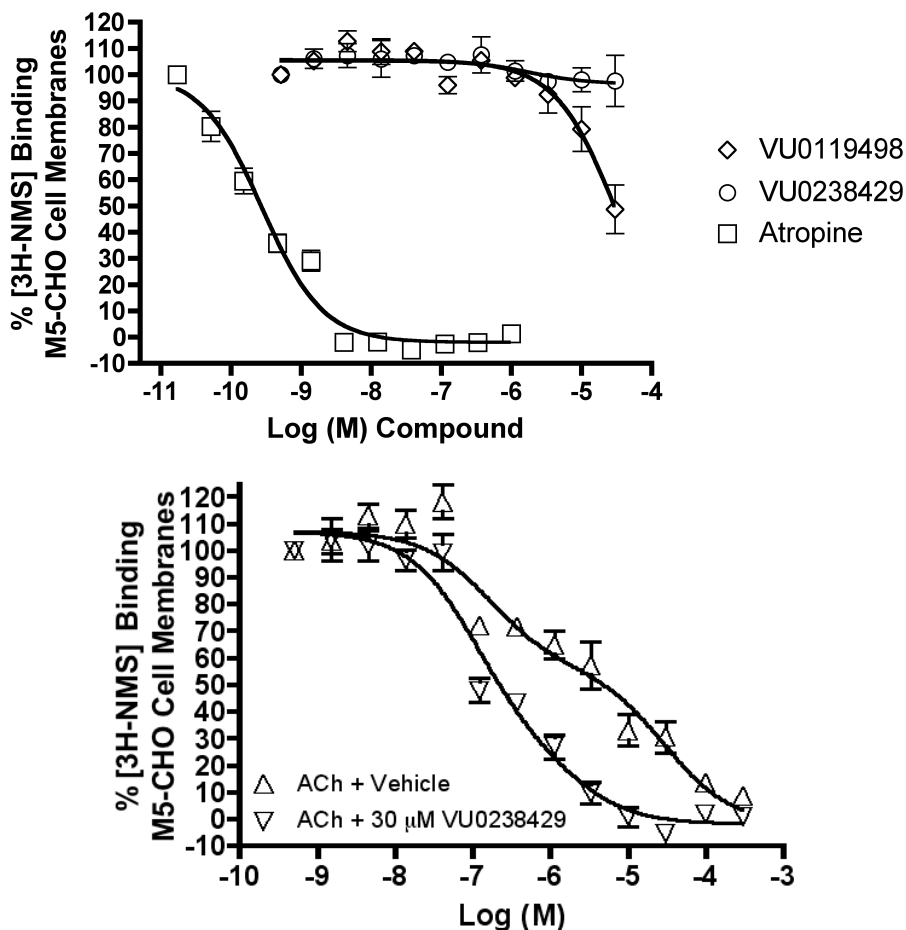


**Figure 9**. Competition binding experiments with VU0238429 and compound VU0119498. [$^3$H]-NMS (0.1 nM) binding to $M_5$ cell membranes was measured (top panel) in the presence of increasing concentrations of test compounds or atropine control ($K_i$ = 0.21 nM). [$^3$H]-NMS (0.1 nM) binding to $M_5$ cell membranes was used to obtain CRCs of ACh in the presence and absence of compound 42 (30 μM, bottom panel). ACh curves exhibited two-site competition (+Vehicle: $K_i$ high affinity = 123 nM, $K_i$ low affinity = 22 μM; +VU0238429: $K_i$ high affinity = 81 nM, $K_i$ low affinity = 1.39 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

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# *In vivo* rat pharmacokinetics of $M_5$ PAM VU0238429

We then determined rat *in vivo* pharmacokinetics and brain penetration of the $M_5$ PAM to evaluate its ability to serve as an *in vivo* tool compound. Unfortunately, VU0238429 exhibited poor systemic absorption after I.P. administration with maximum concentration in plasma (161.7 ng/ml) being achieved within 1 hour (**Fig. 10**). However, it was eliminated relatively slowly from systemic circulation with an elimination half-life of 4.7 hours, which is desirable for *in vivo* studies (**Fig. 10**). Although quickly taken up in the brain, it displayed poor brain penetration with an AUC-brain/AUC-plasma value of 0.2514 (**Fig. 10**).
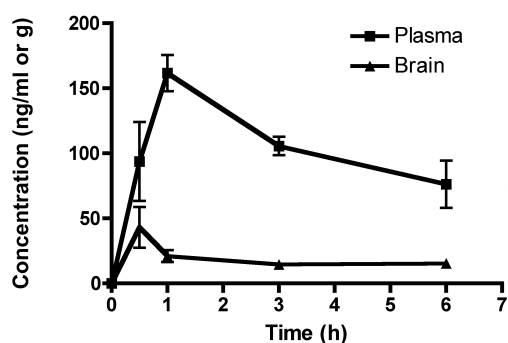


| PK Parameter | Plasma | Brain |
|---|---|---|
| $C_{max}$ (ng/ml or g) (mean±SD) | 161.7 ± 13.9 | 43.08 ± 15.68 |
| $T_{max}$ (h) | 1 | 0.5 |
| Elimination half life (h) | 4.7 | 3.69 |
| AUC $_{(0-6h)}$ (ng.h/ml or g) (mean±SD) | 621.1 ± 39.9 | 158.62 ± 47.23 |
| CL/F (ml/min) | 145 | |
| V/F (L/kg) | 59.6 | |
| $AUC_{brain}/AUC_{plasma}$ | 0.25 | |

**Figure 10**. Rat pharmacokinetic PBL study of VU0238429 and calculated PK parameters. Concentration of compound in brain and plasma over a 6-hour time course was measured by LC/MS/MS following a single 10 mg/kg (IP) dose using male Spraque-Dawley rats. Data represent the Mean ±S.E.M. with *N*=3. Study performed by S. Jadhav.

**Optimization of M$_5$ PAM VU0238429**

Despite the relatively limited scope of the initial matrix analog library in terms of structural modification, we found clear SAR associated with this novel mAChR PAM chemotype, with 5-OCF$_3$ modification of the isatin core and 3- or 4- position OMe substitutions of the benzyl ring conferring strong M$_5$ PAM preference versus M$_1$-M$_4$. In contrast, virtually all modifications conferring increased M$_1$ PAM activity simultaneously increased M$_5$ PAM activity. As exemplified in the case of compound **56**, 7-Cl substitution of the isatin core carried comparably high M$_1$/M$_5$ potency and efficacy, but also caused degeneration into a pan muscarinic potentiator as revealed by CRC profiling at all five subtypes. Based on these initial findings from the matrix library and the identification of VU0238429 as the first M$_5$-preferring PAM, we began a more comprehensive optimization campaign focused on VU0238429 as the lead structure.

The rationale for further optimization included a desire to improve potency, efficacy, and subtype-selectivity, as well as to decrease potential DMPK liabilities of the lead compound. An iterative parallel analog synthesis approach was used to drive the VU0238429 optimization as this methodology provides a more focused and efficient route compared to generation of matrix libraries or singleton syntheses because global structural diversity and random SAR exploration was no longer a priority based on the results from the initial 92-member matrix library around VU0119498. As introduced previously (Chapter I), iterative libraries are well suited to optimizations aimed at improving specific pharmacological or physiochemical characteristics of a lead compound because they are hypothesis-driven and less random than matrix-based or

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other medicinal chemistry optimization strategies.  Based on the initial VU0119498 SAR

and from consideration of chemistry/synthetic route accessibility and efficiency, we

outlined a number of modifications to VU0238429 to explore (**Fig. 11**).
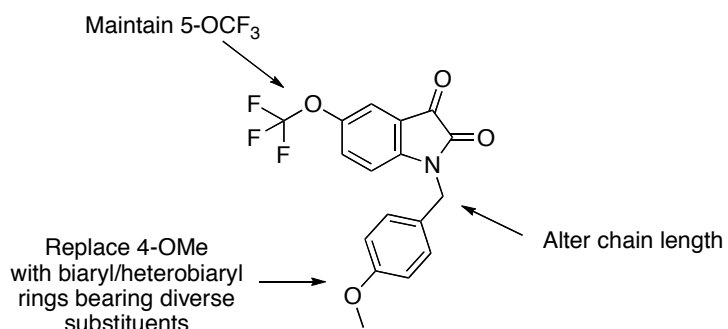


**Figure 11**.  Initial optimization strategy for $M_5$ PAM VU0238429.

Given the importance of the 5-$OCF_3$ moiety of the isatin core to $M_5$ subtype-

selectivity, it was held constant while the *N*-alkyl chain was lengthened and while the

benzyl ring was substituted at the *para* position with different aryl/heteroaryl rings in

place of the methoxy group of the lead structure.  Libraries were prepared according to

the scheme depicted in **Figure 12**, wherein commercial 5-(trifluoromethoxy)indoline-2,3-

dione (compound **4**) was alkylated with *p*-bromobenzylbromide to deliver key

intermediate (compound **5**).  An 11-member Suzuki library was then prepared to explore

the effect introducing the biaryl and heterobiaryl motifs into VU0238429 (compounds **6**).

In parallel, compound **4** was alkylated with functionalized phenethyl bromides

(compounds **7**) to probe the effect of chain homologation (compounds **8**).  Compound

libraries were then triaged by a singlepoint (10 μM) screen for their ability to potentiate

an ~$EC_{20}$ of ACh in $M_1$ and $M_5$ cells using $Ca^{2+}$ mobilization assay with VU0238429

included as a positive control (**Fig 12**).
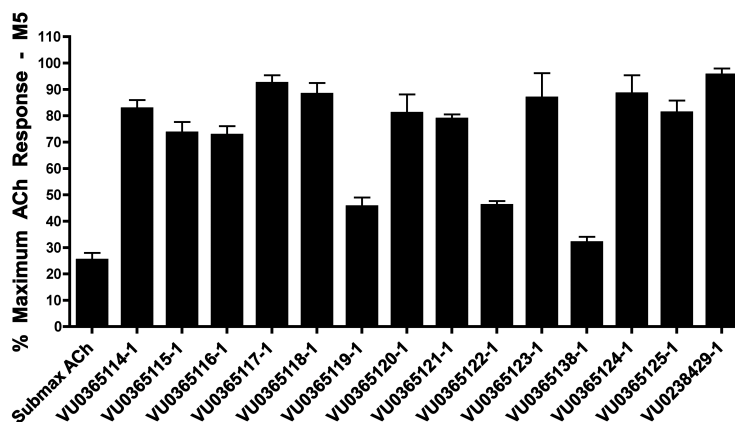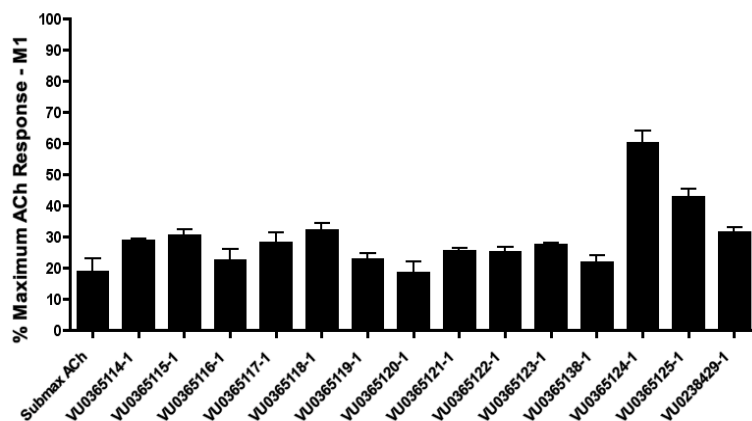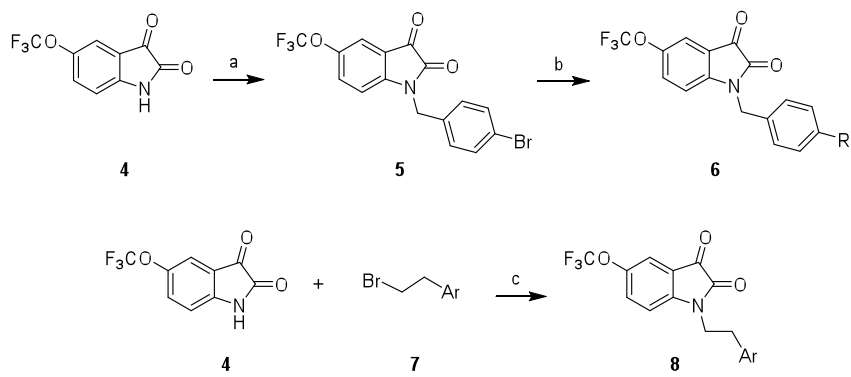
**Figure 12**. Synthesis and $M_1/M_5$ singlepoint screening of biaryl/heterobiaryl and phenethyl analogs of VU0238429. 13 compounds were synthesized (top panel) by a) p-bromobenzylbromide, $K_2CO_3$, KI, ACN, rt, 16 h (99%); b) R-B(OH)$_2$, Pd(Pt-Bu$_3$)$_2$, Cs$_2$CO$_3$, THF:H$_2$O, mw, 120 °C, 20 min (10-90%); c) $K_2CO_3$, KI, ACN, rt, 16 h (50-90%). $Ca^{2+}$ mobilization assays with $M_1$ and $M_5$ cells were used to obtain singlepoint (10 μM) potentiation responses for each analog in the presence of a submaximal (~$EC_{20}$) concentration of ACh (middle and bottom panels). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

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From these singlepoint screens at $M_1$ and $M_5$, select compounds were assayed using full CRCs based on the general high $M_5$ versus $M_1$ potentiation in terms of % ACh max efficacy observed with numerous members of this library. The potency SAR and associated efficacy data from the singlepoint screens at $M_1$ and $M_5$ for this library is presented in **Table 4**. In general, chain homologation (compounds **8**, i.e. VU0365124 and VU0365125) proved unsuccessful as potency and $M_5$-selectivity was compromised despite retention of PAM efficacy at higher concentrations. For example, the direct phenethyl analog of VU0238429 (compound **8a**, i.e. VU0265124) possessed a $M_5$ $EC_{50}$ of 4.9 μM and 78% ACh max response and a $M_1$ $EC_{50}$ >10 μM and 90 % ACh max response (**Table 4**). Biaryl and heterobiaryl analogs (compounds **6**) proved far more productive, affording a number of $M_5$ PAMs with high selectivity versus $M_1$ (>30 uM $M_1$ $EC_{50}$s) and low micromolar $M_5$ $EC_{50}$s (**Table 4**). All other analogs possessed $M_5$ $EC_{50}$s >10 μM. In general, both 5- and 6-membered heterocycles were tolerated as was simple phenyl substitution (compound **6a**). Potency was very similar for most of these analogs ($M_5$ $EC_{50}$s 2.7 μM to 4.8 μM) as were % ACh max values (70-85%). Shallow SAR was again noted with compounds either being active in this potency range or inactive as $M_5$ PAMs.

**Table 4**. M$_1$ and M$_5$ potency SAR for biaryl/heterobiaryl and phenethyl analogs of VU0238429. Ca$^{2+}$ mobilization assays with M$_1$ and M$_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~EC$_{20}$) concentration of ACh. n/d = potency not determined (no CRCs and the % ACh max values represent singlepoint screen data). Data represent the Means of at least 3 independent experiments.
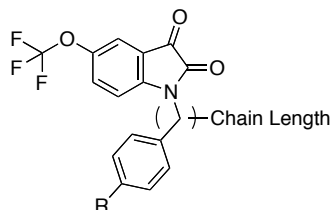


| Cmpd | VU# | Chain Length | R | M5 EC$_{50}$, ACh max | M1 EC$_{50}$, ACh max |
|------|-----|--------------|---|----------------------|----------------------|
| 6a | 0365114 | CH$_2$ | | 2.7 uM, 85% | >30 uM, n/a |
| 6b | 0365115 | CH$_2$ | | n/d, 73% | n/d, 30% |
| 6c | 0365116 | CH$_2$ | | n/d, 73% | n/d, 22% |
| 6d | 0365117 | CH$_2$ | | 2.8 uM, 90% | >30 uM, n/a |
| 6e | 0365118 | CH$_2$ | | 4.8 uM, 98% | >30 uM, n/a |
| 6f | 0365119 | CH$_2$ | | n/d, 46% | n/d, 23% |
| 6g | 0365120 | CH$_2$ | | n/d, 81% | n/d, 18% |
| 6h | 0365121 | CH$_2$ | | 3.6 uM, 88% | >30 uM, n/a |
| 6i | 0365122 | CH$_2$ | | n/d, 46% | n/d, 25% |
| 6j | 0365123 | CH$_2$ | | 3.3 uM, 85% | >30 uM, n/a |
| 6k | 0365138 | CH$_2$ | | n/d, 31% | n/d, 21% |
| 8a | 0365124 | C$_2$H$_4$ | | 4.9 uM, 78% | >10 uM, 90% |
| 8b | 0365125 | C$_2$H$_4$ | | n/d, 81% | n/d, 42% |

Given the relatively high potency and efficacy of compounds **6a** and **6d**, and the highest efficacy observed from compound **6e**, these three analogs were chosen for characterization in ACh CRC fold-shift (**Fig. 13**) and $M_3$ CRC selectivity (**Fig. 13**) via $Ca^{2+}$ mobilization assays. $M_3$ selectivity was determined due to the lower likelihood that $M_2$ and $M_4$ activity would present itself in light of previous SAR for this chemical series and the fact that $M_5$ shares highest amino acid sequence identity with $M_3$, thus rendering it the most obvious of the remaining mAChR subtypes to check for selectivity first. As seen in **Figure 13**, compound **6a** (VU0365114) exhibits very high PAM selectivity for $M_5$ versus $M_1$ and $M_3$, which is even greater than that of the parent compound VU0238429. Interestingly, the 3-thiophene substitution of compound **6d**, which is a common and well-recognized bio-isosteric replacement for a phenyl ring, exhibited a much more narrow selectivity window versus $M_3$ than compound 6a (**Fig. 13**). This serves as a clear example of the idiosyncratic and often-unpredictable SAR observed with many GPCR allosteric modulators. Likewise, the chloropyridine moiety of compound **6e** is electronically homologous (and modestly similar sterically) to the phenyl ring of compound **6a**; however, it too confers an approximately equal loss of selectivity versus $M_3$ as the thiophene of compound **6d** (CRC data for compound **6e** not shown).

In terms of ACh CRC fold-shift, these biaryl/heterobiaryl congeners proved far superior to VU0238429 with robust ACh potency left-shift of >50x compared to the 14x left-shift induced by VU0238429 (**Fig. 13**). As observed with other mAChR PAMs such as BQCA (discussed in more detail in Chapter V), intrinsic agonist activity was present with both compound **6a** and **6d**, which is reflected by baselines that begin at (~25-50% ACh max) for the ACh CRCs obtained in the presence of 30 μM compound (**Fig. 13**).
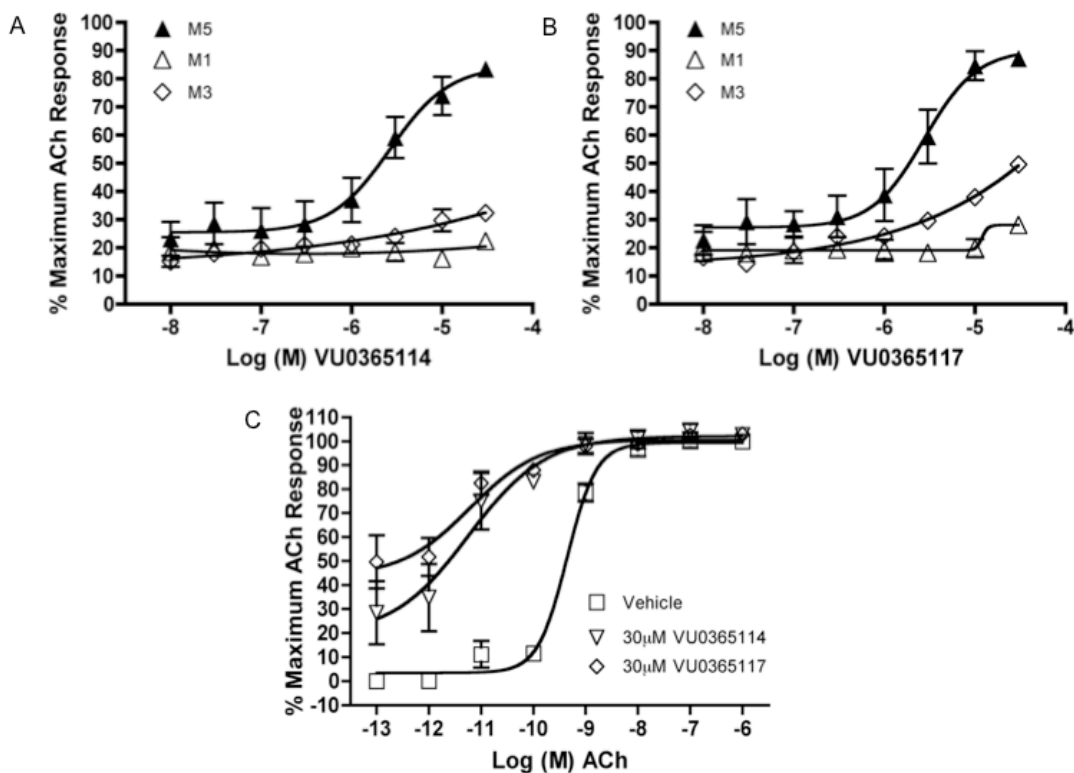
**Figure 13**. $M_1$, $M_3$, and $M_5$ subtype-selectivity and ACh CRC fold-shift of compound **6a** (VU0365114) and **6d** (VU0365117). $Ca^{2+}$ mobilization assays with $M_1$, $M_3$, and $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal ($\sim EC_{20}$) concentration of ACh (top panels, VU0365114 $EC_{50}$ values: $M_5$ = 2.7 µM 85% ACh max, $M_3$ = >10 µM, $M_1$ = >30 µM, VU0365117 $EC_{50}$ values: $M_5$ = 2.8 µM 90% ACh max, $M_3$ = >10 µM, $M_1$ = >30 µM). $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain ACh CRCs in the presence and absence of test compounds (bottom panel). Actual fold-shift values not determined due to intrinsic agonist activity of test compounds and therefore reported as >50x left-shift for both compounds. Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Encouraged by the potency, high ACh CRC fold-shift, and subtype-selectivity of VU0365114 (compound **6a**), we hypothesized that a hybrid analog of this compound and the parent VU0238429 lead in the form of a bi-aryl ether on the southern portion of the scaffold may provide to a more optimal $M_5$ PAM. We also sought to explore bio-isosteric replacements of the ketone at the 3-position of the isatin core or possibly

deletion/replacement of the entire keto-amide moiety, which is not a common drug-like structure and may represent a DMPK liability as an efflux pump (e.g. Pgp) substrate, potentially underlying the poor *in vivo* PK profile of VU0238429. The strategy for this library iteration is presented in **Figure 14**.
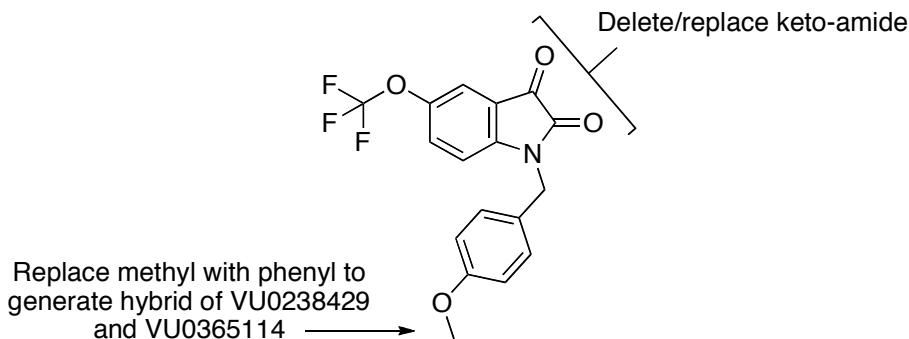


**Figure 14**. Strategy for second round of $M_5$ PAM analog library iterations.

The hybrid analog was synthesized according to the same general alkylation procedure described previously for earlier compounds (**Fig. 4** and **Fig. 12**). Briefly, commercial 5-trifluoromethoxyisatin was treated with *p*-phenoxybenzyl bromide in the presence of $K_2CO_3$ and subjected to microwave irradiation at 160 °C for 10 minutes to afford the desired compound as VU0400265 (68% yield). This analog was then profiled in CRCs at $M_1$-$M_5$ (in the presence of an ~$EC_{20}$ of ACh) using $Ca^{2+}$ mobilization assays (**Fig. 15**). The potency of VU0400265 (1.9 μM) and % ACh max (75%) for this analog was comparable to that of VU0238429 and VU0365114; however, it exhibited complete selectivity for $M_5$ with no elevation of the ACh ~$EC_{20}$ at any other mAChR subtype at up to 30 μM (**Fig. 15**). This represented the most selective $M_5$ PAM to date; however, it remains unknown whether or not this compound binds $M_1$-$M_4$.
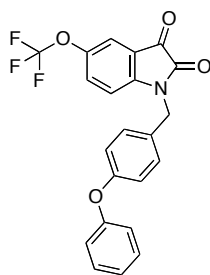
VU0400265
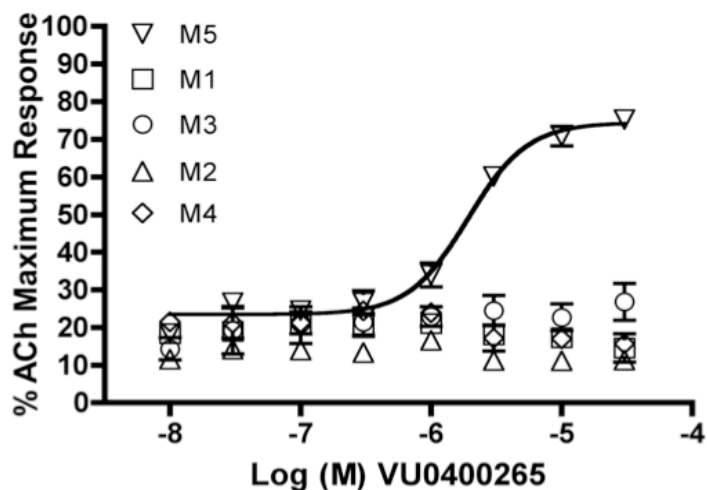
**Figure 15**. Structure, mAChR subtype-selectivity profile, and ACh CRC fold-shift for VU0400265, the first $M_5$-selective PAM representing a hybrid of VU0238429 and VU0365114. $Ca^{2+}$ mobilization assays with $M_1$-$M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: $M_5$ = 1.9 μM, 75% ACh max, $M_1$-$M_4$: >30 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

During the preparation of this phenoxybenzyl analog (VU0400265), we simultaneously generated a library of substituted indole and oxindole analogs to test the importance of the keto-amide moiety (2-,3-position carbonyls) of the isatin headpiece as part of the strategy outlined in **Figure 14**. To this end, 14 compounds were synthesized using previously described alkylation conditions (data not shown), which were then screened at 30 μM in $M_1$ and $M_5$ cells in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization assay, similar to previous singlepoint screens (**Fig. 16**). In both cell lines, VU0119498 and VU0238429 were used as positive controls.

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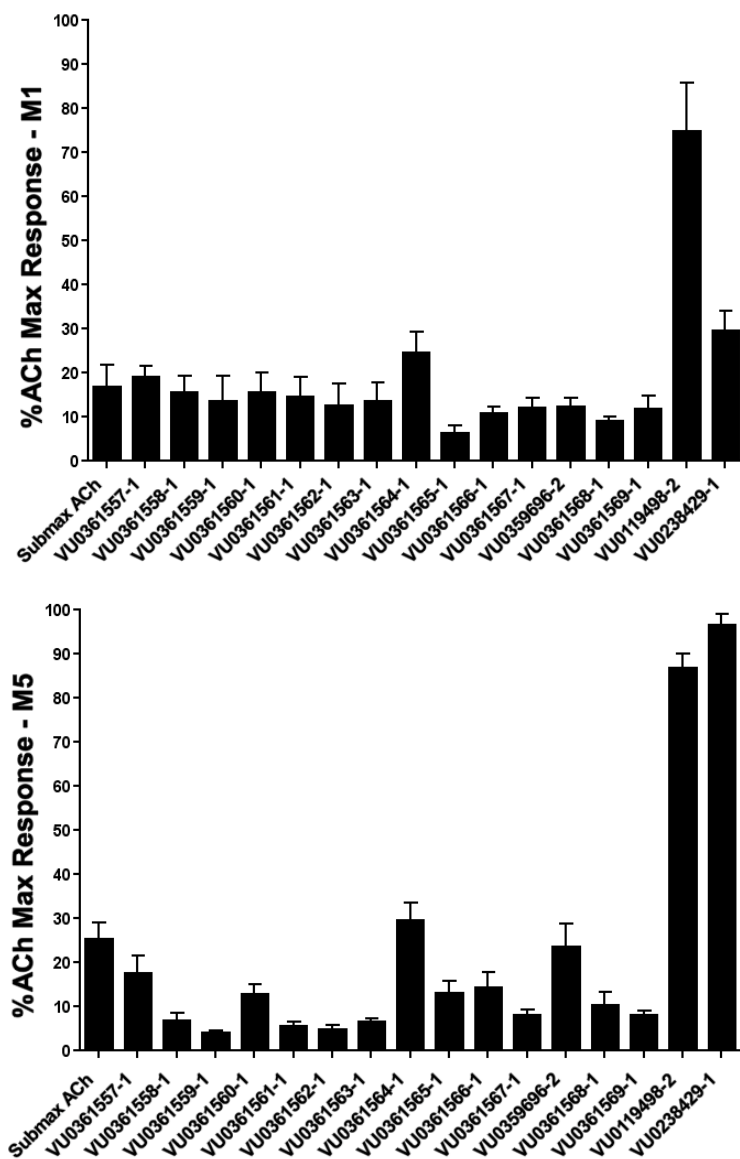
**Figure 16**. $M_1/M_5$ singlepoint screening of substituted indole and oxindole analogs. $Ca^{2+}$ mobilization assays with $M_1$ and $M_5$ cells were used to obtain singlepoint (30 μM) potentiation responses for each analog in the presence of a submaximal (~$EC_{20}$) concentration of ACh. $M_1/M_3/M_5$ HTS hit VU0119498 and $M_5$ PAM lead VU0238429 were included as positive controls. Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

However, none of these modifications were tolerated for PAM activity at either

receptor subtype (regardless of the southern benzyl substitution present), and some

appeared to induce modest inhibition of the ACh response at $M_5$, suggesting possible antagonism or negative allosteric modulation. Other attempts at replacing the 2-position carbonyl by introducing an eastern carboxylic acid in this position included synthesis of a small set of benzylated scaffolds (data not shown); however, these compounds were extremely unstable and degraded quickly at room temperature, thus they were not tested for mAChR PAM activity.

Having then established the importance of the keto-amide structure of the isatin core, we turned to specific bio-isoteric replacements for the 3-position ketone, which included gem di-fluoro, spirocyclopropyl, and a chiral tertiary alcohol. These structural changes are well-known mimetics for ketones as they retain the general electronic and steric characteristics of a ketone carbonyl, with the exception of the tertiary alcohol due to its hydrogen bond donation property (as compared to the hydrogen bond accepting property of a ketone). These three analogs were synthesized using the VU0238429 template (i.e. retention of *p*-methoxybenzyl on the southern portion) as shown in the scheme of **Figure 17**.
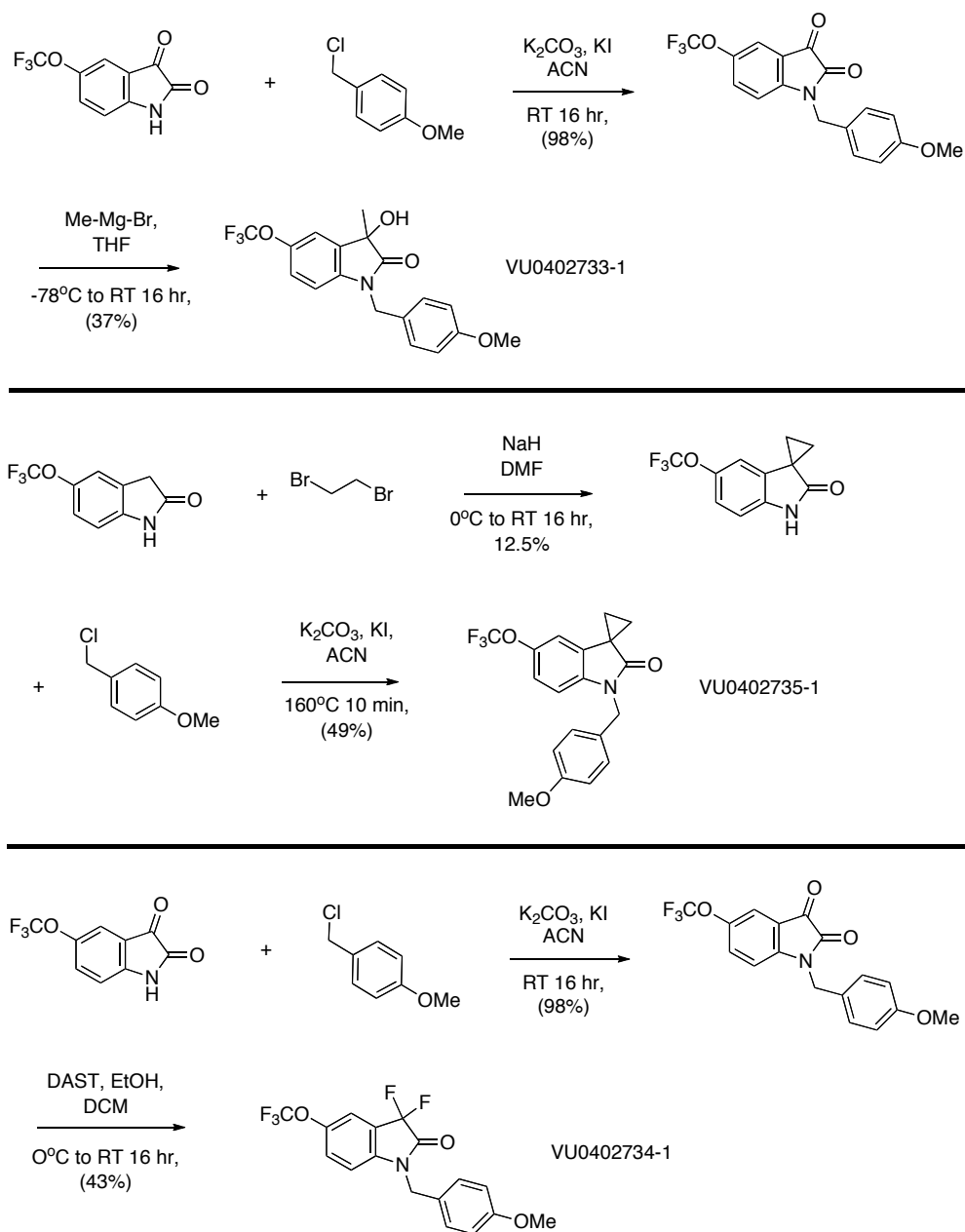
**Figure 17**. Synthesis of oxindole $M_5$ PAM analogs VU0402733, VU0402734, VU0402735.

Each compound was then tested via $Ca^{2+}$ mobilization in CRCs at $M_5$ in the presence of an ACh $\sim EC_{20}$ (**Fig. 18**). Gem-difluoro substitution (VU0402734) was extremely well tolerated and provided a modest increase in $M_5$ PAM potency, whereas spirocyclopropyl substitution (VU0402735) completely abolished $M_5$ PAM activity (**Fig.**

**18**).    Interestingly, the chiral tertiary alcohol analog (VU0402733) exhibited an approximately ~10x right-shift in potency compared to that of the parent $M_5$ PAM VU0238429 (**Fig. 18**).   These SAR data indicated that gem-difluoro would be ideal for subsequent analog generation, and it may confer an improved *in vivo* PK profile to this $M_5$ PAM series in contrast to retention of the isatin-based headpiece of VU0238429. Furthermore, this modification was synthetically favorable as any isatin-containing analog could be fluorinated in a simple one-step manner (**Fig. 17**).
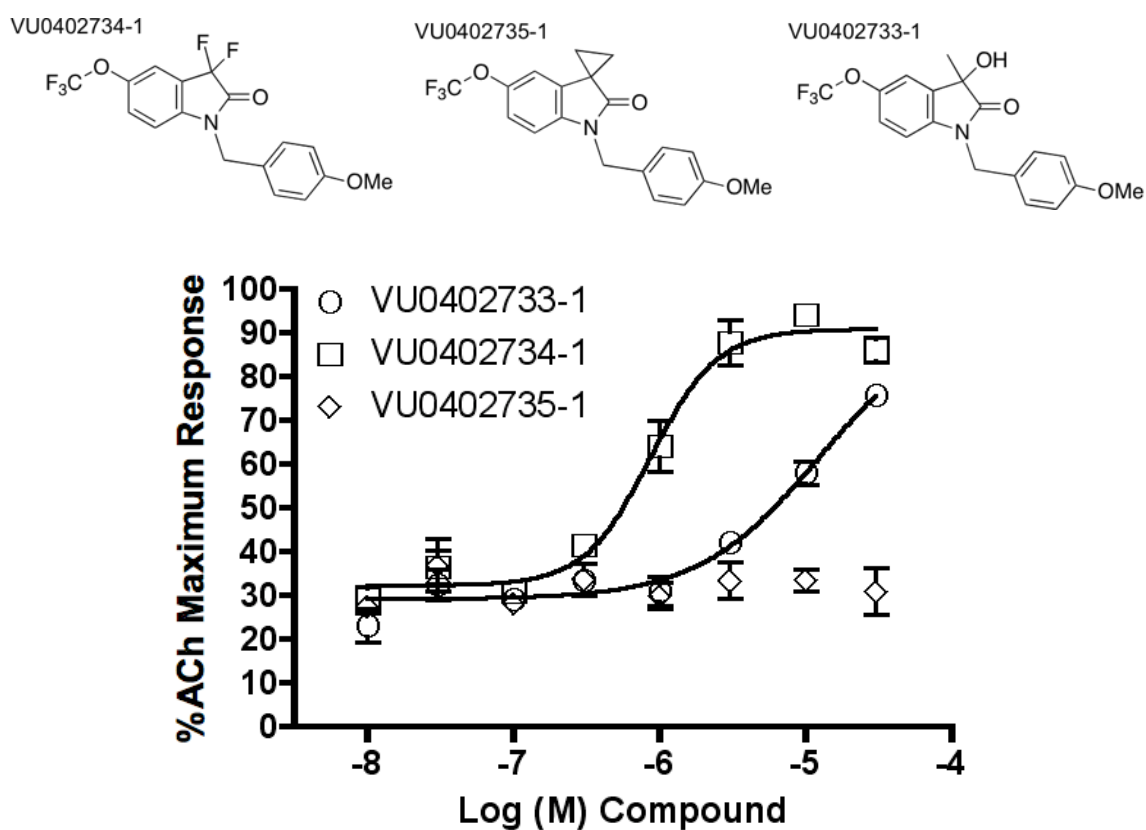


**Figure 18**.   Structures and CRCs for $M_5$ PAM analogs bearing 3-position ketone replacements. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: VU0402734 = 870 nM, 90% ACh max, VU0402733 = >10 μM, VU0402735 = >30 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Following this, VU0402734 was profiled in CRCs assays at $M_1$ and $M_3$ in the presence of an ACh ~$EC_{20}$ to determine if this modification altered subtype-selectivity (**Fig. 19**). Not only did gem-difluorination of the $M_5$ PAM scaffold result in greater potency, it also decreased PAM activity at $M_1$ (relative to the VU0238429 parent subtype-selectivity profile) and exhibited similarly low activity at $M_3$ (**Fig. 19**), which provided further confidence that this modification would be worth retaining for a final optimized scaffold.
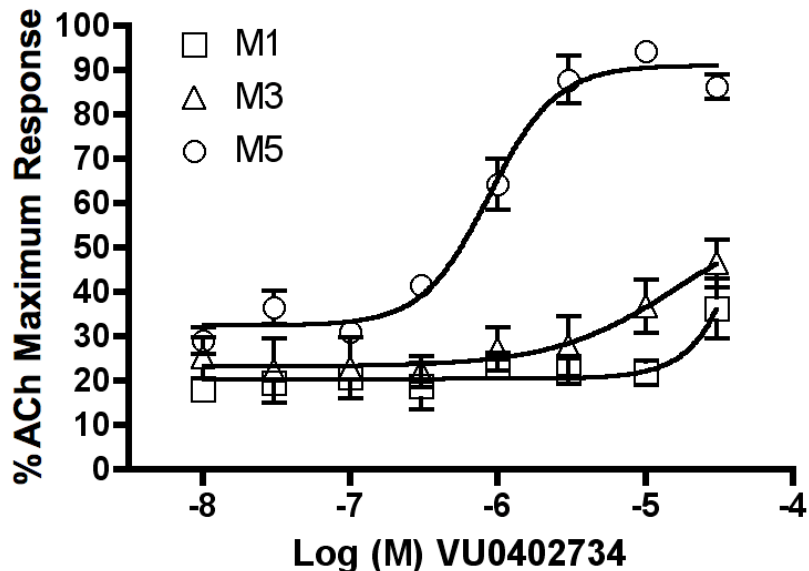


**Figure 19**. $M_1$, $M_3$, and $M_5$ subtype-selectivity profile for VU0402734, the gem-difluoro analog of $M_5$ PAM VU0238429. $Ca^{2+}$ mobilization assays with $M_1$, $M_3$, and $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: $M_5$ = 870 nM, 90% ACh max, $M_1$ = >30 μM, $M_3$ = >30 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Having completed the optimization strategy outlined in **Figure 14** and establishing important SAR demonstrating the favorable effect of the southern bi-aryl ether on $M_5$ PAM subtype-selectivity with no loss of potency as well as the northern

gem-difluoro substitution on both $M_5$ PAM subtype-selectivity and potency, a third iteration of parallel synthesis was planned (**Fig. 20**). At this point, movement of the 5-position $OCF_3$ group on the isatin core, introduction of mono or difluorines to the ortho position(s) on the southern benzyl ring, and southern 3-position biaryl/heterobiaryl extensions were planned in order to further investigate SAR around the $M_5$ PAM scaffold (**Fig. 20**). Rotation from the 5- to 6-position of the $OCF_3$ moiety was specifically investigated due to the fact that no 6-position substitutions were present in the initial 92-member matrix library around VU0119498. Impetus for fluorination of the southern benzyl ring in the *ortho* position(s) stemmed from SAR findings with $M_1$ PAMs derived from BQCA and from VU0119498 as part of a parallel but separate optimization project (discussed in Chapter V), which revealed an increase in PAM potency via fluorination in this position. Finally, biaryl/heterobiaryl extension at the 3-position of the southern benzyl ring was sought due to the fact that 3-methoxybenzyl (compound 41 from the 92-member matrix library) was well tolerated based on its $M_5$ versus $M_1$ PAM potency (**Table 2**) as well as the finding that a piperonyl group (VU0366628, synthesized using one-step *N*-alkylation method as previously described) in the southern region was well-tolerated for $M_5$ PAM activity (**Fig. 21**). Given that the gem-difluoro or ketone of the 3-position on the core both appear similar in pharmacology, the latter was maintained temporarily to increase speed of synthesis (i.e. reduce by one step).
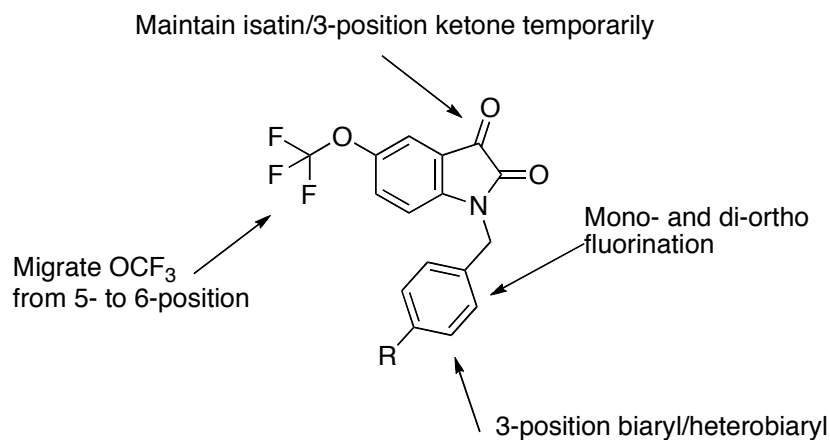
**Figure 20**. Optimization strategy for third round of $M_5$ PAM library iterations.
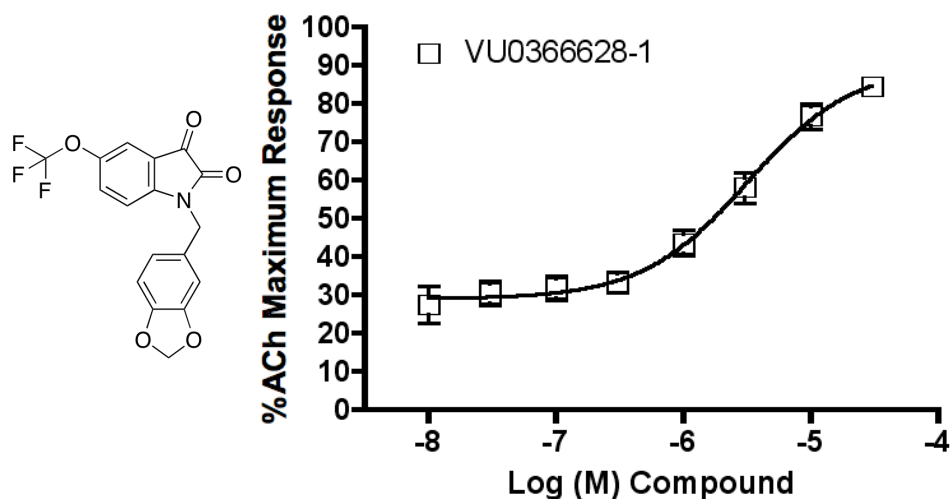


**Figure 21**. Structure and $M_5$ PAM activity of piperonyl analog VU0366628. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal ($\sim EC_{20}$) concentration of ACh ($EC_{50}$ values: $M_5$ = 3.2 μM, 87% ACh max). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Synthesis of mono and di-ortho fluorinated analogs was carried out using the same general alkylation conditions described previously with the naked 4-bromobenzyl

halide reactant(s) being replaced by the fluorine substituted congeners that were then Suzuki coupled with phenyl boronic acid (**Fig. 12**) to afford the desired products as VU0366625 and VU0366626 (**Fig. 22**). The *p*-phenylbenzyl southern moiety was used due to better commercial availability of the fluorinated bromobenzyl halide in lieu of the corresponding fluorinated methoxybenzyl and also because this moiety (based on VU0365114) is comparable if not superior to that of VU0238429. Both new fluorinated analogs were evaluated in CRCs at $M_5$ by $Ca^{2+}$ mobilization assay in the presence of an ACh $\sim EC_{20}$ (**Fig. 22**). Remarkably, di-fluorination of the benzyl ring in the *ortho* positions completely abolished $M_5$ PAM activity while mono-fluorination resulted in a substantial decrease in both potency and efficacy.
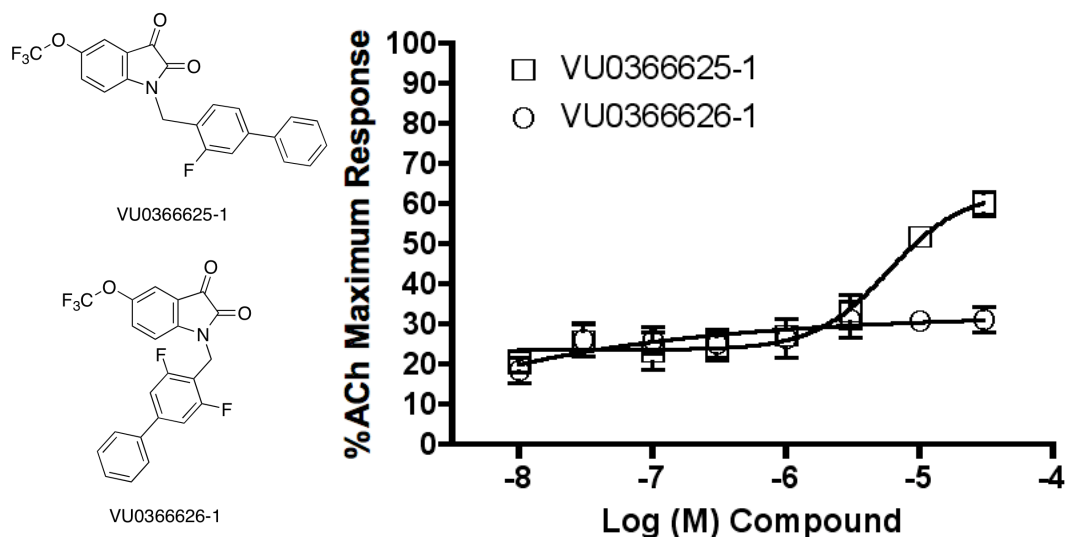


**Figure 22**. Structures and CRCs for *ortho*-fluorinated $M_5$ PAMs based on VU0365114 lead structure. $Ca^{2+}$ mobilization assays $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal ($\sim EC_{20}$) concentration of ACh ($EC_{50}$ values: VU0366625 = 5.9 µM 60% ACh max, VU0366626 = >30 µM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Fluorine atoms, which are approximately the same size in as hydrogen atoms, exhibit partial hydrogen-bond accepting properties, withdraw electron density from aromatic rings, and increase lipophilicity (i.e. calculated logP [cLogP]). One or more of these properties carried by these specific fluorinations compromises either binding to $M_5$ and/or functional PAM activity, although it is difficult to ascertain the exact reason(s) underlying this effect given an absence of structural knowledge for the putative allosteric site at which this $M_5$ PAM series binds. Given these SAR data, no subsequent libraries were generated with *ortho*-fluorine substitution(s).

Returning temporarily to the biaryl/heterobiaryl extension investigation, we simultaneously prepared analogs bearing direct phenyl, 2-thiophene, 3-thiophene, or phenoxy substitutions at the *meta* position of the southern benzyl ring, as outlined in the strategy presented in **Figure 20**. These analogs were synthesized according to the same alkylation procedure described previously using commercially available benzyl halides bearing the desired meta-substitutions, thus Suzuki couplings were not necessary. All of these analogs were devoid of $M_5$ PAM activity up to 10 μM and showed only a moderate elevation of an ACh $\sim EC_{20}$ at 30 μM in $Ca^{2+}$ mobilization assays with $M_5$ cells (data not shown, *N*=1). Based on these results, further derivatizations in the form of alternate heterocycles in this meta-benzyl position were not performed.

In parallel and again in accordance with the aims of the third round of SAR exploration as outlined in **Figure 20**, 6-OCF$_3$ analogs of VU0365114 and VU0238429 (the two lead $M_5$ PAMs containing the isatin headpiece) were prepared using the same synthetic route described previously, starting in this case with the commercially available 6-substituted isatin material. Both compounds were then tested in CRCs at $M_5$ in the

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presence of an ACh $\sim EC_{20}$ by $Ca^{2+}$ mobilization assays (**Fig. 23**). $M_5$ potency was moderately decreased in the case of VU0400267 (5.7 μM) but unchanged in the case VU0400266 (2.7 μM); however, efficacy was enhanced, with % ACh max values approaching 95-100% for both analogs (**Fig. 23**). Interestingly, subtype-selectivity eroded (**Fig. 23**) when these compounds were tested in similar CRC assays at $M_1$-$M_4$.



VU0400266-1
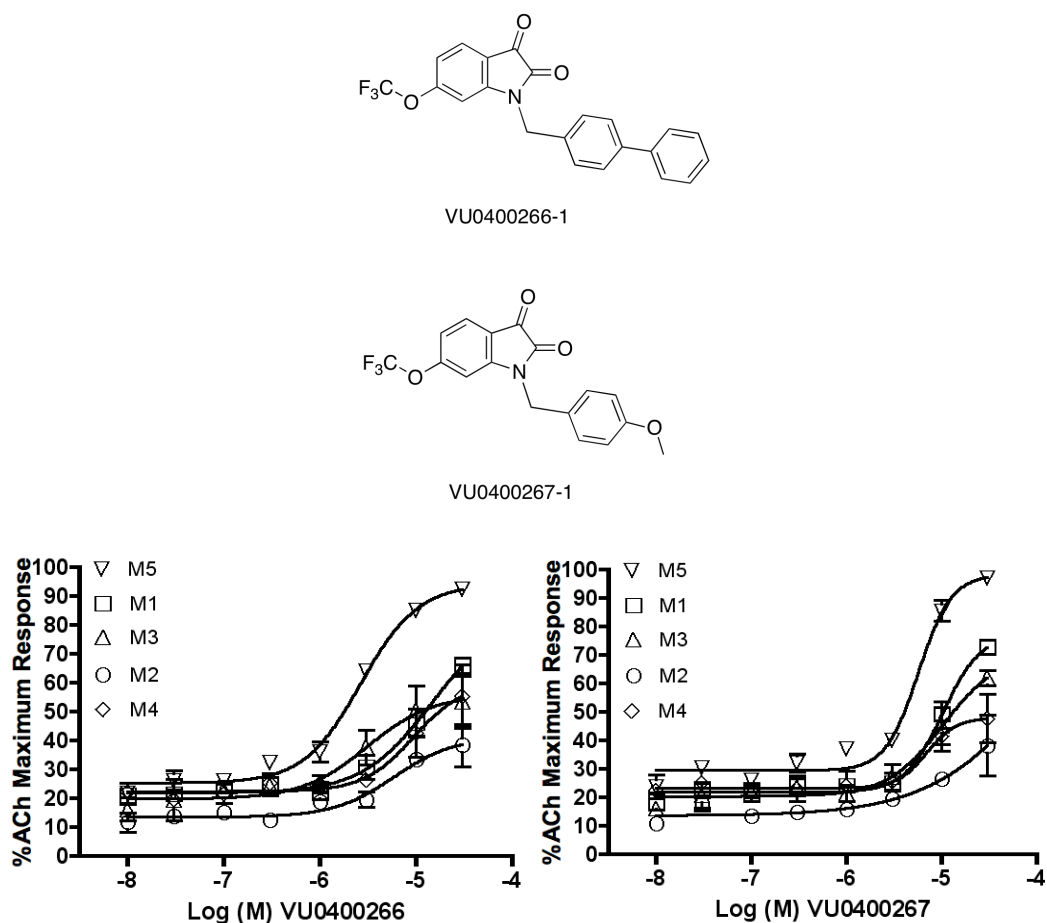
VU0400267-1

**Figure 23**. Subtype-selectivity profiles for VU0400266 and VU0400267. $Ca^{2+}$ mobilization assays with $M_1$-$M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal ($\sim EC_{20}$) concentration of ACh ($EC_{50}$ values: VU0400266 $M_5$ = 2.7 μM, 95% ACh max, $M_1$-$M_4$ >10 μM; VU0400267 $M_5$ = 5.7 μM 98% ACh max, $M_1$-$M_4$ >10 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

These SAR clearly suggested that 6-position migration of the $OCF_3$ group was not favored going forward, yet it was unknown whether or not the phenoxybenzyl southern moiety of earlier analogs could rescue the selectivity of a 6-$OCF_3$ template given that it provided a completely $M_5$-selective PAM (VU0400265, **Fig. 15**). To test this hypothesis, we synthesized a small 2x2 matrix library containing 5-$OCF_3$ and 6-$OCF_3$ substituted headpieces with gem-difluorines at the core's 3-position and each bearing either 3- or 4-phenxoxybenzyl southern moieties. The gem-difluoro versions were prepared because they too increased subtype-selectivity (although only modestly) and because the phenoxybenzyl analog VU0400265 had never been di-fluorinated. The 3-phenoxybenzyl substitutions were made due to the possibility that rotation of the $OCF_3$ from the 5- to 6-position may no longer be favored with southern *para*-substitution (e.g. due to rotation of the scaffold in the binding site). Again, synthesis proceeded as described previously using *N*-alkylation conditions on the respective commercially available starting materials with the substituted benzyl halides. These four analogs were tested in CRCs at $M_5$ in the presence of an ACh ~$EC_{20}$ via $Ca^{2+}$ mobilization assays (**Fig. 24**). As expected, the gem-difluorinated 5-$OCF_3$ headpiece bearing a southern 4-phenoxybenzyl substitution (VU0403751) exhibited potent $M_5$ PAM activity with an $EC_{50}$ of 1.6 µM and a 69% ACh max (**Fig. 24**). Likewise, the corresponding 6-$OCF_3$ analog (VU0403753) exhibited an $EC_{50}$ of 3.6 µM and an 81% ACh max (**Fig. 24**). In contrast, both *meta*-phenoxybenzyl analogs (VU0403752 and VU0403754) were devoid of $M_5$ PAM activity at up to 30 µM (**Fig. 24**), further confirming that the *para*-position is more optimal for the southern benzyl substitutions.

VU0403751-1

VU0403753-1

VU0403752-1

VU0403754-1
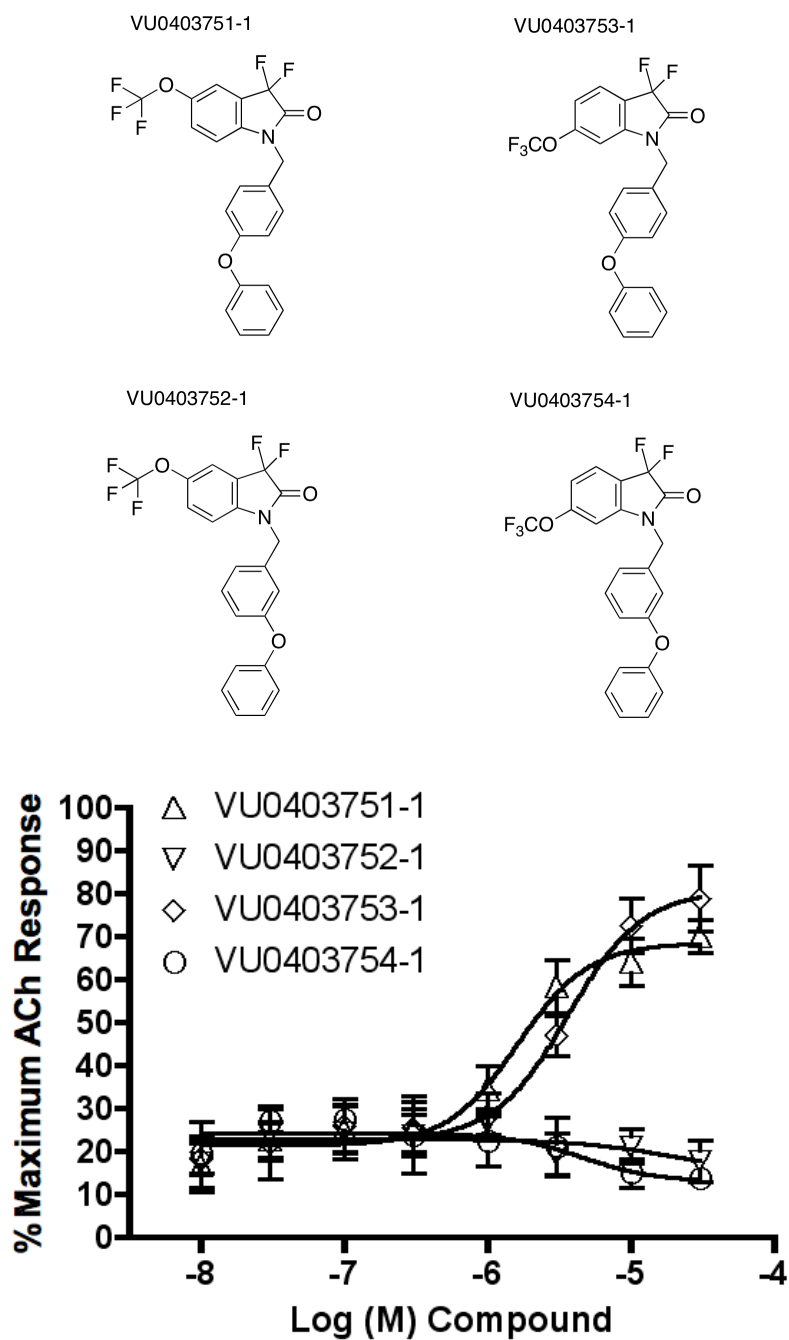


**Figure 24.** Structures and $M_5$ CRCs for 2x2 matrix library of phenoxybenzyl gem-difluoro analogs. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: VU0403751 = 1.6 μM, 69% ACh max; VU0403753 = 3.6 μM, 81% ACh max; VU0403752 = >30 μM; VU0403754 = >30 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Next, both VU0403751 and VU0403753 were profiled for subtype-selectivity in CRCs at $M_1$ and $M_3$ (**Fig. 25**), with the former maintaining its high $M_5$-selectivity comparable to the parent VU0400265 isatin-based compound and the latter exhibiting an increase in $M_5$-selectivity engendered by the gem-difluoro and phenoxybenzyl substitutions. However, because the 6-$OCF_3$ group in VU0403753 still introduced $M_1$ and $M_3$ potentiation at higher concentrations despite the selectivity-engendering substitutions made to its core and southern portions, further optimization did not utilize the 6-position of the core headpiece despite its favorable effect on $M_5$ PAM efficacy.
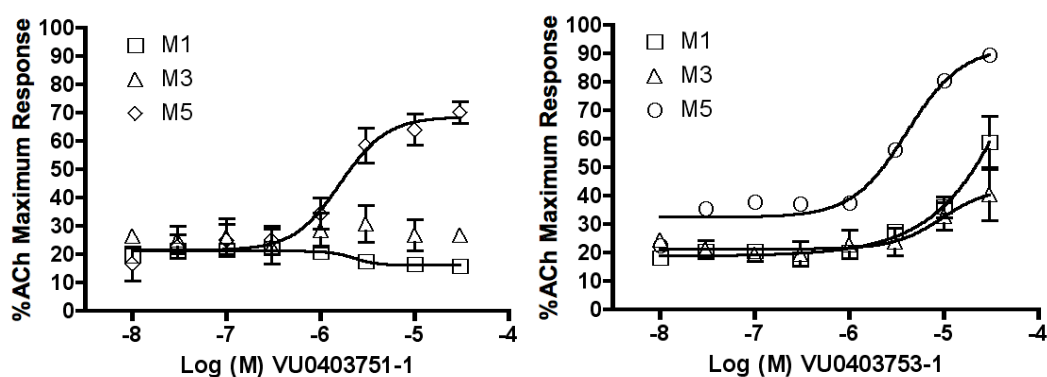


**Figure 25.** $M_1$, $M_3$, and $M_5$ CRCs for VU0403751 and VU0403753. $Ca^{2+}$ mobilization assays with $M_1$, $M_3$, and $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: VU0403751 $M_5$ = 1.6 μM, 69% ACh max; VU0403751 $M_1$-$M_4$ >30 μM; VU0403753 $M_5$ = 3.6 μM, 81% ACh max; VU0403753 $M_1$ and $M_3$ = >10 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Having then further established the favorable effect of gem-difluoro substitution at the 3-position of the scaffold's core on $M_5$ PAM potency and subtype-selectivity, the VU0403751 and earlier VU0402734 lead analogs bearing gem-difluorines were tested in ACh CRC fold-shift experiments by $Ca^{2+}$ mobilization assays with $M_5$ cells (**Fig. 26**). At 30 μM, these compounds induced approximately 6x and 23x left-shifts of the ACh curve,

respectively, with the stronger potentiator VU0402734 inducing moderate intrinsic agonist activity (**Fig. 26**). These results agreed well with previous SAR from the homologous isatin sister analogs of these two compounds and added additional confirmation that gem-difluorination conferred increased PAM efficacy.
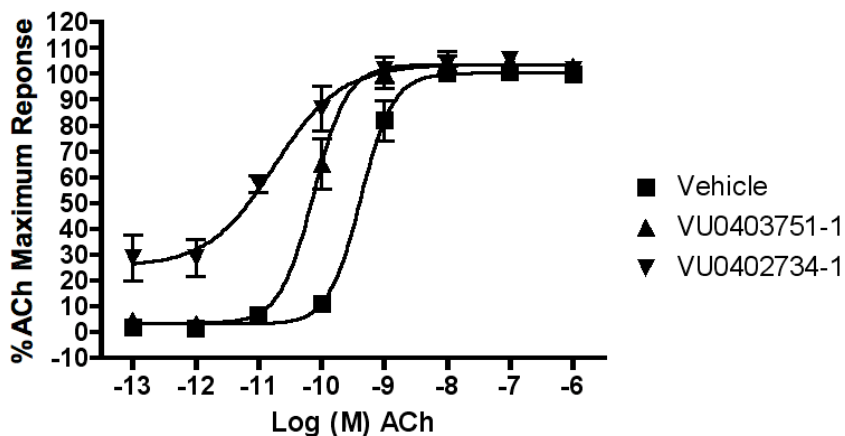


**Figure 26**. ACh CRC fold-shift effect of gem-difluoro lead analogs VU0403751 and VU0402734. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain ACh CRCs in the presence and absence of test compounds. VU0403751 induced an approximately 6x left-shift and VU0402734 induced an approximately 23x left-shift with moderate intrinsic agonism at 30 μM. Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

To be comprehensive and before concluding that only 5-position substitution on the core was favorable, we synthesized two tricyclic di-fluoromethyldioxylene analogs, which represented hybrids of 5-$OCF_3$ and 6-$OCF_3$ groups in terms of general electronics, lipophilicity, and to a lesser extent steric bulk. These compounds were synthesized using one-step alkylation methods utilizing commercial 2,2-difluoro[1,3]dioxolo[4,5-f]indole-6,7-dione as starting material, again with 4- and 3-phenoxybenzyl halides. The structures and $M_5$ CRCs for these compounds in $Ca^{2+}$ mobilization assays in the presence of an ACh ~$EC_{20}$ are shown in **Figure 27**. Neither compound exhibited robust PAM activity and therefore this tricyclic template was abandoned (**Fig. 27**).

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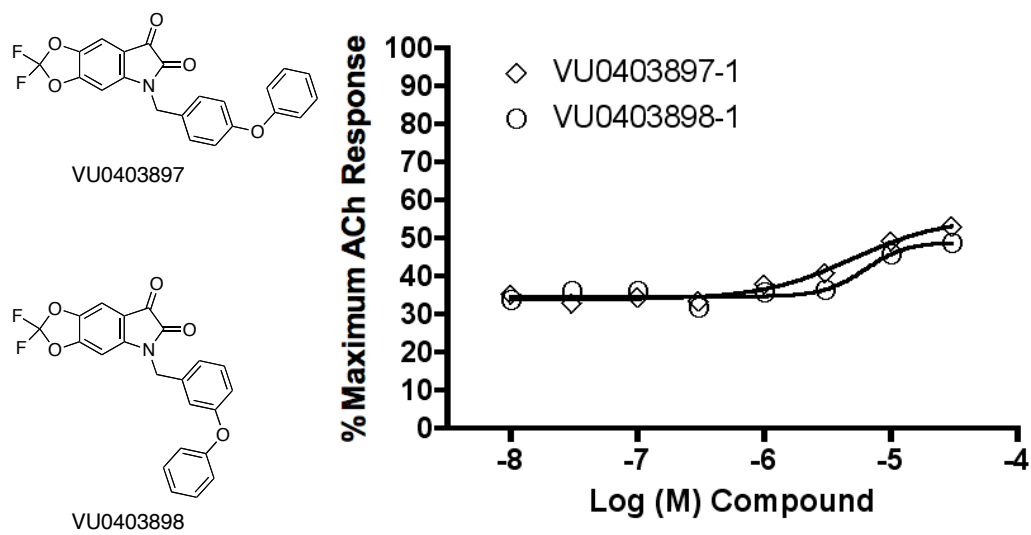
**Figure 27**. $M_5$ CRCs for tricyclic analogs VU0403897 and VU0403898. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh ($EC_{50}$ values: VU0403897 and VU0403898 = >10 μM). Data represent the results of one experiment.

For the fourth iteration of parallel libraries, the western 5-$OCF_3$ group was chosen as a point of derivation (**Fig. 28**). Although movement of this moiety to the 6-position of the core was found to erode subtype-selectivity (despite enhancement of $M_5$ PAM efficacy), other substitutions beyond those present in the initial 92-member matrix library (5-$OCF_3$, 5-Cl, 5-F, 5-Me) to the 5-position had not been explored. In particular, alkyl extensions of the trifluoro group in the form of an ethoxy or propoxy ether and deletion of the trifluoro group altogether via synthesis of the 5-methoxy analog was planned. These structures would provide important insight to the significance of the $OCF_3$ group. Beyond these three specific changes, we also sought an extensive SAR survey though synthesis of a diverse set of benzyl, heteroaryl, alkyl, substituted alkylamine, etc... off of a 5-position ether. Most of these analogs could be accessed synthetically via generation of the 5-hydroxy starting material, which would involve methyl-deprotection of a 5-

methoxy substrate.  In light of this synthetic route, the *p*-phenylbenzyl moiety of VU0365114 was chosen as the southern piece to be held constant for these libraries, since its mAChR pharmacology confers it with "lead" status comparable or perhaps greater than that of the methoxybenzyl southern moiety in VU0238429, and its use would not result in problematic bis-hydroxyl production during the methyl-deprotection step, unlike VU0238429.  The synthetic scheme for this set of libraries is presented in **Figure 28**, and a complete list of structures generated is shown in **Table 5**.  The rationale for the broad range of substituents was simply to increase the likelihood of uncovering useful SAR since no previous analogs investigated such modification in the western region of this general $M_5$ PAM scaffold.
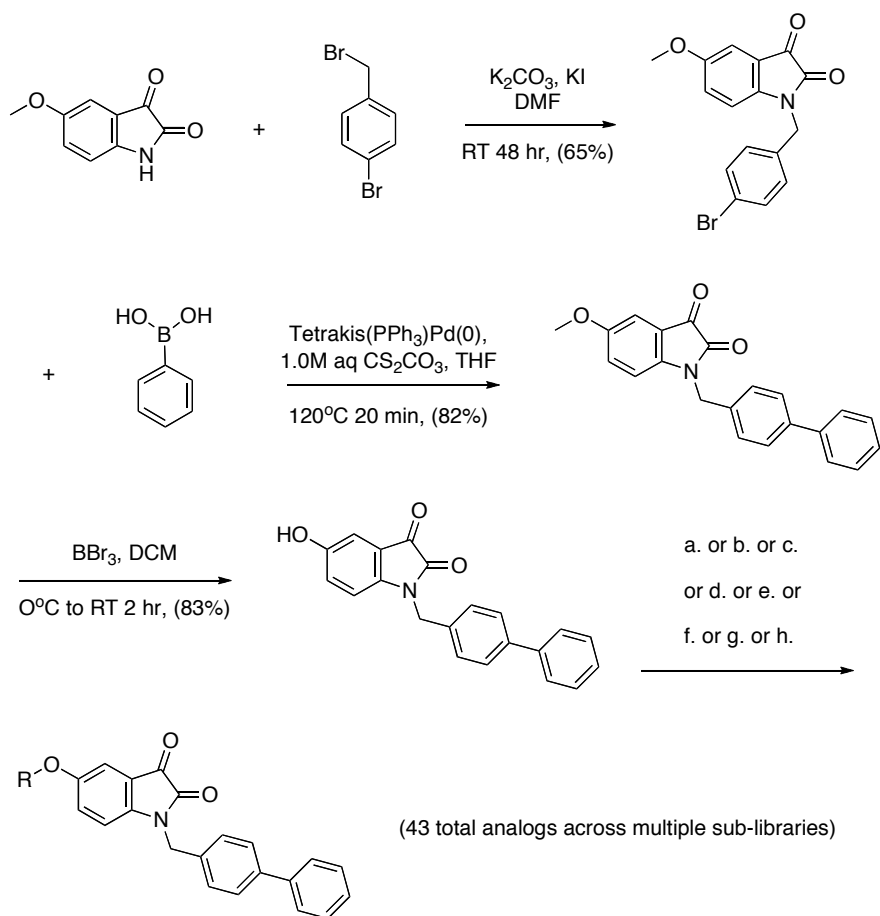
a.: R-X, CS₂CO₃, KI, DMF, 120°C 30 min, (avg. 20%), R = 27 analogs

b.: (1) ethylchloroacetate, CS₂CO₃, KI, DMF, 120°C 30 min, (16%)
   then (2) LiOH, THF, H₂O, 120°C 10 min, (43%)

c.: R-OH, PS-PPh₃, DIAD, THF, RT 16 hr, (avg. 4%), R = 10 analogs

d.: 4-(2-chloroethyl)morpholine HCl, NaH, KI, DMF, 55°C 24 hr (4%)

e.: 3-chloromethyl-1-methylpiperidine HCl, CS₂CO₃, KI, DMF 180°C 30 min, (1%)

f.: 1,1,1-trifluoro2-iodoethane, K₂CO₃, DMF, 160°C 10 min, (4%)

g.: 2-fluoropyridine, CS₂CO₃, DMF, 160°C 20 min, (3%)

h.: 2-chloropyridine, K₂CO₃, DMF, 100°C 30 min, (14%)

**Figure 28**. Synthetic scheme for generation of a diverse set of M₅ PAM analogs based on VU0365114 bearing western ethers. Analogs were synthesized by utilization of eight sub-library/singleton chemistry routes (a.-h.) using the 5-hydroxy(*N*-substituted)isatin starting material following methyl-deprotection.

**Table 5**. Structures of western ether analog library based on M$_5$ PAM VU0365114. All analogs were devoid of robust M$_5$ PAM activity in 10 µM singlepoint screen (**Fig. 29**).
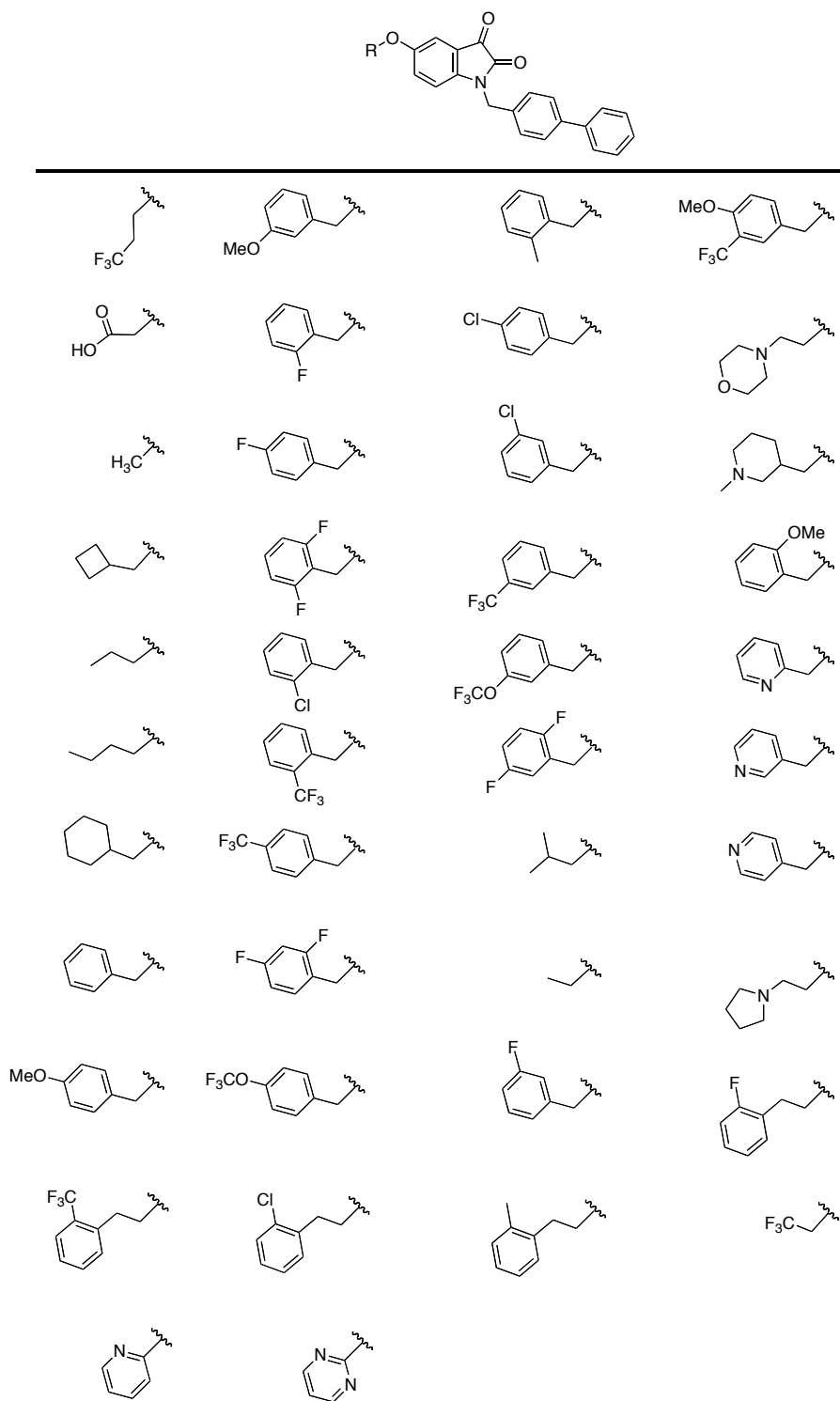
Following synthesis and purification, the compounds were evaluated in a singlepoint PAM (10 μM) $Ca^{2+}$ mobilization assay in the presence of an ACh ~$EC_{20}$ with $M_5$ cells (**Fig. 29**). Remarkably, all analogs were devoid of any considerable PAM activity, suggesting in agreement with all previous SAR that the 5-$OCF_3$ group is absolutely required for activity at $M_5$ and even the subtle derivation to trifluoroethoxy in this position causes loss of the PAM effects (**Fig. 29**). Intriguingly, a subset of analogs in this screen induced partial inhibition of the submaximal ACh concentration, which may reflect either orthosteric antagonism or possible NAM activity (**Fig. 29**). Each of these analogs bore basic amines on similarly long alkyl tethers to the oxygen at the 5-position of the isatin core, with the three most basic analogs inducing the greatest inhibition (**Fig. 30**).
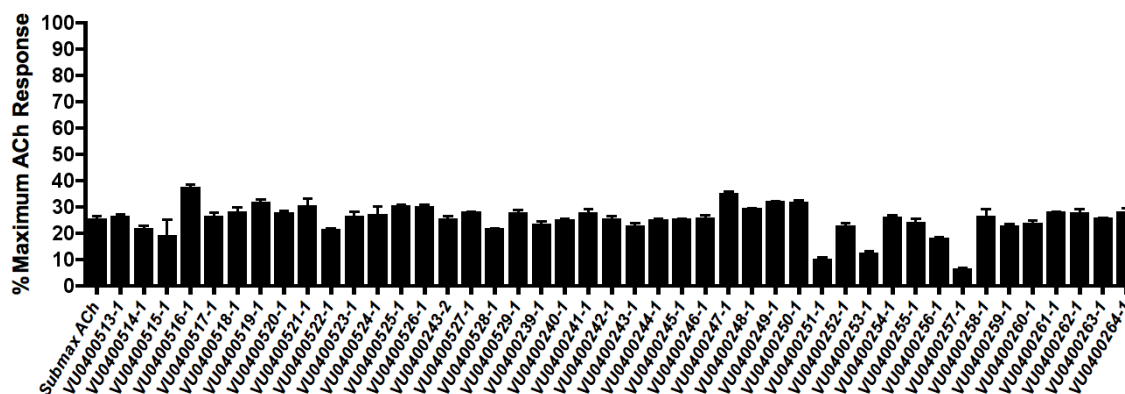


**Figure 29**. $M_5$ singlepoint screening of western ether analogs (**Table 5**). $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain singlepoint (10 μM) potentiation responses for each analog in the presence of a submaximal (~$EC_{20}$) concentration of ACh. Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.
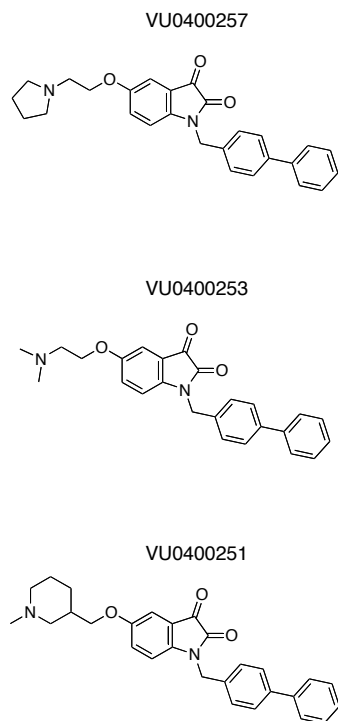
Figure 30. Basic-amine containing western ether analogs that partially inhibited an ACh ~$EC_{20}$ response in $M_5$ PAM screen shown in Figure 29, suggesting potential $M_5$ NAM or antagonism.

Based on these data, an exploratory optimization project was launched in hopes of discovering $M_5$ NAMs or antagonists (of which there are none at present reported in the international scientific or patent literature), which has identified a number of additional compounds maintaining the western basic alkylamine that exhibit complete inhibition of an ACh ~$EC_{80}$ with upper micromolar-level potency in CRCs using $M_5$ cells by $Ca^{2+}$ mobilization. Although not a focus of this thesis, this discovery may eventually lead to $M_5$-preferring or selective NAMs or antagonists suitable as research tools, which would provide invaluable insight to $M_5$ neurobiology across a range of possible studies (discussed further in Chapter VI). However, given the structure of ACh itself, it is possible that the basic amines of these putative $M_5$ NAMs/antagonists may be inhibiting the ACh response via orthosteric antagonism, especially due to the comparable distance

between the nitrogen and the oxygen of the ether. If orthosteric antagonism does in fact underly these data, $M_5$-preference or selectivity may still be achievable due to the remainder of the scaffold. Whether or not such a mechanism for selectivity is present with these analogs is currently unknown, as no radioligand binding studies have been performed to assess allosteric versus orthosteric modes of modulation.

Returning to the $M_5$ PAM SAR revealed by this large library, we opted to maintain the 5-$OCF_3$ moiety of the scaffold's core indefinitely, as it alone appeared to provide the $M_5$ potentiation effect. This extremely flat SAR (around the western area of the scaffold) is common with many GPCR allosteric modulators and can be a difficult impediment to optimization projects. In the case of these western ether analogs, a number of modifications were explored and importantly the crucial 5-methoxy analog (VU0366627), which was synthesized and also tested separately in $Ca^{2+}$ mobilization CRCs at $M_5$ (**Fig. 31**). This compound provided a stark example of the narrow steric and electronic tolerance(s) of the receptor-binding interaction of the scaffold as this analog lacked $M_5$ PAM activity at up to 10 μM with only modest potentiation occurring at 30 μM (**Fig. 31**). Whether the hydrophobic properties of the three fluorines, their electronic attenuation of the hydrogen-bond accepting potential of the neighboring oxygen, and/or their own partial hydrogen-bond accepting characteristics underlies this tight SAR remains unknown.
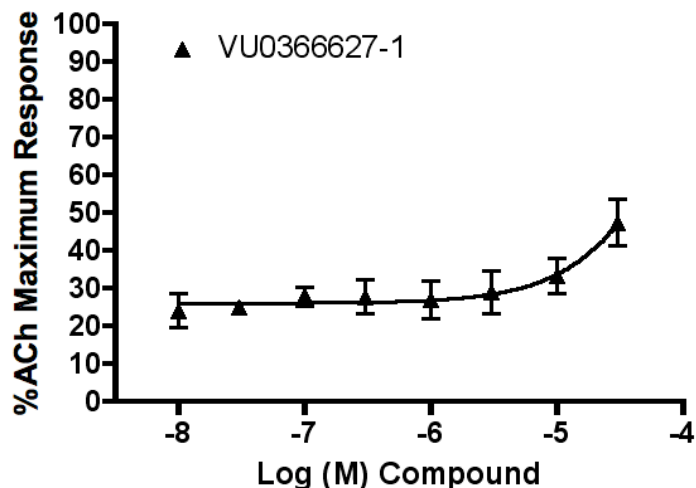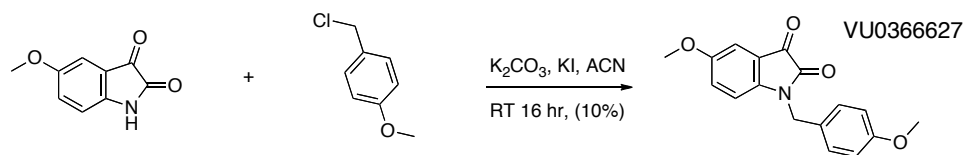
**Figure 31**. Synthesis and $M_5$ CRC for 5-methoxy analog VU0366627. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRC in the presence of a fixed submaximal ($\sim EC_{20}$) concentration of ACh ($EC_{50}$ = >10 μM). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

## Ancillary pharmacology and physiochemical optimization of M₅ PAMs

Having investigated a large number of structural modifications to the general VU0238429 $M_5$ PAM scaffold across four rounds of targeted libraries and uncovering important SAR to obtain a set of optimized lead compounds, we temporarily halted the medicinal chemistry efforts and determined ancillary off-target pharmacological activity via radioligand binding screens at MDS Pharma/Ricerca. Both the isatin containing and gem-difluoro congeners of the two most potent and most subtype-selective compounds (VU0238429, VU0402734, VU0400265, VU0403751, respectively), as well as the moderately potent and highly subtype-selective intermediate lead VU0365114 were

chosen for testing, which involved $N$=2 singlepoint (10 μM) screening across approximately 75 different GPCRs, ion channels, transporters, nuclear hormone receptors, enzymes, and RTKs. For any off-target binding that resulted in >50% inhibition, the protein and % inhibition is reported for each compound in **Table 6**.

**Table 6**. Ancillary/off-target binding screening results for five $M_5$ PAM leads (10 μM). Data represent the Mean of 2 independent experiments with similar results. Experiments performed by MDS Pharma/Ricerca.
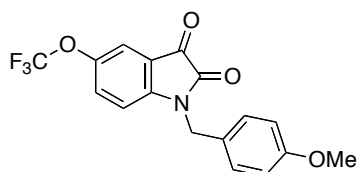


VU0238429

| Target/Protein | Species | % Inhibition |
|---|---|---|
| Adenosine $A_3$ | Human | 65 |
| Adrenergic $\alpha_{2A}$ | Human | 99 |
| NE Transporter | Human | 66 |
| Cannabinoid $CB_1$ | Human | 84 |
| Dopamine $D_{4.2}$ | Human | 61 |
| Histamine $H_1$ | Human | 62 |
| Histamine $H_2$ | Human | 55 |
| Opiate μ | Human | 70 |
| hERG $K^+$ Channel | Human | 60 |
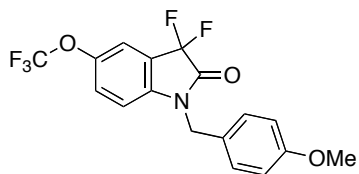


VU0402734

| Target/Protein | Species | % Inhibition |
|---|---|---|
| Adenosine $A_3$ | Human | 68 |
| Adrenergic $\alpha_{2A}$ | Human | 98 |
| NE Transporter | Human | 71 |
| Cannabinoid $CB_1$ | Human | 64 |
| Dopamine $D_3$ | Human | 58 |

| | | |
|---|---|---|
| Dopamine $D_{4.2}$ | Human | 57 |
| Histamine $H_1$ | Human | 68 |
| Histamine $H_2$ | Human | 59 |
| Opiate μ | Human | 73 |
| hERG $K^+$ Channel | Human | 65 |
| PDE-4/Rolipram | Rat | 60 |
| $Na^+$ Channel Site-2 | Rat | 60 |
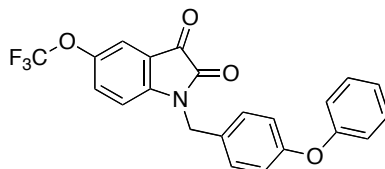
VU0400265

| Target/Protein | Species | % Inhibition |
|---|---|---|
| Adenosine $A_1$ | Human | 50 |
| Adenosine $A_3$ | Human | 97 |
| Adrenergic $\alpha_{1A}$ | Rat | 79 |
| Adrenergic $\alpha_{1D}$ | Human | 66 |
| Adrenergic $\alpha_{2A}$ | Human | 100 |
| Adrenergic $\beta_1$ | Human | 89 |
| NE Transporter | Human | 93 |
| Bradykinin $B_1$ | Human | 52 |
| $Ca^{2+}$ L-Type Channel | Rat | 74 |
| Cannabinoid $CB_1$ | Human | 109 |
| Dopamine $D_1$ | Human | 99 |
| Dopamine $D_{2S}$ | Human | 85 |
| Dopamine $D_3$ | Human | 79 |
| DA Transporter | Human | 90 |
| GABA Transporter | Rat | 56 |
| Histamine $H_1$ | Human | 99 |
| Histamine $H_2$ | Human | 101 |
| Imidazoline $I_2$ | Rat | 50 |
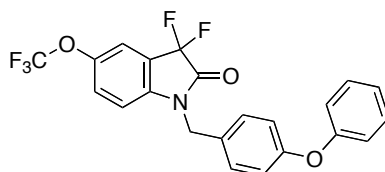
VU0403751

| Target/Protein | Species | % Inhibition |
|---|---|---|

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| | | |
|---|---|---|
| Adenosine $A_1$ | Human | 55 |
| Adenosine $A_{2A}$ | Human | 51 |
| Adenosine $A_3$ | Human | 97 |
| Adrenergic $\alpha_{1A}$ | Rat | 62 |
| Adrenergic $\alpha_{1D}$ | Human | 67 |
| Adrenergic $\alpha_{2A}$ | Human | 98 |
| Adrenergic $\beta_1$ | Human | 86 |
| NE Transporter | Human | 91 |
| Bradykinin $B_1$ | Human | 60 |
| $Ca^{2+}$ L-Type Channel | Rat | 79 |
| Cannabinoid $CB_1$ | Human | 106 |
| Dopamine $D_1$ | Human | 100 |
| Dopamine $D_{2S}$ | Human | 86 |
| Dopamine $D_3$ | Human | 76 |
| DA Transporter | Human | 86 |
| GABA Transporter | Rat | 56 |
| Histamine $H_1$ | Human | 93 |
| Histamine $H_2$ | Human | 96 |
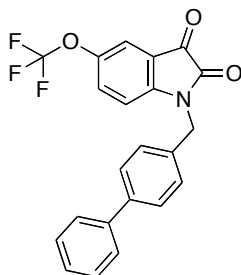


VU0365114

| Target/Protein | Species | % Inhibition |
|---|---|---|
| Adenosine $A_3$ | Human | 98 |
| Adrenergic $\alpha_{1B}$ | Rat | 51 |
| Adrenergic $\alpha_{1D}$ | Human | 70 |
| Adrenergic $\alpha_{2A}$ | Human | 99 |
| Adrenergic $\beta_1$ | Human | 86 |
| NE Transporter | Human | 86 |
| $Ca^{2+}$ L-Type Channel | Rat | 73 |
| Cannabinoid $CB_1$ | Human | 68 |
| Dopamine $D_1$ | Human | 104 |
| Dopamine $D_{2S}$ | Human | 89 |
| Dopamine $D_3$ | Human | 75 |
| DA Transporter | Human | 74 |
| Endothelin $ET_A$ | Human | 92 |
| Endothelin $ET_B$ | Human | 51 |

| Histamine $H_1$ | Human | 102 |
|---|---|---|
| Histamine $H_2$ | Human | 103 |
| Leukotriene $CysLT_1$ | Human | 104 |
| Muscarinic $M_1$ | Human | 78 |
| Muscarinic $M_2$ | Human | 96 |
| Muscarinic $M_3$ | Human | 62 |
| Opidate $\kappa$ | Human | 98 |
| Opidate $\mu$ | Human | 100 |
| hERG $K^+$ Channel | Human | 69 |
| Prostanoid $EP_4$ | Human | 72 |
| Serotonin 5-$HT_{2B}$ | Human | 67 |
| Serotonin Transporter | Human | 64 |

These ancillary pharmacology data clearly indicated that the southern methoxybenzyl moiety (VU0238429 and VU0402734) was favored over the phenoxybenzyl (VU0400265 and VU0403751), as the latter introduced a large number of off-target activities, including pronounced binding to DAT and multiple DA receptors, which would pose serious difficulties in many $M_5$ neurobiology experiments (particularly those focused on the VTA, SNc, and/or striatum). Gem di-fluorination (VU0402734 and VU0403751) dialed in modest $D_3$ receptor (58% inhib.), PDE-4 (60% inhib.), and sodium channel site-2 (60% inhib.) activity, which did not raise substantial concerns for most potential $M_5$ studies. VU0365114, bearing the highly lipophilic linear bi-phenyl southern motif on the 5-$OCF_3$ isatin core, suffered from the highest number of off-target activities.

Given these results, further optimization of the southern biaryl ether moiety was performed in order to reduce/eliminate its associated off-target activities and further explore SAR. Specifically, introduction of more polar and heteroatomic moieties to the southern ether would likely reduce off-target activities that may be due to the increased lipophilicity of the distal phenyl ring in VU0400265 and VU0403751 as compared to the

methyl group of VU0238429 and VU0402734.  Furthermore, a reduction in the relatively high cLogPs of these compounds would likely aid in the overall physiochemical and drug-like properties of the scaffold, particularly if introducing basic amines to allow HCl salt formation is tolerated.

To this end, we replaced the distal phenyl ring with various 5- or 6-member naked or mono-substituted heterocyclic rings while holding constant the remainder of the $M_5$ PAM scaffold.  Due to the challenging synthesis of these analogs (**Fig. 32**), which required derivatization at the first rather than the last step as well as laborious reaction conditions and purifications, only four compounds were generated to explore this SAR (**Fig. 32**).



$R_1$: 2-fluoropyridine
$R_2$: 2,6-difluoropyridine
$R_3$: 2-chloro-1,3-thiazole
$R_4$: 2-fluoro,6-methoxypyridine
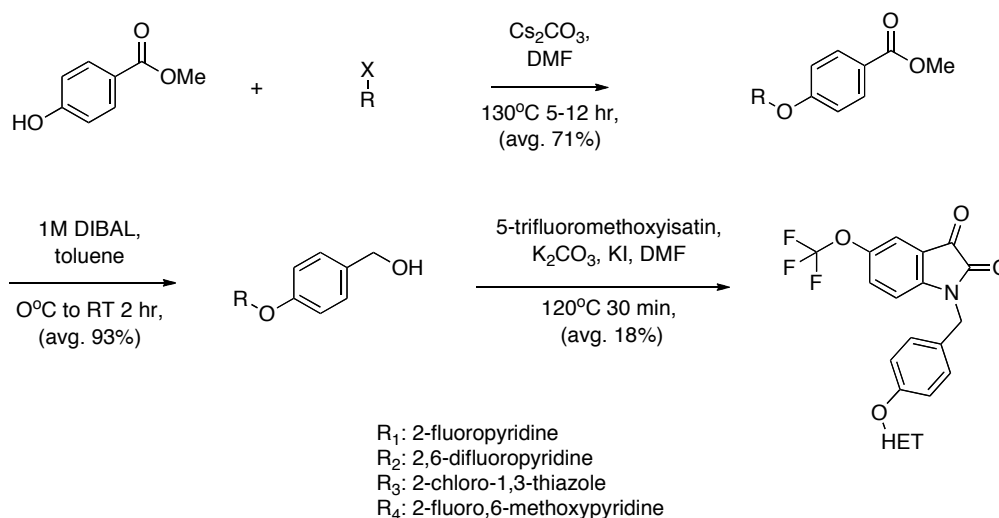
**Figure 32**.  Synthesis of four southern heterobiaryl ether analogs based on VU0400265.

These four southern heterobiaryl ether analogs were then evaluated in CRCs at $M_5$ in the presence of an ACh $\sim EC_{20}$ by $Ca^{2+}$ mobilization assay and exhibited PAM activity with comparable potency (1.5 µM to 3.2 µM) to the phenoxybenzyl analog VU0400265

(**Fig. 33**). These compounds proved advantageous given our goal of improving the physiochemical properties of VU0400265 (cLogP = 5.8, true logP = 4.6) as lipophilicity was decreased approximately by a full order of magnitude (cLogPs = 4.1-5.1) and the presence of a basic amine afforded HCl salt formation. However, their efficacies (46-62% ACh max) were moderately lower than that of the parent VU0400265 (75% ACh max) compound (**Fig. 33**).
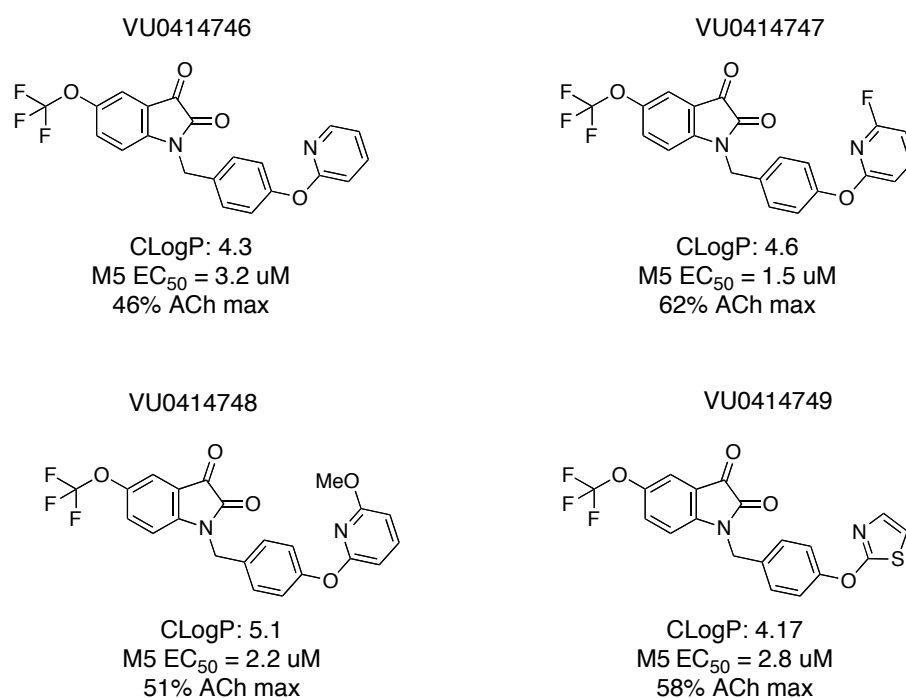


Figure 33. Structures, lipophilicities, and $M_5$ potency/efficacy for four heterobiaryl ether $M_5$ PAMs. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh. Data represent the Mean of at least 3 independent experiments with similar results.

The fluoropyridine ether analog VU0414747 was selected as the most potent and efficacious compound from this small 4-member library for evaluation in ACh CRC fold-shift assays (**Fig. 34**). At 30 μM, VU0414747 induced a modest 6x left-shift of the ACh

CRC and was devoid of intrinsic agonism, similar to the parent VU0400265 (**Fig. 34**).

Although this degree of maximal potentiation is significant, previous analogs bearing

straight biaryl/heterobiaryl southern motifs (i.e. without the ether linkage, such as

VU0365114) exhibited much broader fold-shift values and %ACh max responses at 30

μM (**Fig. 13**).
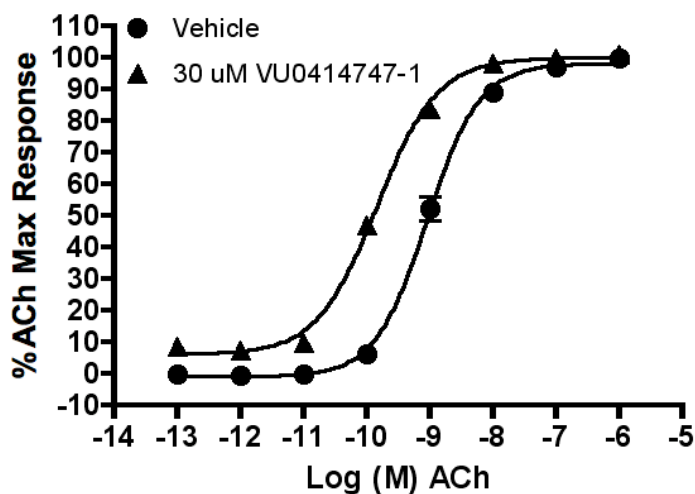


**Figure 34**. $M_5$ ACh CRC foldshift assay with analog VU0414747. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain ACh CRCs in the presence and absence of test compound (6x left-shift). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

Therefore we returned to the straight biaryl/heterobiaryl southern scaffold and

likewise explored introduction of basic amines in the form of un-substituted pyridines in

the proximal and/or distal locations of the linear southern motif (**Fig. 35**). In order to

survey multiple combinations of regio-isomeric pyridines, a matrix-like library approach

was utilized for this synthesis (**Fig. 35**). Standard *N*-alkyation conditions provided the

*para* –Cl or –Br intermediates, which were then subjected to Suzuki-couplings with the

various heterocyclic boronic acids to afford the final products (**Fig. 35**). A similar route

was utilized to obtain VU0415485 as a singleton, which contained a distal 3,5-pyrimidyl

*para* substitution off of a proximal benzyl ring (**Fig. 35**). The final compounds (as well

as the Cl-bearing intermediate VU0414754) were then profiled in $M_5$ CRCs in the

presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization assay to obtain potency and efficacy data

(**Fig. 35**).
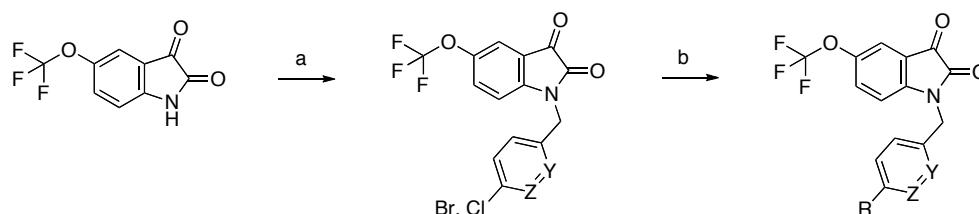


| Compound | Y, Z | R | $M_5$ $EC_{50}$, ACh max |
|----------|------|---|------------------------|
| VU0415483 | CH, CH | 3-pyridyl | 1.6 μM, 51% |
| VU0415484 | CH, CH | 4-pyridyl | 2.8 μM, 60% |
| VU0415485 | CH, CH | 3,5-pyrimidyl | 2.1 μM, 53% |
| VU0414754 | CH, N | Cl | 2.4 μM, 80% |
| VU0415478 | CH, N | phenyl | 3.8 μM, 76% |
| VU0415479 | CH, N | 3-pyridyl | 4.0 μM, 53% |
| VU0415480 | CH, N | 4-pyridyl | 4.4 μM, 50% |
| VU0415481 | N, CH | phenyl | 3.1 μM, 47% |
| VU0415487 | N, CH | 3-pyridyl | >30 μM, n/a |
| VU0415482 | N, CH | 4-pyridyl | >30 μM, n/a |

**Figure 35**. Synthesis and SAR for southern heterobiaryl amine-containing $M_5$ PAM analogs. Reagents and conditions: (a) 4-bromobenzyl bromide, $K_2CO_3$, KI, DMF, rt, 48 h (99%), OR 5-bromo-2-(bromomethyl) pyridine OR 5-(bromomethyl)2-chloropyridine, $K_2CO_3$, KI, DMF, rt, 48 h (98%); (b) Het-B(OH)$_2$, 5 mol % Pd(PPh$_3$)$_4$, 1.0 M aq. $Cs_2CO_3$, THF, mw, 120 °C, 20 min (5-18%). $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$) concentration of ACh. Data represent the Mean of at least 3 independent experiments with similar results.

This effort produced a number of moderately-highly potent $M_5$ PAM compounds with a wide range of efficacies (**Fig. 35**). Importantly, these SAR data suggested that 3-pyridyl in the proximal ring location was uniformly favored over 2-pyridyl in the same position, as the former exhibited compounds with the best balance of potency/efficacy (of this library) while the latter was virtually inactive regardless of the distal ring substitution present (**Fig. 35**). This provided success similar to that of the heterobiaryl ether analog library in that $M_5$ PAM potency/efficacy was generally retained while cLogP values were simultaneously reduced by 1-1.5 orders of magnitude (true logP for VU0415478 = 3.6), and likewise providing the ability to formulate HCl salts.

Furthermore, the *p*-phenyl substituted 3-pyridyl analog VU0415478 exhibited a robust 14x left-shift of an $M_5$ ACh CRC at 30 μM in $Ca^{2+}$ mobilization assays and displayed modest intrinsic agonist activity, which agreed well with previous SAR suggesting that a linear biaryl/heterobiaryl southern moiety confers greater ACh CRC fold-shift (and associated % ACh max values in CRC assays) than related analogs containing a biaryl/heterobiaryl ether (**Fig. 36**).
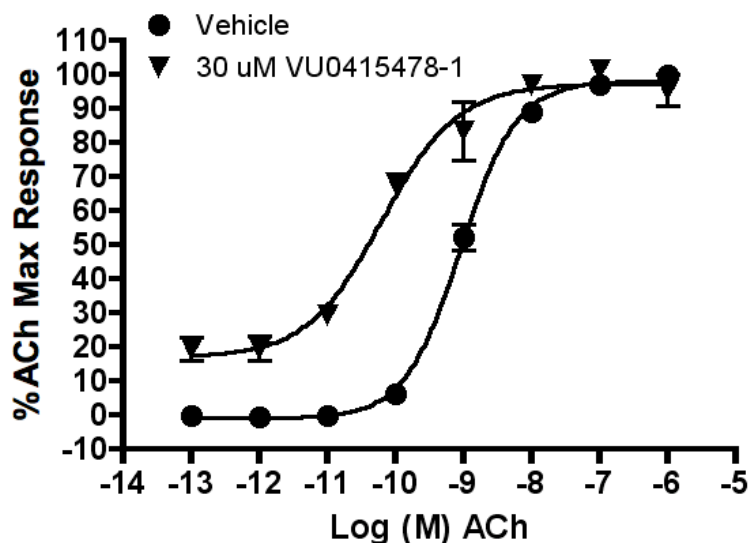
**Figure 36**. M$_5$ ACh CRC fold-shift assay with analog VU0415478. Ca$^{2+}$ mobilization assays with M$_5$ cells were used to obtain ACh CRCs in the presence and absence of test compound (14x left-shift). Data represent the Mean ±S.E.M. of at least 3 independent experiments with similar results.

In light of the well-tolerated 3-pyridyl replacement for the southern benzyl ring, which increased polarity and thereby decreased lipophilicity for the heterobiaryl sub-series of compounds, we hypothesized that this nitrogen substitution would be similarly tolerated in the original M$_5$ PAM lead VU0238429 structure bearing a southern *p*-methoxy moiety. Thus the 3-pyridyl congener was synthesized via one-step standard microwave alkylation conditions with the commercially available chloromethyl-methoxypyridine and with the 5-trifluromethoxyisatin starting material as previously described, although the final yield (4%) was very low due to poor recovery from reverse-phase HPLC purification (i.e. not due to poor % conversion of starting material to product) (**Fig. 37**). This compound (VU0415486) exhibited 3.2 μM M$_5$ potency and 47% ACh max efficacy in CRC assays using Ca$^{2+}$ mobilization (**Fig. 37**). Due to the relatively low efficacy, VU0415486 was not further characterized in the form of ACh CRC fold-shift or subtype-selectivity assays was not performed.

VU0415486

M5 EC$_{50}$: 3.2 uM, 47% ACh max
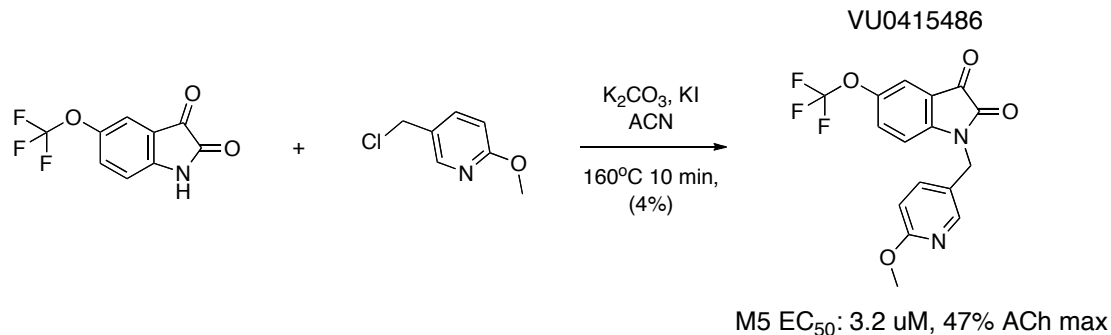
**Figure 37**. Synthesis and M$_5$ potency/efficacy of VU0415486, the 3-pyridyl congener of VU0238429. Ca$^{2+}$ mobilization assays with M$_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~EC$_{20}$) concentration of ACh. Data represent the Mean of at least 3 independent experiments with similar results.

Finally, to more comprehensively explore SAR in the southern region of the M$_5$ PAM scaffold for the purpose of identifying additional compounds with improved physiochemical properties and reduced ancillary/off-target activities, we synthesized a small library of analogs bearing either acidic or basic moieties at R shown in **Figure 38**, as well as the de-methylated phenol congener of the original *p*-methoxybenzyl motif. For each of these analogs, the gem-difluorinated core was used for reasons of convenience related to starting material availability at the time. Starting with the methoxybenzyl-bearing VU0402734, methyl de-protection afforded the phenol (VU0414750), which was used as starting material for subsequent *O*-alkylations (detailed conditions described in Chapter II) with the corresponding alkyl halides to obtain the three compounds of **Figure 38**. Each analog was profiled in M$_5$ CRCs with an ACh ~EC$_{20}$ by Ca$^{2+}$ mobilization assays (**Fig. 38**). However, the ethylpyrrolidine (VU0414751) exhibited a fully efficacious (~100% ACh max) agonist effect at 30 μM despite absence of any potentiation of the ACh ~EC$_{20}$, which suggests possible orthosteric agonism that may be

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conferred by the ACh-like structure of this particular motif (i.e. basic amine with ethyl tether to oxygen). By contrast, the morpholine (VU0414752) and carboxylic acid (VU0414753) analogs lacked both agonism and PAM activity. Interestingly, the phenol (VU0414750) exhibited concentration-dependent inhibition of the ACh $\sim EC_{20}$, suggesting potential NAM or antagonist activity.
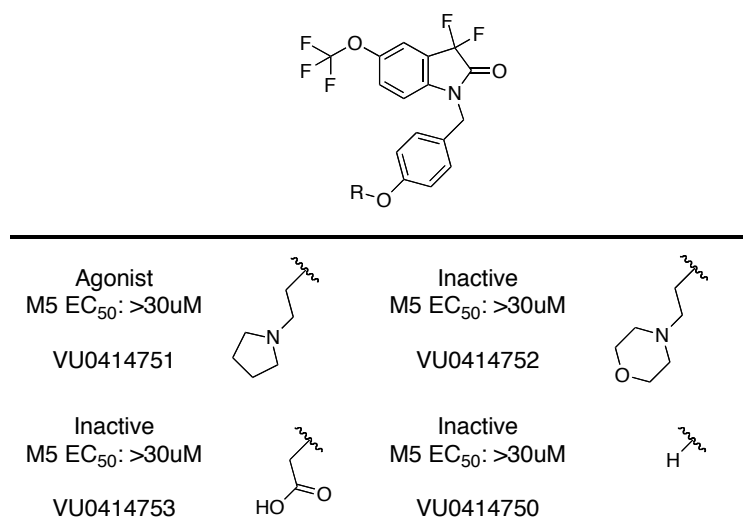


**Figure 38**. Structures and $M_5$ potency/efficacy of acidic and basic moieties in the distal southern region of the $M_5$ PAM scaffold. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal ($\sim EC_{20}$) concentration of ACh. Data represent the Mean of at least 3 independent experiments with similar results.

In order to further investigate the potential NAM activity of VU0414750, similar CRC assays were performed using $Ca^{2+}$ mobilization with $M_5$ cells in the presence of an $\sim EC_{50}$ and $\sim EC_{80}$ of ACh (**Fig. 39**). These experiments revealed similar concentration-dependent inhibition of the ACh response, which is consistent but not necessarily suggestive of true NAM activity for this phenol compound. Due to the greater focus on optimizing $M_5$ PAMs, further examination of this compound in radioligand-binding or

other assays was not performed. However, given these present data and in light of the compound's structure, VU0414750 may represent the first $M_5$ NAM.
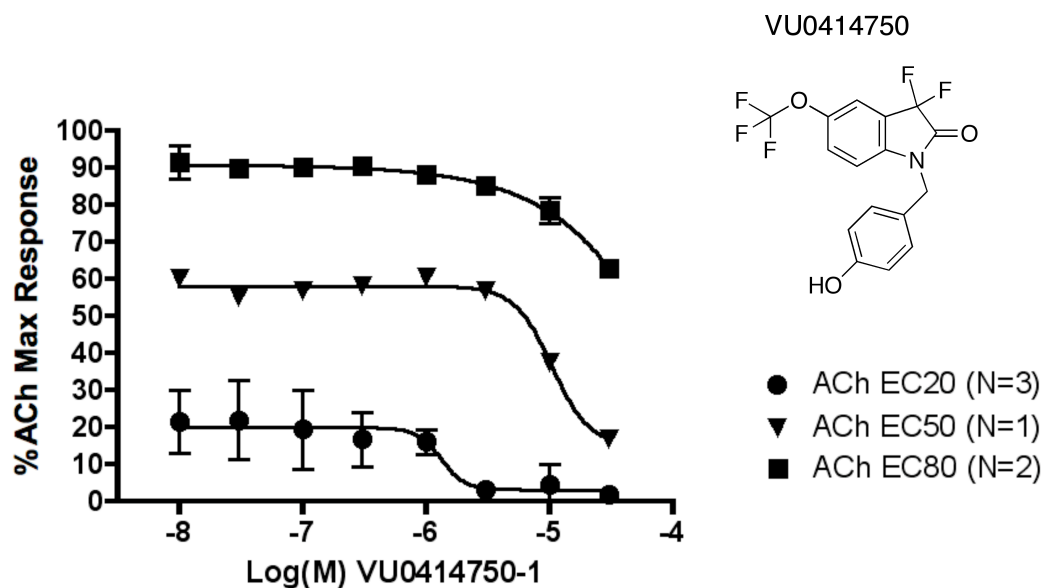
VU0414750



**Figure 39**. $M_5$ ACh inhibition CRCs for VU0414750, a potential $M_5$ NAM. $Ca^{2+}$ mobilization assays with $M_5$ cells were used to obtain compound CRCs in the presence of a fixed submaximal (~$EC_{20}$, ~$EC_{50}$, and ~$EC_{80}$) concentrations of ACh. Data represent the Mean ±S.E.M. or results of 1-3 experiments.

The switch in VU0414750 from methoxy to hydroxy at the *para* benzylic position is relatively modest; however, a growing number of "molecular switches" that confer dramatic changes to the pharmacological mode of modulation of a given scaffold have appeared in recent GPCR allosteric modulator medicinal chemistry literature[133,134]. Given that the methoxy moiety possesses hydrogen-bond accepting properties while the phenol is a hydrogen-bond donor, it is possible that hydrogen-bond mediated attraction or repulsion of a key residue/motif in the $M_5$ receptor allosteric binding site is responsible for switching this PAM scaffold into a NAM scaffold. This hypothesis will require further experimental exploration, as discussed in more detail in Chapter VI.

Having thus surveyed a wide range of modifications to the southern region of the M$_5$ PAM scaffold with the aim of decreasing lipophilicity/cLogP while simultaneously maintaining potency, efficacy, and subtype-selectivity, we re-assessed ancillary off-target activity for VU0414747 and VU0415478. These were chosen because they represented the most potent and efficacious compounds with reduced cLogP values from the most recently discussed heterobiaryl ether and straight heterobiaryl libraries, respectively. Unfortunately, in the same assays described previously for ancillary pharmacology screening (performed by MDS Pharma/Ricerca, **Table 6**), which used a large panel of off-targets in competition binding at 10 μM, both compounds exhibited a wide range of activities (**Table 7**).

**Table 7**.　Ancillary/off-target binding screening results for physiochemically-optimized M$_5$ PAMs VU0414747 and VU0415478 (10 μM). Data represent the Mean of 2 independent experiments with similar results.　Experiments performed by MDS Pharma/Ricerca.
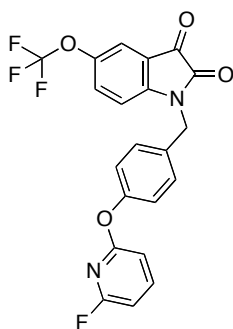


VU0414747

| Target/Protein | Species | % Inhibition |
|---|---|---|
| Adenosine A$_3$ | Human | 75 |
| Adrenergic α$_{1D}$ | Human | 61 |
| Adrenergic α$_{2A}$ | Human | 101 |
| Adrenergic β$_1$ | Human | 70 |
| NE Transporter | Human | 106 |
| Ca$^{2+}$ L-Type Channel | Rat | 68 |
| Cannabinoid CB$_1$ | Human | 93 |

| | | |
|---|---|---|
| Dopamine $D_1$ | Human | 89 |
| Dopamine $D_{2S}$ | Human | 58 |
| Dopamine $D_3$ | Human | 78 |
| Dopamine $D_{4.2}$ | Human | 53 |
| Endothelin $ET_A$ | Human | 79 |
| Histamine $H_1$ | Human | 100 |
| Histamine $H_2$ | Human | 96 |
| Leukotriene $CysLT_1$ | Human | 99 |
| Muscarinic $M_1$ | Human | 51 |
| Muscarinic $M_2$ | Human | 75 |
| Tachykinin $NK_1$ | Human | -72 |
| Opiate $\kappa$ | Human | 61 |
| Opidate $\mu$ | Human | 91 |
| hERG $K^+$ Channel | Human | 69 |
| Serotonin 5-$HT_{2B}$ | Human | 56 |
| $Na^+$ Channel Site-2 | Rat | 64 |
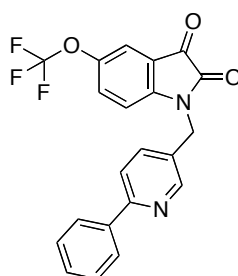


VU0415478

| Target/Protein | Species | % Inhibition |
|---|---|---|
| Adenosine $A_3$ | Human | 73 |
| Adrenergic $\alpha_{1D}$ | Human | 76 |
| Adrenergic $\alpha_{2A}$ | Human | 97 |
| Adrenergic $\beta_1$ | Human | 59 |
| NE Transporter | Human | 106 |
| Bradykinin $B_2$ | Human | -53 |
| $Ca^{2+}$ L-Type Channel | Rat | 56 |
| Cannabinoid $CB_1$ | Human | 70 |
| Dopamine $D_1$ | Human | 88 |
| Dopamine $D_{2S}$ | Human | 66 |
| Dopamine $D_3$ | Human | 87 |
| Endothelin $ET_A$ | Human | 79 |
| GABA Transporter | Rat | 57 |
| Histamine $H_1$ | Human | 88 |
| Histamine $H_2$ | Human | 92 |
| Leukotriene $CysLT_1$ | Human | 86 |

| Muscarinic $M_2$ | Human | 78 |
|---|---|---|
| Opidate $\mu$ | Human | 90 |
| hERG $K^+$ Channel | Human | 63 |
| $Na^+$ Channel Site-2 | Rat | 51 |

Despite the substantially reduced cLogP values for these analogs versus earlier leads within the series, broad off-target activity was observed (**Table 7**), which suggests, in light of the previous ancillary pharmacology SAR shown in **Table 6**, that the southern heterobiaryl moiety (with or without an internal ether linkage) engenders promiscuous off-target binding. These results, and the dopamine $D_1$ and $D_2$ receptor binding in particular, were surprising and present an impediment to utilization of these two advanced lead compounds in $M_5$-related neurobiology studies. Nonetheless, both compounds (VU0414747 and VU0415478) and their parent libraries revealed useful SAR and may serve as starting points for a more exhaustive optimization effort to survey a larger range of heterobiaryl and heterobiaryl substitutions that may be devoid of some or ideally all of these off-target liabilities (discussed in more detail in Chapter VI).

### *In vitro* characterization of $M_5$ PAMs at rat $M_5$ receptor

For all *in vitro* $M_5$ assays performed throughout this optimization, cell lines expressing the human receptor were used. Because species-specific differences in PAM activity have been found with other mAChR allosteric modulators (e.g. $M_4$ PAMs described in Chapter III)[126], and because future $M_5$ electrophysiology experiments and *in vivo* studies will be performed using rats, we determined the effect of early lead compound VU0238429 at the rat $M_5$ receptor. Increasing fixed concentrations of

VU0238429 resulted in concentration-dependent left-shifting of an ACh CRC in rM$_5$ cells by Ca$^{2+}$ mobilization assay (**Fig. 40**), with a maximum fold-shift of approximately 30x at the top concentration (30 μM).
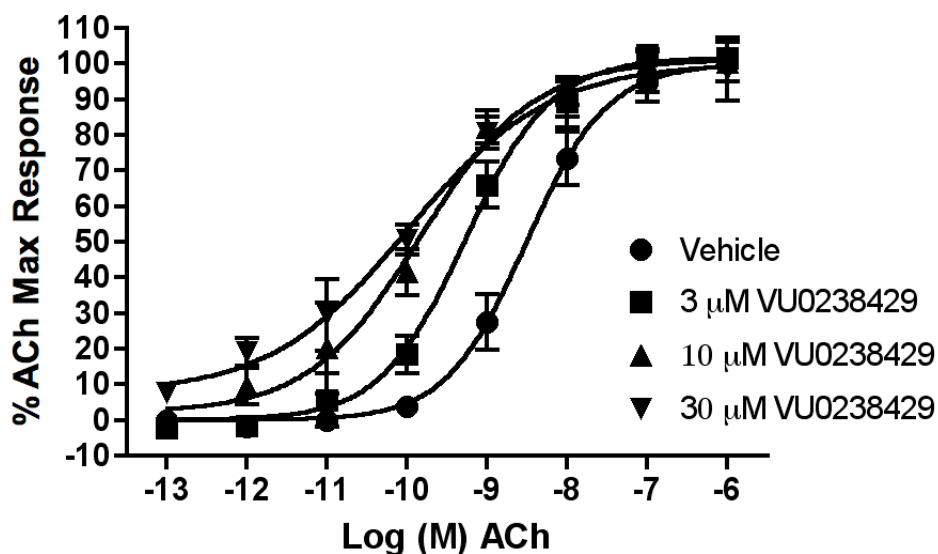


**Figure 40**. Rat M$_5$ ACh CRC fold-shift assay with early lead compound VU0238429. Ca$^{2+}$ mobilization was used to obtain CRCs of ACh in the absence and presence of increasing fixed concentrations of test compound (30 μM maximum fold-shift = 30x). Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by Dan Foster.

These data suggest that this novel series of M$_5$ PAMs is not only active at the rat M$_5$ receptor but may also be more efficacious, as the maximum fold-shift observed for VU0238429 at hM$_5$ was 14x. Further examination of VU0238429 and other lead compounds in rM$_5$ assays, including CRCs to determine potencies, will be required to confirm this possibility. Likewise, subtype-selectivity across all rat mAChRs will be important to confirm prior to initiation of neurobiology experiments. Nonetheless, it was gratifying to find that VU0238429 was active in this rM$_5$ PAM experiment.

It is possible that differences in receptor expression/reserve between our rat and human mAChR cell lines could influence the relative activity of a given PAM, resulting in an illusory greater or lesser activity. In the case of our findings with VU0238429 at $rM_5$ and $hM_5$, it is unlikely that differences in receptor expression/reserve between cell lines have confounded the data interpretation for several reasons. First, $Ca^{2+}$ mobilization readouts under the assay conditions used with our mAChR CHO cells, which have been generated using identical stable transfection techniques, are typically less sensitive to such effects because receptor expression is extremely high (i.e. large reserve) across each cell line. Correspondingly, the potency of ACh in parallel CRC assays using rat versus human $M_5$ cells is essentially identical (0.5-3 nM ACh $EC_{50}$ for both), which further supports the hypothesis that the larger ACh CRC fold-shift observed at $rM_5$ reflects truly comparable or perhaps greater PAM efficacy at the rat versus human receptor.

## Summary

In summary, this first and presently only class of highly subtype-selective $M_5$ positive allosteric modulators was discovered from the optimization of an $M_1$ PAM HTS hit (VU0119498). A large matrix-analog library was generated around VU0119498, which quickly provided SAR that conferred $M_5$-preference. This led to discovery of an early lead compound, VU0238429, which displayed approximately 1 μM $M_5$ PAM potency with >30x subtype-selectivity versus $M_1$-$M_4$ by *in vitro* $Ca^{2+}$ mobilization assay. This compound was found to potentiate ACh activation of $M_5$ via allosteric enhancement of agonist affinity in radioligand binding studies using $M_5$ cell membranes and induced

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an approximately 14x left-shift of an ACh CRC in $M_5$ cells by $Ca^{2+}$ mobilization. However, VU0238429 possessed relatively poor *in vivo* PK properties and required further optimization to confer more drug-like characteristics. Ultimately, a number of subsequent lead compounds were discovered, which maintained low micromolar to sub-micromolar $M_5$ potency and high subtype-selectivity but with improved DMPK and physiochemical properties. However, problematic ancillary off-target binding for many of these compounds will require that future neurobiological experiments be carefully planned and controlled (such as evaluation of the $M_5$ PAM alone in the absence of agonist). In initial experiments using the rat $M_5$ receptor, this series of PAMs retained equal or possibly greater ACh CRC fold-shift efficacy than at human $M_5$, suggesting that these compounds are suitable for future rat neurobiology studies as they do not exhibit an undesirably loss of activity between the two species.

At present, these novel compounds represent the only tools available to the mAChR research field to pharmacologically investigate the role(s) of selective $M_5$ activation in native/WT neurobiological systems. Furthermore, this optimization effort represents part of the unprecedented finding that selective $M_5$ or $M_1$ (as discussed in Chapter III) PAM activity can be engendered via structural modification of the same lead compound displaying multiple mAChR PAM activities (VU0119498). Future experiments and potential optimization efforts are presented in detail in Chapter VI.

**Chapter V**

**DISCOVERY, OPTIMIZATION, AND CHARACTERIZATION OF
NOVEL SUBTYPE-SELECTIVE MACHR1 LIGANDS**

**$M_1$ PAMs derived from VU0119498**

Based on the successful discovery of numerous $M_5$-preferring and selective PAMs from SAR exploration and optimization of the $M_1/M_3/M_5$ PAM HTS hit VU0119498 (Chapter IV), we hypothesized that this *N*-benzyl isatin scaffold could likewise be used as a starting point for discovery of novel $M_1$-preferring or selective PAMs[135]. The broad SAR observed, for example in the initial 92-member matrix library, which explored the effect of various simple substitutions to the isatin core and southern benzyl moiety, as well as the structural similarity between VU0119498 and the Merck $M_1$ PAM series based on BQCA (discussed later in this Chapter), provided further rationale for this hypothesis.

Our initial optimization strategy is outlined in **Figure 1**, and as SAR with allosteric ligands is often shallow, we employed an iterative parallel synthesis approach. From our $M_5$ PAM optimization work where we often counter-screened on $M_1$, we quickly learned that most substitutions (with the exception of 5-$OCF_3$) on the isatin ring led to either loss of activity or pan-mAChR activation profiles with various degrees of potency, efficacy, and subtype-selectivity (Chapter IV). Thus, our first libraries employed a naked isatin core and surveyed diversity on the southern benzyl moiety. Modification of chain length from benzyl to phenethyl linkage as well as

biaryl/heterobiaryl extension in the para position was performed based primarily on SAR

findings from the M5 PAM series (Chapter IV).
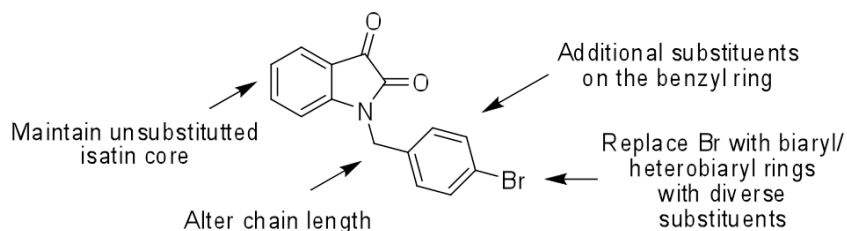


**Figure 1**. Initial optimization strategy for VU0119498 aimed at discovering modifications that engender increased $M_1$ PAM activity and subtype-selectivity.


Libraries were prepared according to the scheme shown in Figure 2, wherein

commercial indoline-2,3-dione (compound **7**) was alkylated with p-bromobenzylbromide

to deliver key intermediate (compound **8**). A 10-member Suzuki library was then

prepared to explore the effect of introduction of biaryl and heterobiaryl motifs into

VU0119498 providing analogs (compounds **9**). In parallel, the isatin starting material

(compound 7) was also alkylated with functionalized phenethyl bromides (compounds

10) to probe the effect of chain homologation (compound **11**).
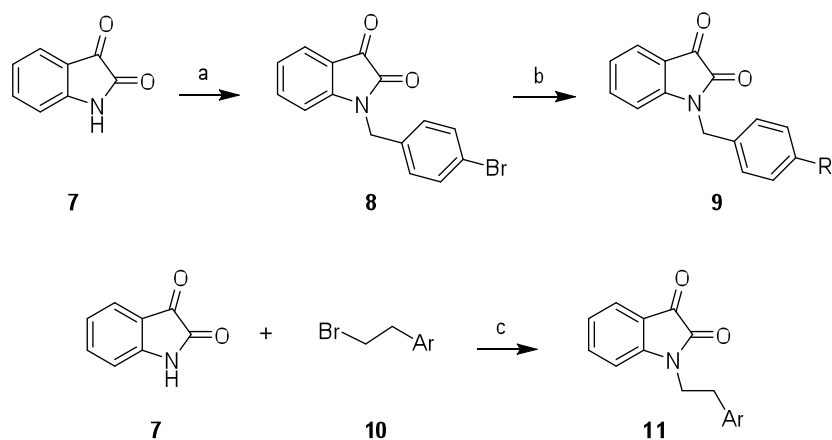
**Figure 2**. Synthesis of initial biaryl/heterobiaryl library based on VU0119498. Reagents and conditions: a) *p*-bromobenzylbromide, $K_2CO_3$, KI, ACN, rt, 16 h (97%); b) RB(OH)$_2$, Pd(P*t*-Bu$_3$)$_2$, $Cs_2CO_3$, THF:H$_2$O, mw, 120°C, 20 min (15-90%); c) $K_2CO_3$, KI, ACN, rt, 16 h (50-90%). B(OH)$_2$ reactants used to generate 10-member parallel library in b): 2,5-difluorophenyl, 6-fluoropyridin-3-yl, 4-methoxyphenyl, pyrazole, *N*-methylpyrazole, 3,4-dichlorphenyl, 2-methoxyphenyl, 6-chloropyridin-3-yl, 4-propoxypheyl, and 4-chlorophenyl. Br-ethyl-Ar reactants used in c): 4-methoxyphenyl and 4-bromophenyl.

This 12-member (total) library was then triaged by a singlepoint (10 μM) screen that examined the compounds' ability to potentiate an ~EC$_{20}$ concentration of ACh by $Ca^{2+}$ mobilization assay with M$_1$ and M$_5$ CHO cells (**Fig. 3**). Only one analog (VU0365137, compound **9a**) demonstrated robust M$_1$ versus M$_5$ potentiation. All other analogs exhibited weak M$_1$ potentiation and/or comparable activity at M$_5$, with the exception of VU0365110 (the 2,5-difluorophenyl congener), which exhibited robust dual M$_1$/M$_5$ activity. In follow-up CRC assays at M$_1$, M$_3$, and M$_5$, VU0365137 (compound **9a**), which possessed an *N*-methylpyrazole in the 4-position of the southern benzyl ring, displayed an M$_1$ EC$_{50}$ of 2.3 μM, and high selectivity versus M$_3$ and M$_5$ (**Fig. 4**). Moreover, this analog afforded a ~5-x left-shift of the M$_1$ ACh CRC at 10 μM, and a larger ~14x left-shift at 30 μM, with ~30% intrinsic allosteric agonism by $Ca^{2+}$ mobilization assay.
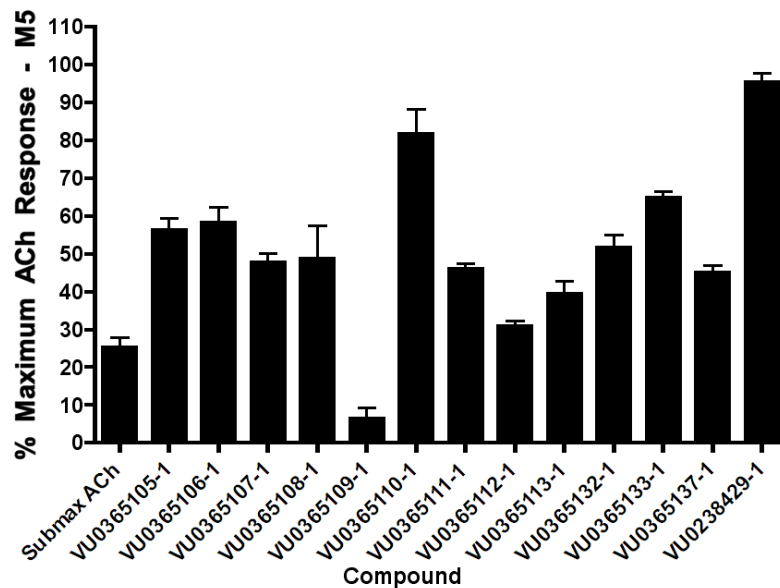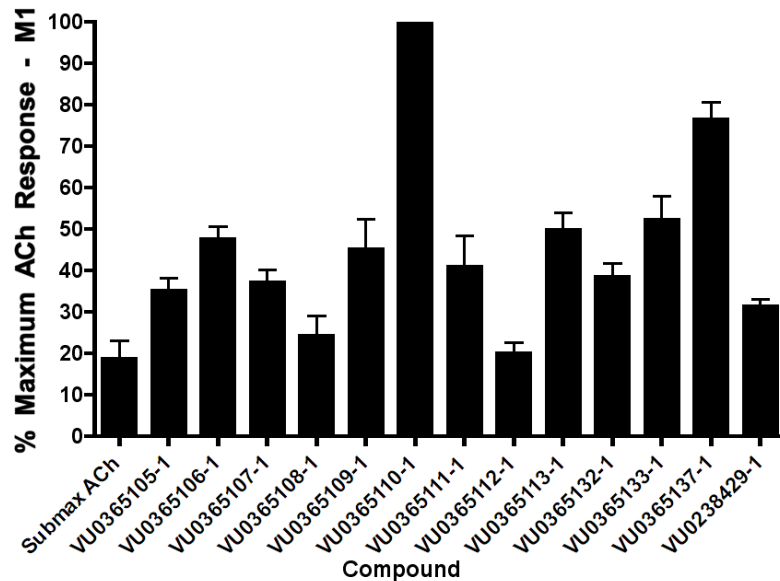
**Figure 3**. Singlepoint (10 μM) $M_1/M_5$ screen of initial 12-member biaryl/heterobiaryl and phenethyl analog library based on VU0119498. $Ca^{2+}$ mobilization assays were performed to obtain % ACh max values induced by each test compound in the presence of an ACh $\sim EC_{20}$ with $M_1$ (top panel) and $M_5$ (bottom panel) CHO cells. Data represent Means +/- SEM from at least three independent determinations.
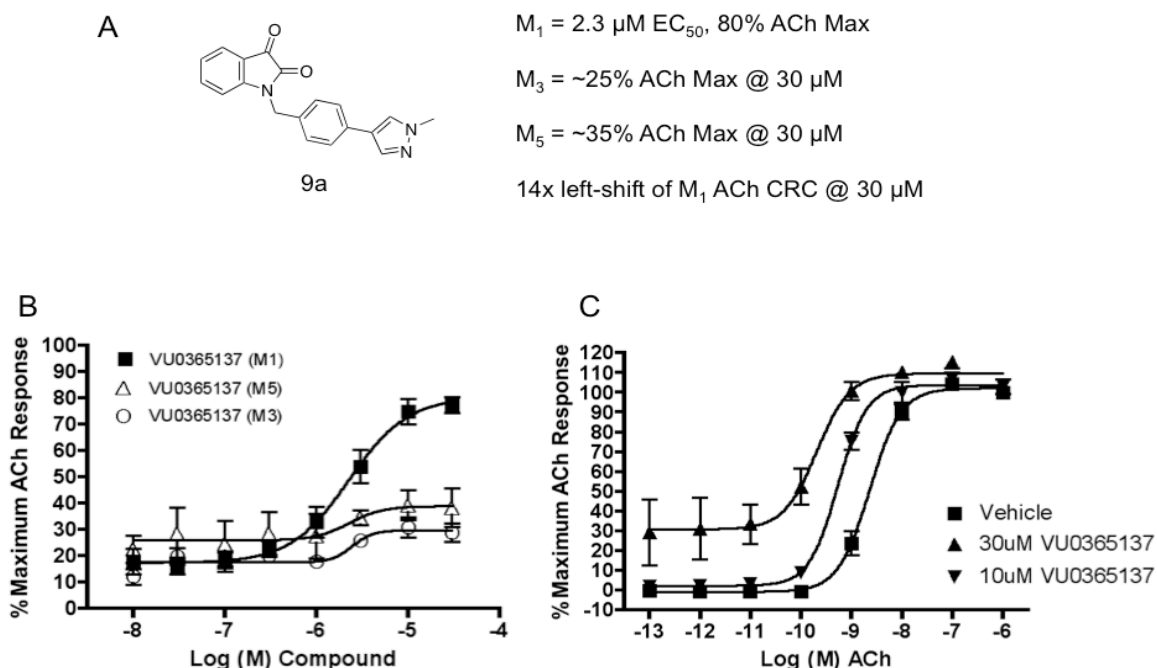
**Figure 4.** A) Structure and PAM activity of VU0365137; B) CRCs for VU0365137 in the presence of a ACh ~$EC_{20}$ at $M_1$, $M_3$ and $M_5$; C) ACh CRC fold-shift assay at $M_5$ with 10 µM and 30 µM VU0365137, providing an ~5x and ~14x left-shift, respectively. Data represent means of at least three independent determinations with similar results using mobilization of intracellular calcium in $M_1$, $M_3$, or $M_5$ CHO cells.

Intriguingly, the 5-$OCF_3$ congener of VU0365137 (compound **9a**) was an equipotent $M_5$-preferring PAM (as reported in Chapter IV), highlighting the 'molecular switch' to engender $M_5$ preference. Despite this shallow SAR, it was exciting to see that we could rapidly obtain an $M_1$-preferring PAM from our initial $M_1$, $M_3$, $M_5$ PAM lead via this small analog library.

We next incorporated subtle changes, in the form of fluorine atoms, to the lead VU0365137 scaffold. The rationale for these modifications stemmed in part from SAR around the Merck $M_1$ PAM BQCA series, which demonstrated that mono- or di-fluoro substitution in the *ortho* positions(s) of the southern benzyl ring and/or on the western aryl ring of the core headpiece conferred increased potency/efficacy (discussed later in

this chapter).  Following the synthetic route outlined previously in **Figure 2**, analogs with

fluorine on both the isatin core and the southern benzyl ring were readily prepared and

evaluated for their ability to potentiate an ~$EC_{20}$ of ACh at $M_1$ via $Ca^{2+}$ mobilization.

This SAR revealed that fluorine substitution was well tolerated on both the isatin

core (4,7-difluoro or 7-fluoro) and on the benzyl ring (2-fluoro and 2,6-difluoro), as

shown in **Table 1**.   The addition of a single fluorine atom to the 2-position of the benzyl

ring delivered VU0366369 with an $M_1$ $EC_{50}$ of 830 nM (65% ACh max) – comparable to

BQCA ($M_1$ $EC_{50}$ = 845 nM) and advantageously without the problematic carboxylic acid

moiety (**Table 1**).  This single change caused an approximately 3-fold increase in potency

over VU0365137.  A 2,6-difluorobenzyl congener (VU0366368) provided equivalent $M_1$

potency with a slightly diminished ACh max response (60%), as shown in **Table 1**.

However, as fluorine content increased (compounds **12c-12e**; fluoro-substitutions on both

the isatin core and benzyl ring) ACh max values decreased (40-55%) despite retention of

comparable potencies (**Table 1**).

**Table 1.** SAR of fluorine-bearing analogs of lead compound VU0365137. $Ca^{2+}$ mobilization assays were used to obtain CRCs for each analog in $M_1$ cells in the presence of an ACh $\sim EC_{20}$. Data represent Mean values from at least three independent determinations.



**12**

| Cmpd | VU Number | Compound | $M_1$ $EC_{50}$ $(\mu M)$ [a] | % ACh Max[a] |
|------|-----------|----------|-------------------------------|--------------|
| **12a** | 0366369 | | 0.83 | 65 |
| **12b** | 0366368 | | 0.86 | 60 |
| **12c** | 0366370 | | 2.3 | 55 |
| **12d** | 0366367 | | 1.1 | 40 |
| **12e** | 0366372 | | 1.2 | 50 |

Gratifyingly, VU0366369 was found to be a highly selective $M_1$ PAM, with minimal/no activation of $M_2$-$M_5$ up to 30 μM (**Fig. 5a-b**). However, in $M_1$ ACh CRC fold-shift experiments, this compound as well as the di-fluoro congener (compound **12b**) displayed only a subtle effect, increasing the potency of ACh by only 3x and 2x

respectively, at 30 μM (**Fig. 5c**). Lack of correlation between PAM potency and fold-shift is commonly observed within series of mAChR allosteric modulators and underscores the importance of determining both parameters when establishing SAR.
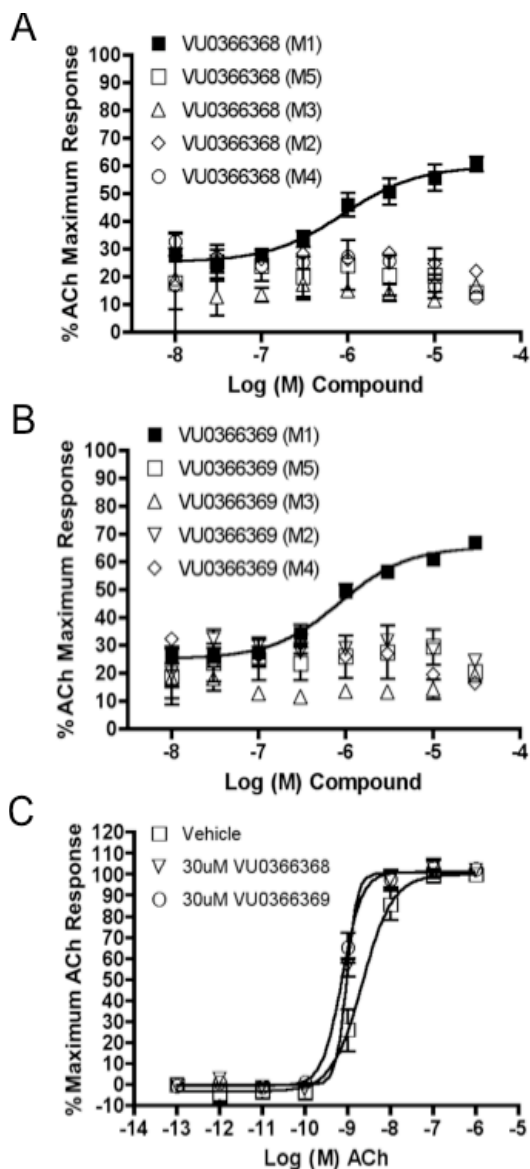


**Figure 5**. A) and B) CRCs for VU0366368 (compound **12b**) and VU0366369 (compound **12a**) in the presence of a ACh ~$EC_{20}$ at $M_1$, $M_2/G_{qi5}$, $M_3$, $M_4/G_{qi5}$ and $M_5$; C) $M_1$ ACh CRC fold-shift assay with 30 μM of VU0366369 and VU0366368, providing an approximately 3- and 2-fold-shift, respectively. Data represent Mean of at least two independent determinations with similar results using mobilization of intracellular calcium in $M_1$, $M_2/G_{qi5}$, $M_3$, $M_4/G_{qi5}$ and $M_5$ CHO cells.

Thus, optimization of the $M_1$, $M_3$, $M_5$ PAM HTS hit VU0119498, which previously led to the discovery of the first selective $M_5$ PAM (VU0400265), provided VU0366369, a highly selective and potent $M_1$ PAM, which represented only the second known $M_1$ PAM chemotype; however, it has modest efficacy. Furthermore, the ability to dial in or out $M_1$ and $M_5$ PAM activity within a single scaffold is unprecedented.

Interestingly, gem-difluorination of the 3-position on the isatin core within this series of $M_1$ PAMs resulted in substantially reduced potency and efficacy (data not shown), in stark contrast with the increased potency, efficacy, and subtype-selectivity conferred by this modification in the related $M_5$ PAM series (Chapter IV). However, replacement of the naked isatin core with a homologous cyclic carbamate ring system within the original VU0365137 $M_1$ PAM lead scaffold using the same alkylation conditions shown in **Figure 2** provided VU0366367. In $Ca^{2+}$ mobilization CRC experiments at $M_1$ in the presence of an ACh $\sim EC_{20}$, this analog exhibited similar potency and efficacy as the isatin-bearing parent, with an $EC_{50}$ of 1.4 μM and 65% ACh max response (**Fig. 6**). This is a highly desirable modification to the scaffold as the cyclic carbamate core is more drug-like and does not carry the DMPK-related liabilities of the isatin core. Further optimization of this cyclic carbamate series in the form of additional southern biaryl/heterobiaryl substitutions is currently underway; however, early data suggest that SAR around this series is relatively flat and no subtype-selectivity is known.
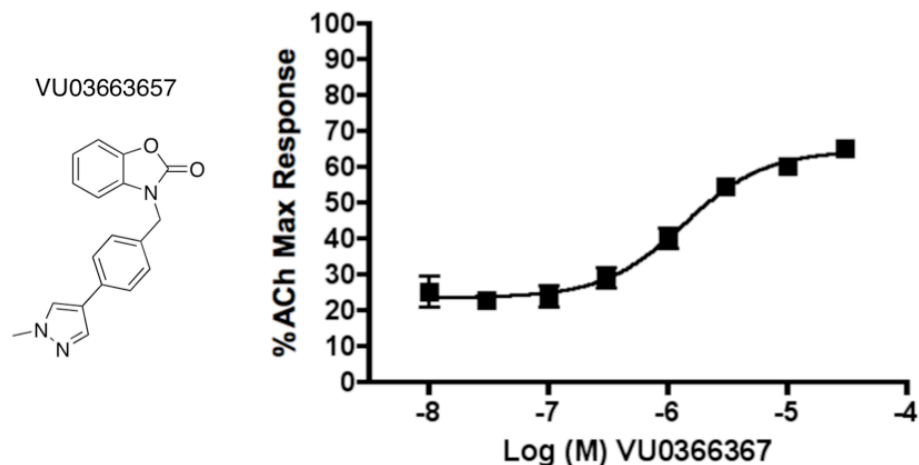
**Figure 6**. Structure and $M_1$ PAM CRC for VU03663657. $Ca^{2+}$ mobilization assays were performed to obtain a CRC of VU0366367 in the presence of an ACh $\sim EC_{20}$ with $M_1$ cells. Data represent Means +/- SEM from at least three independent determinations.

## $M_1$ PAMs VU0028767 and VU0090157

As in Chapter IV, other novel $M_1$ PAMs such as VU0028767 and VU0090157 were identified by same HTS that identified VU0119498 (**Fig. 7**)[27]. Optimization of these compounds was of lesser priority and a limited investigation into SAR around these two hits found extremely shallow SAR. Any change to the VU0028767 structure resulted in a complete loss of $M_1$ PAM activity (data not shown), and resynthesis of the hit itself following HTS identification produced spurious results that may have been due to chemical instability of the structure (**Fig. 7**). For these reasons, further attempts at VU0028767 optimization were not pursued. In a similar vein, SAR revealed by a series of VU0090157 analogs was quite flat; however, replacement of the nitro group on VU0090157 with bromine was tolerated for $M_1$ PAM activity, as was deletion of the southern *N*-methyl moiety (data not shown).

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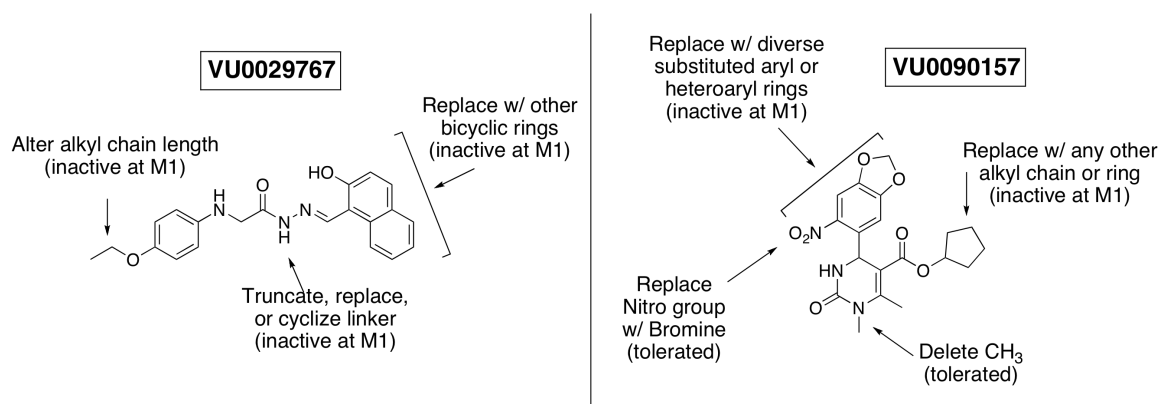
**Figure 7**. SAR summary for $M_1$ PAM HTS hits VU0029767 and VU0090157.

Any changes to the eastern ester side-chain in the form of alternative linear alkyl (e.g. ethyl, propyl, etc…) or cyclo alkyl (e.g. cyclobutyl, cyclohexyl, etc…) substituents resulted in complete loss of $M_1$ PAM activity (data not shown). Likewise a more exhaustive exploration of replacements for the nitro-piperonyl headpiece in the form a diverse 17-member library revealed that modification to this northern region compromises $M_1$ PAM activity (**Fig. 8, Fig. 9**).
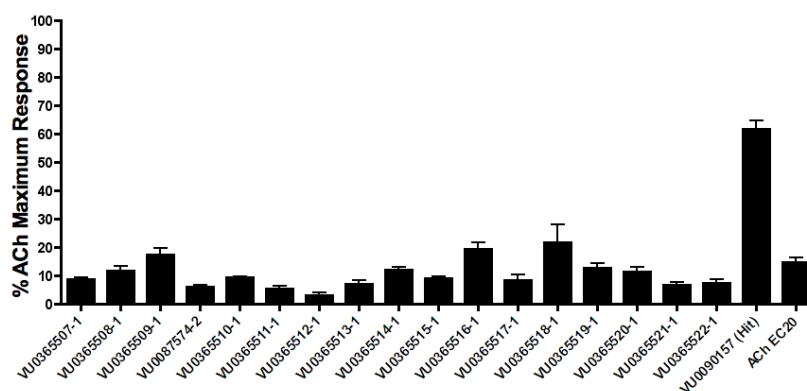


**Figure 8**. Singlepoint (10 μM) $M_1$ PAM screen of VU0090157 analogs bearing diverse northern substitutions. $Ca^{2+}$ mobilization assays with $M_1$ cells were used to obtain % ACh max values for each compound in the presence of an ACh ~$EC_{20}$. Parent hit compound included as positive control. Data represent Mean +/- SEM from at least three independent determinations.
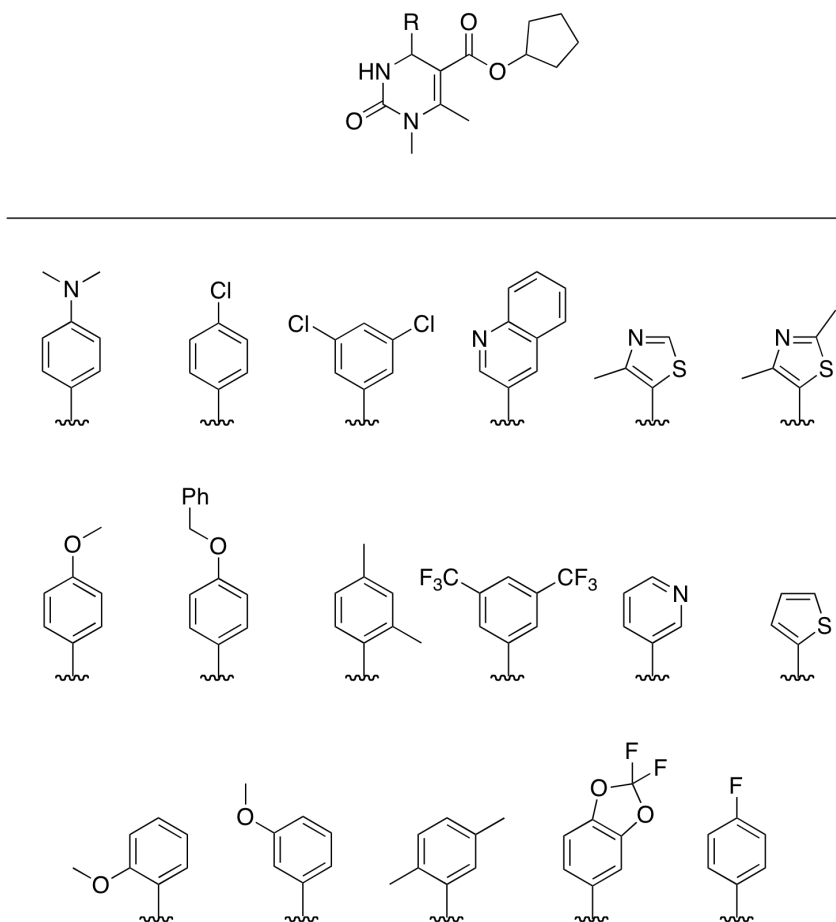
**Figure 9**. Structures of VU0090157 analogs generated to explore SAR at the northern headpiece.

## M₁ PAM BQCA and analogs

Concomitant with discovery of the novel and diverse M$_1$ PAM scaffolds from the VU HTS campaign and the subsequent optimization of the VU0119898 to obtain the series of M$_1$ PAMs (e.g. VU0365137 and VU0366369), Merck disclosed a series of novel M$_1$ PAMs based on a substituted benzyl quinolone carboxylic acid template[136]. The main exemplified compound bore a *p*-methoxybenzyl substitution on the naked quinolone carboxylic acid core and was subsequently termed BQCA[137]. This compound was

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reported to exhibit robust PAM activity at human $M_1$ with an $EC_{50}$ of 845 nM and a 50% ACh max intrinsic agonist activity at 100 μM in $Ca^{2+}$ mobilization assays performed in the presence of a fixed ACh ~$EC_{20}$[137]. In similar assays with human $M_1$ cells, BQCA was reported to induce a 130x left-shift of an ACh CRC at 100 μM[137]. However, GPCR PAMs can display species specificity (as exemplified in the case of the $M_4$ PAM series discussed in Chapter III), and the activity of BQCA at the rat $M_1$ receptor were not initially reported. As discussed in the Introduction (Chapter I), a lack of truly-subtype selective $M_1$ activators with drug-like properties has precluded *in vivo* investigations into the role $M_1$ in both basic neurobiology and in the context of various CNS disorders such as Alzheimer's disease and schizophrenia. Therefore, attainment of an $M_1$ PAM suitable as an *in vivo* tool for use in basic and pre-clinical rodent studies would provide a major advancement to the field.

Thus, in order to determine whether BQCA and/or related compounds have properties needed for use in rodent studies, we resynthesized BQCA and a panel of 20 analogs to identify selective PAMs for the rat $M_1$ receptor[138]. Three libraries were generated each consisting of seven compounds possessing the same *N*-benzyl substitutions on either an 8-fluorinated quinolone carboxylic acid (compounds **Ia-Ig**), a quinolone carboxylic acid (compounds **IIa-IIg**, including BQCA), or a 5,8-difluorinated quinolone carboxylic acid (compounds **IIIa-IIIg**) template, respectively (**Fig. 10**).
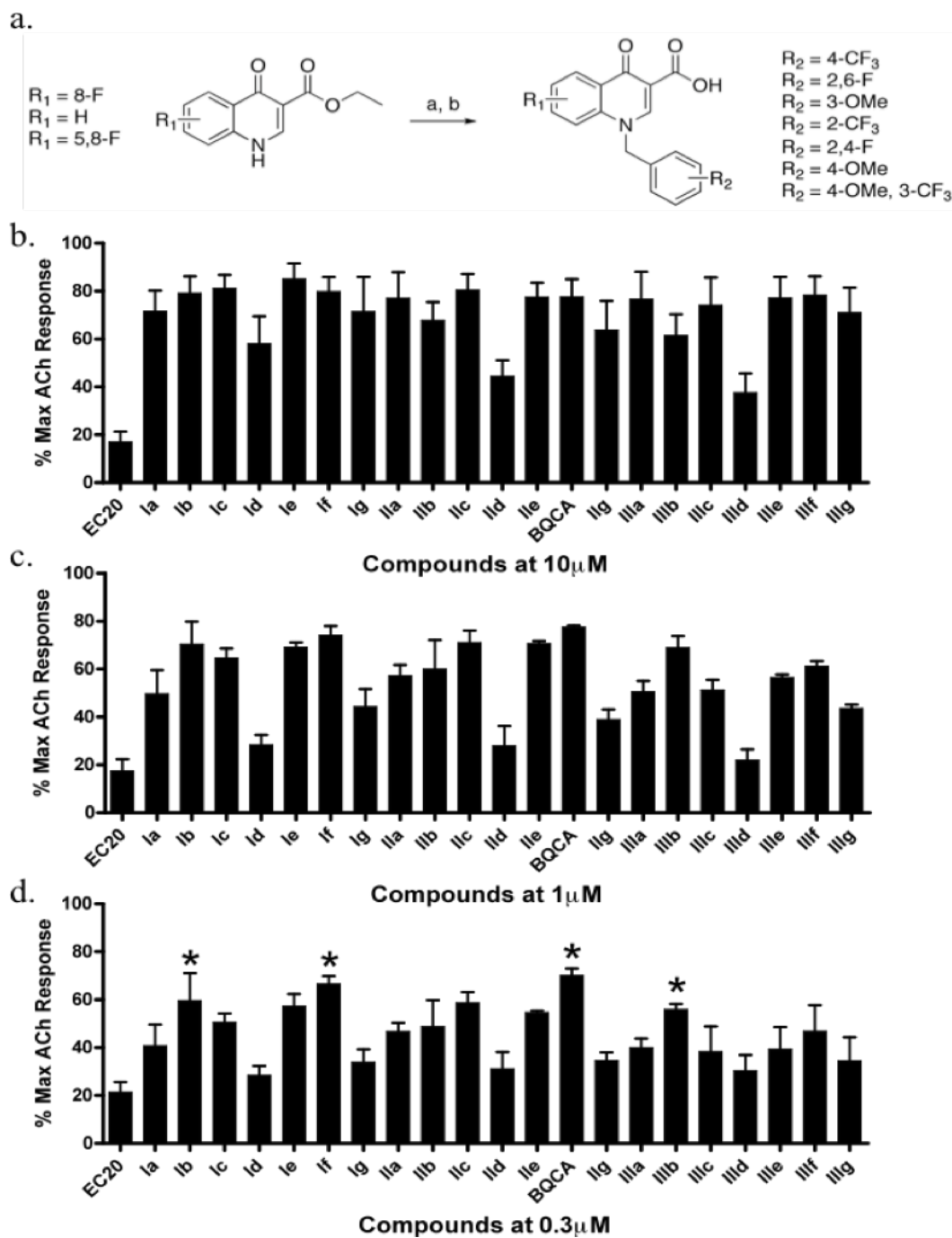
**Figure 10**. Synthesis and screening of BQCA and BQCA analogs at rat $M_1$. a.) reagents and conditions: a. $K_2CO_3$, KI, R-Br, DMF, 24 hr. at r.t., b. LiOH, 9:1 $THF:H_2O$ 10 min. 120 °C µwave. $Ca^{2+}$ mobilization assays with rat $M_1$ cells were used to obtain singlepoint % ACh max values for each compound at b.) 10 µM, c.) 1 µM, or d.) 0.3 µM in the presence of an ACh $\sim EC_{20}$. Four compounds, denoted by an asterisk (*), were selected for further evaluation based on their structural diversity and efficacy at 0.3 µM. Data represent the mean ± S.E.M. of 3 independent determinations. Experiments performed by A. Brady.

The activity of BQCA and related compounds was evaluated by measuring effects on $Ca^{2+}$ mobilization of each compound at 10, 1, or 0.3 $\mu$M (**Fig. 10b-d**) prior to the addition of an ~$EC_{20}$ concentration of ACh. From the panel, four compounds (one of which was parent BQCA) that exhibited robust potentiator activity at 0.3 $\mu$M were selected for further evaluation based on their structural diversity (**Fig. 10d**). All four compounds had similar potencies at the $rM_1$ receptor, with $EC_{50}$ values in the 200-400 nM range as determined by CRCs of each compound in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization assay (**Fig. 11a**). Each of these four compounds elicited a robust increase in ACh potency at rat $M_1$, as manifest by 10-23x left-shifts of an ACh CRC when applied at 3 $\mu$M in $Ca^{2+}$ mobilization assays (**Fig. 11b**)
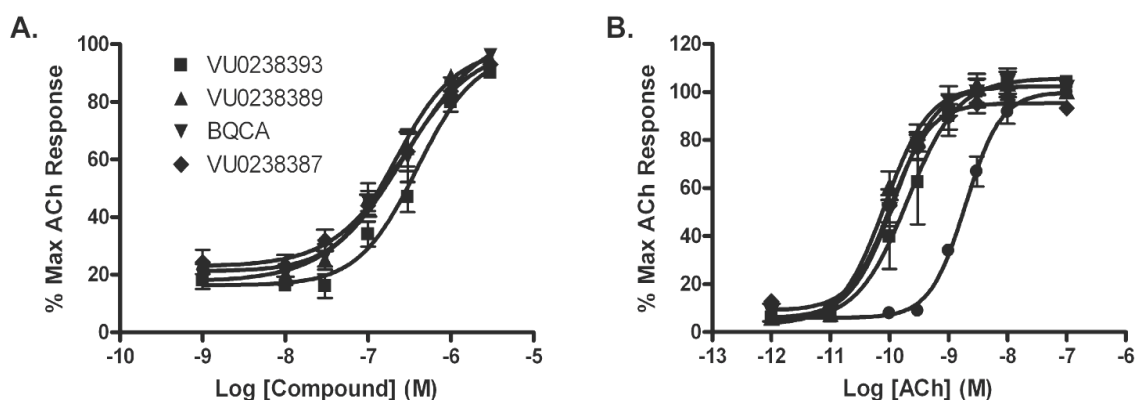


**Figure 11**. a) CRCs of BQCA and three analogs in the presence of an ACh ~$EC_{20}$ at rat $M_1$ in $Ca^{2+}$ mobilization assays ($EC_{50}$ values: VU0238389 = 187 ± 39 nM; VU0238393 = 404 ± 59 nM; BQCA = 316 ± 102 nM; and VU0238387 = 284 ± 32 nM. b.) $Ca^{2+}$ mobilization was used to obtain ACh CRCs in the absence and presence of each compound at 3 $\mu$M (fold-shifts: VU0238389 = 23x, VU0238393 = 10x, BQCA = 16x, and VU0238387 = 19x). Data represent the Mean ± S.E.M. of 3 independent determinations. Experiments performed by A. Brady.

Following this survey of BQCA analog SAR at rat $M_1$, which found a number of well tolerated southern benzylic substitutions as well as mono- and di-fluoro substitutions to the quinolone core, further *in vitro*, *ex-vivo*, and *in vivo* experiments were performed

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using the parent BQCA compound due to the comparable potency/efficacy across these analogs. In radioligand binding experiments using rat $M_1$ cell membranes, BQCA lacked competition with the orthosteric antagonist [$^3$H]-NMS at up to 10 μM and increased the affinity of ACh for rat $M_1$ by 31x at 3 μM (data not shown). These data provide strong evidence that BQCA potentiates ACh-induced $M_1$ activation via an allosteric enhancement of ACh affinity. Furthermore, BQCA lacked any agonist, PAM, or antagonist activity at a panel of 15 other GPCRs (including $M_2$-$M_5$) in $Ca^{2+}$ mobilization assays performed by Millipore's GPCR Profiler Service™ (data not shown). In a single-dose (10 mg/kg IP) rat PK/PBL study, BQCA exhibited sub-optimal yet satisfactory pharmacokinetics with a brain:plasma AUC ratio of 0.09, a brain $C_{max}$ of 1181 ng/mL at $T_{max}$ 1 hr, and a brain $T_{1/2}$ of 2 hr (data not shown).

Having thus determined that BQCA possessed acceptable rat $M_1$ potency/efficacy, selectivity, and systemic pharmacokinetics rendering it suitable for rodent electrophysiology experiments and behavioral studies, no further optimization or investigation into additional SAR for this series was performed. Nonetheless, these analogs and initial characterization experiments provided crucial evidence and rationale for the utility of BQCA as an acceptable small molecule tool for selective activation of rat $M_1$. A large-scale (30 g) batch of BQCA was prepared according to the scheme in **Figure 10a** as the $Na^+$-salt, which was jet-milled and made available to collaborators in order to facilitate a range of $M_1$ neurobiology studies. Such investigations found that activation of $M_1$ induced a robust inward current and increased spontaneous excitatory post-syanptic currents (EPSCs) in medial pre-frontal cortical (mPFC) pyramidal cells in rodent brain slices, and that BQCA-mediated $M_1$ activation increased firing of mPFC

pyramidal cells and restored discrimination reversal learning in a transgenic mouse model of AD *in vivo*[138].

The BQCA $M_1$ PAM medicinal chemistry work was primarily focused on screening/assays with $M_1$ cells with the exception of subtype-selectivity confirmation at $M_2$-$M_5$ for BQCA itself. Analogs generated in the 20-member panel were not screened at any of the other mAChRs and it remained a question as to whether any structural modifications to the BQCA scaffold could confer PAM activity for subtypes other than $M_1$, similar to SAR findings with the VU0119498 *N*-benzyl isatin series of mAChR PAMs. Moreover, the fact that $M_1$-preferring/selective compounds as well as $M_5$-preferring/selective compounds could be obtained by subtle modification to the VU0119498 structure (Chapter IV), which is itself highly similar to the BQCA scaffold apart from the latter's carboxylic acid moiety, suggested that it may be possible to dial-in BQCA activity at other mAChRs. Specifically, we hypothesized that appending a western –$OCF_3$ group to the 6-position of the BQCA quinolone core would engender $M_5$ PAM activity in a manner analogous to its effect on the VU0119498-derived PAMs (Chapter IV).

To test this hypothesis, two singleton BQCA analogs were synthesized according to the scheme in **Figure 10a**, starting with the commercial 6-trifluoromethoxy quinolone carboxylate, which was then *N*-alkylated with *p*-methoxy and *p*-phenoxy benzyl halides to afford VU0400268 and VU0400269 (**Fig. 12**). These benzyl groups were selected because they represented two of the most favored/optimal southern moieties within the VU0238429-based $M_5$ PAM series. Each compound was then profiled in CRCs at $M_5$ in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization assay (**Fig. 12**). Surprisingly,

essentially no PAM activity was observed at up to 30 μM for either compound, suggesting that the BQCA template is poorly compatible with $M_5$ binding and/or functional activity (**Fig. 12**).
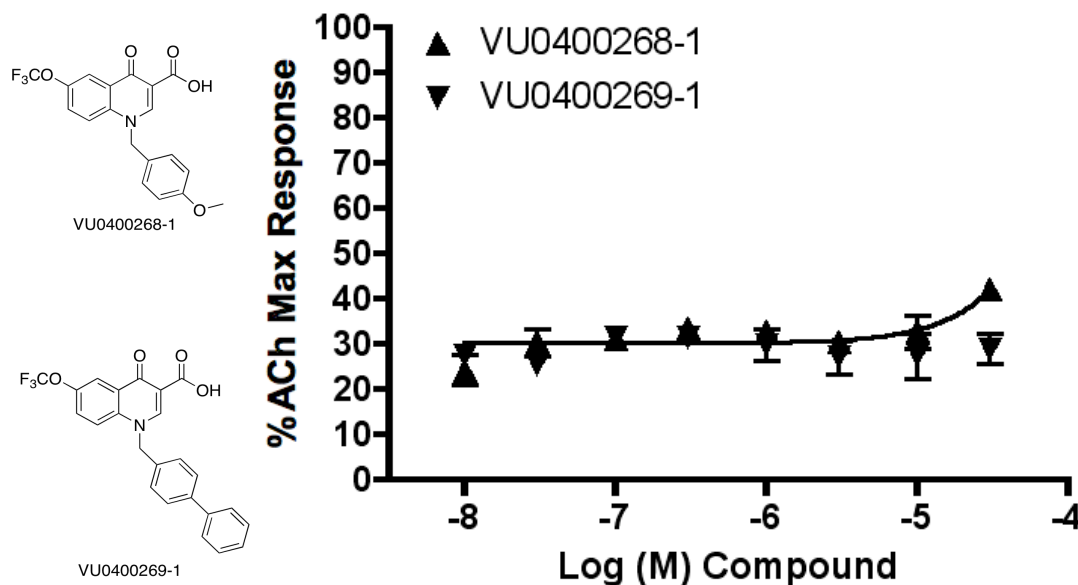


**Figure 12**.  Structure and $M_5$ CRCs for BQCA-based analogs VU0400268 and VU0400269.  $Ca^{2+}$ mobilization was used to obtain CRCs of each compound in the presence of an ACh ~$EC_{20}$ using $M_5$ cells. Data represent Mean +/-SEM from at least three independent determinations.


**$M_1$ PAMs derived from MLPCN probe project**


As part of the NIH Roadmap Initiative, the MLSCN/MLPCN comprises a nationwide consortium of small molecule screening and medicinal chemistry centers aimed at discovering and optimizing novel small molecules for molecular targets that lack known and/or acceptable tool compounds by which to study their role(s) in biology and disease.  Given the  scarcity of selective mAChR small molecules possessing drug-

like characteristics (Chapter I), an HTS of the then 65,000-member MLSCN/MLPCN compound collection was performed to identify novel $M_1$ ligands. This screen was performed while the mAChR PAM projects (Chapters III-V) were underway and prior to most of the associated publications/disclosures arising from our $M_1$ PAM discovery and optimization efforts. Utilizing a functional cell-based fluorescence assay that measured $Ca^{2+}$ mobilization in rat $M_1$ CHO cells during first addition of a test compound followed by an ACh or CCh ~$EC_{80}$, this screen was originally designed to identify $M_1$ antagonist hits. Indeed, a number of novel $M_1$ antagonist series were obtained[139-141], which ultimately led to the discovery of the highly $M_1$-selective antagonist probe compound VU0255035 (discussed later in this Chapter)[140].

Following the HTS and subsequent $M_1$ antagonist medicinal chemistry work, the screening data were re-analyzed to look for compounds that elevated the ACh ~$EC_{80}$. Although this was not an ideal assay format for identification of PAMs, the data mining proved fruitful as approximately 200 compounds flagged as possible potentiators. These putative $M_1$ PAM hits were re-ordered from the commercial vendor and then screened in a singlepoint 30 μM PAM assay in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization in $M_1$ cells (**Fig. 13**). From this screen, only two compounds exhibited robust potentiation activity (VU0165202 and VU0108370) and were thus carried forward to further characterization (**Fig. 13**). Interestingly, both hits were based on the same benzyl-substituted indole sulfonyl amide bearing a distal methyloxazole and differed only a single halogen substitution on the southern benzyl ring (**Fig. 13**).
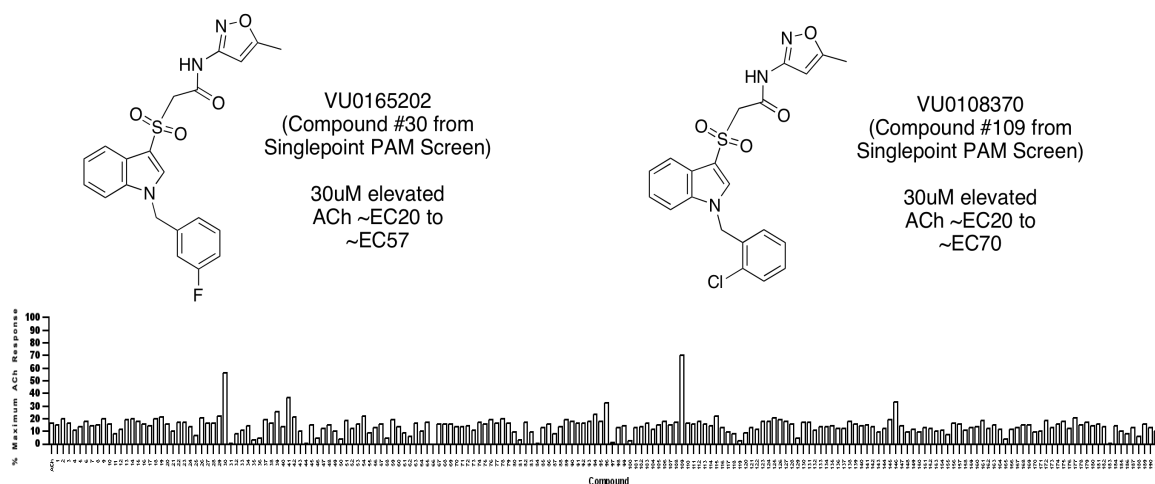
**Figure 13**. Structure of two $M_1$ PAM hits identified by 30 μM singlepoint screen of approximately 200 re-ordered putative PAMs identified from a functional $M_1$ antagonist HTS campaign. $Ca^{2+}$ mobilization was used to obtain % ACh max values for each test compound at 30 μM in the presence of an ACh ~$EC_{20}$ using $M_1$ cells. Data represent a single determination performed in triplicate.

In follow-up CRCs of each compound at $M_1$ in the presence of an ACh ~$EC_{20}$, the 3-fluoro VU0165202 and the 2-chloro VU0108370 exhibited $EC_{50}$ values of 5.5 μM and >10 μM, respectively (**Fig. 14**). These were exciting results as both compounds exhibited clear concentration-dependent potentiation of ACh at $M_1$.
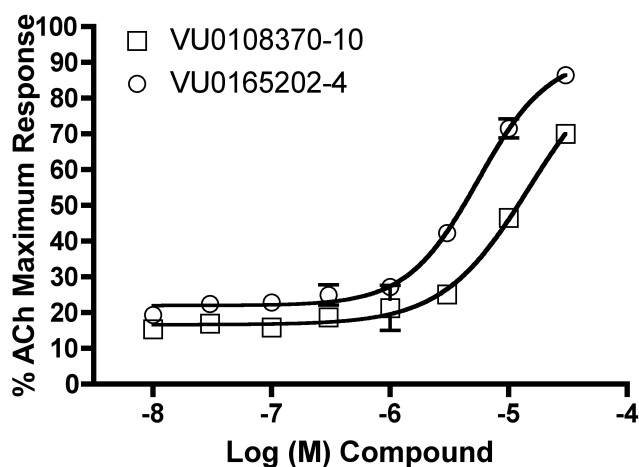


**Figure 14**. $M_1$ CRCs of PAM hits VU0108370 and VU0165202. $Ca^{2+}$ mobilization was used to CRCs for each test compound in the presence of an ACh ~$EC_{20}$ using $M_1$ cells (VU0165202 $EC_{50}$ = 5.5 μM, VU0108370 $EC_{50}$ = >10 μM). Data represent Mean +/-SEM from at least two independent determinations.

Initial optimization efforts focused on a wide range of modifications to the scaffold, including sequential truncation from both the southern and northern termini, as well as a number of diverse benzyl substitutions (**Fig. 15**). A total of 19 analogs as well as parent hit VU0108370 were prepared according to the scheme shown in **Figure 16**, which were then triaged for $M_1$ PAM activity at 10 µM in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization (data not shown).
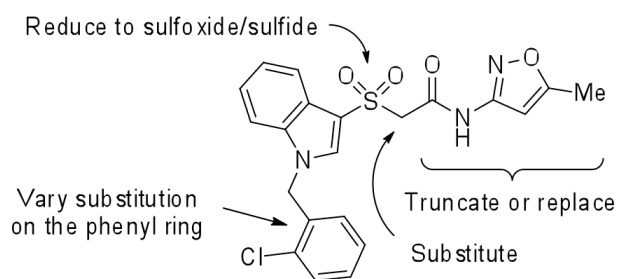


**Figure 15**. Initial optimization strategy for $M_1$ PAM hit VU0108370.
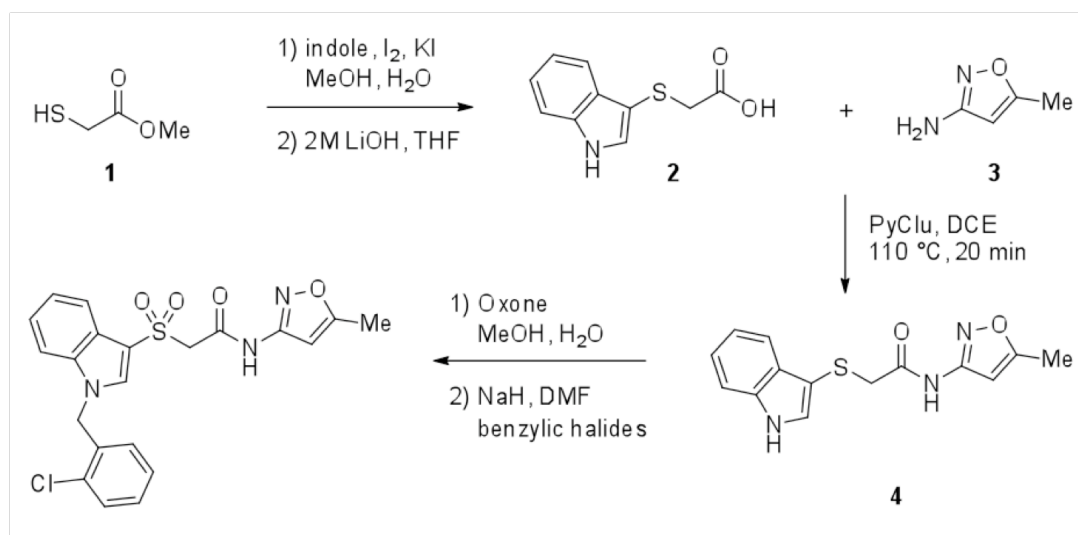


**Figure 16**. Synthesis of $M_1$ PAM hit VU0108370 used to generate southern benzyl-derivatized library. Syntheses performed by P. Reid.

Ultimately, three compounds were chosen for $M_1$ CRC assays to determine potencies (**Fig. 17**). These SAR data revealed that substitution of the *meta* position on the southern benzyl ring was favored, with the *m*-Cl analog specifically exhibiting the highest potency and efficacy (**Fig. 17**).
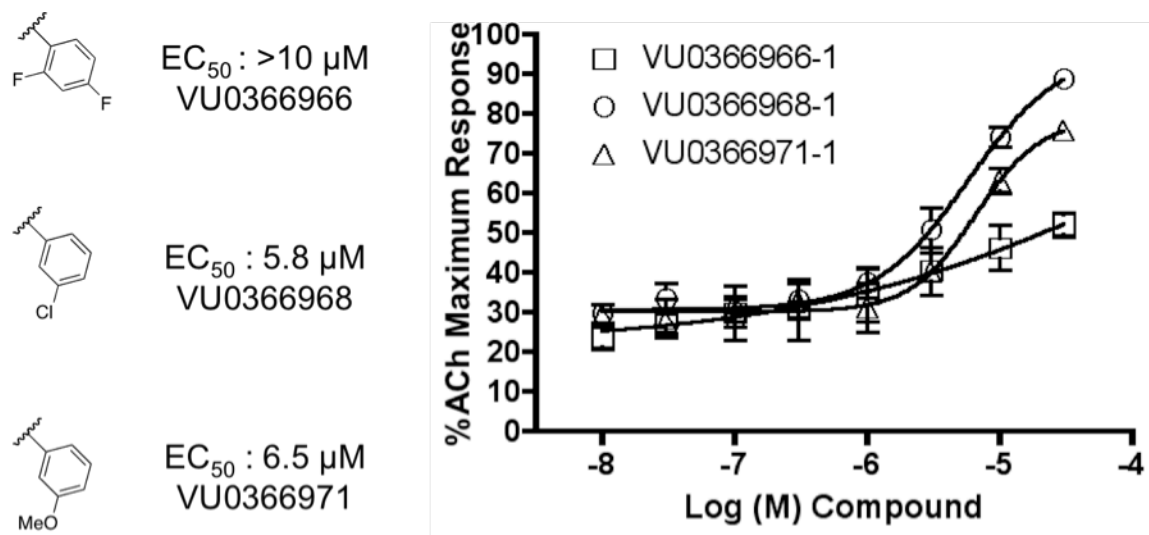


**Figure 17**. Structures and $M_1$ PAM CRCs of southern benzyl analogs based on the VU0108370 / VU0165202 scaffold identified from a larger singlepoint triage assay. $Ca^{2+}$ mobilization was used to obtain CRCs each compound in the presence of an ACh ~$EC_{20}$ using $M_1$ cells. Data represent Mean +/-SEM from at least three independent determinations.

Therefore, additional compounds containing *m*-bromine or *m*-*N*-methylpyrazine substitutions (the latter was chosen based on previous VU0119498-based $M_1$ PAM SAR) on the benzyl ring were synthesized as singletons using the general scheme presented in **Figure 16**. Simultaneously, sulfoxide and thioester congeners of the *m*-bromo compounds were prepared to explore the effect of sequential oxygen deletion from the central linker's sulfone (**Fig. 18**). Each of these compounds were then profiled in CRCs at $M_1$ in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization assay (**Fig. 18**).

Gratifyingly, the *N*-methylpyrazole (VU0402606, $EC_{50}$ = 3.2 μM) increased potency by approximately ~2x over the chlorine-bearing lead (VU0366368, $EC_{50}$ = 5.8 μM) and exhibited a notable 102% maximal ACh response at $M_1$ (**Fig. 18**). The bromine-bearing analog (VU0402604, $EC_{50}$ = 3.8 μM) was only slightly less potent, whereas the sulfoxide and thioether analogs (VU0402605 and VU040260) exhibited moderately weaker potency and inactivity, respectively (**Fig. 18**).
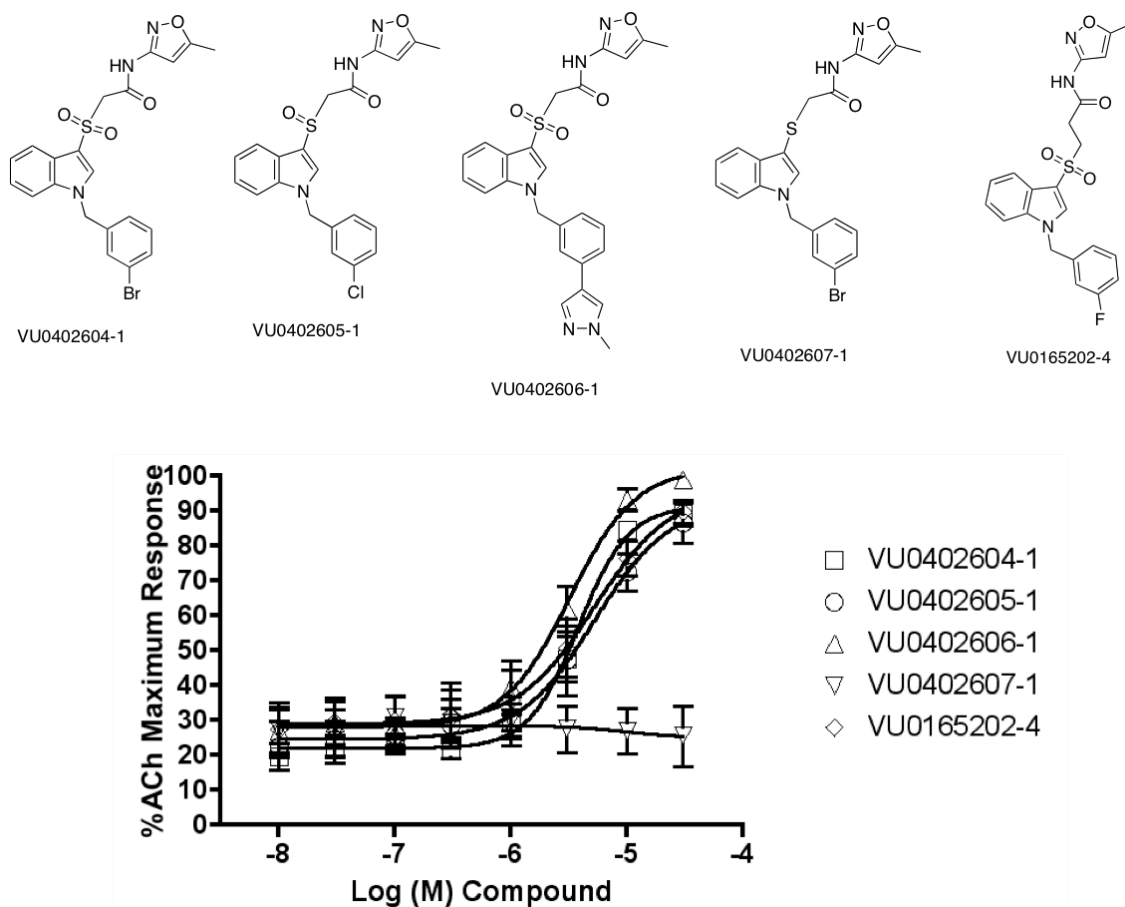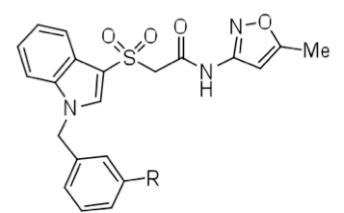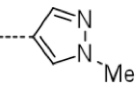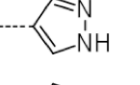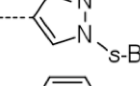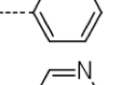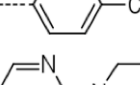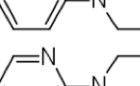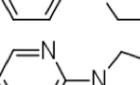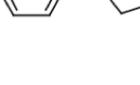


**Figure 18**. Structures and $M_1$ PAM CRCs of *meta*-substituted southern benzyl analogs based on the VU0108370 / VU0165202 scaffold. $Ca^{2+}$ mobilization was used to obtain CRCs each compound in the presence of an ACh ~$EC_{20}$ using $M_1$ cells ($EC_{50}$ values: VU0402604 = 3.8 μM, VU0402605 = 5.2 μM, VU0402606 = 3.2 μM, VU0402607 = >30 μM, VU0165202 included as positive control). Data represent Mean +/-SEM from at least three independent determinations.

These SAR data suggested that, like the VU0119498- and BQCA-related $M_1$ PAMs, biaryl/heterobiaryl southern motifs are favored for increased potency/efficacy. To further explore such substitutions, the *m*-bromo compound was used as starting material for microwave-assisted Suzuki couplings to obtain a small parallel library of biaryl/heterobiaryl analogs using standard conditions. These were then evaluated in CRCs at $M_1$ in the presence of an ACh ~$EC_{20}$ by $Ca^{2+}$ mobilization assay (**Table 2**).

**Table 2.** SAR for 3-position biaryl/heterobiaryl analogs based on VU0402606. $Ca^{2+}$ mobilization was used to obtain compound CRCs in the presence of an ACh ~$EC_{20}$ with $M_1$ cells. Data represent Mean values from at least three independent determinations. Syntheses performed by P. Reid.



| R | $M_1$ $EC_{50}$ (µM) | %ACh Max |
|---|---|---|
| (1-methyl-pyrazol-4-yl) Me | 3.21 | 102 |
| (1H-pyrazol-4-yl) NH | 2.19 | 90 |
| (1-s-Bu-pyrazol-4-yl) s-Bu | >10 | - |
| (phenyl) | >10 | - |
| (chloropyridinyl) Cl | >10 | - |
| (morpholinopyridinyl) N__O | >10 | - |
| (piperazinylpyridinyl) N__NH | >10 | - |
| (pyrrolidinylpyridinyl) N | >10 | - |

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Of these, only the parent *N*-methylpyrazole and its desmethyl congener exhibited robust $M_1$ PAM activity. Interestingly, the deletion of the methyl group conferred a subtle increase in potency but simultaneously reduced efficacy (VU0404010, $EC_{50}$ = 2.2 μM, 90% ACh max). We concluded that the pyrazole would thus be held constant and hypothesized for our next iteration of synthesis that fluorination of the benzyl ring and/or indole core in a manner homologous to that of the VU0119498-derived and BQCA $M_1$ PAMs would confer a modest increase in potency. In order to test this hypothesis, a library of fluorinated analogs was prepared according to general scheme of **Figure 16**. These compounds were then profiled in CRCs at $M_1$ to determine potency (**Table 3**).

**Table 3.** SAR for fluorinated analogs based on VU0404010 lead. $Ca^{2+}$ mobilization was used to obtain compound CRCs in the presence of an ACh $\sim EC_{20}$ with $M_1$ cells. Data represent Mean values from at least three independent determinations. Syntheses performed by P. Reid.
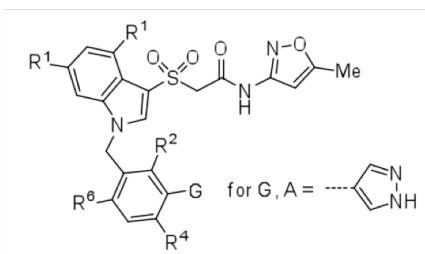


| G | $R^1$ | $R^2$ | $R^4$ | $R^6$ | $M_1$ $EC_{50}$ (μM) | %ACh Max |
|----|----|----|----|----|----|----|
| A | H | H | F | H | > 10 | - |
| Br | H | H | F | H | > 10 | - |
| Cl | H | H | F | H | > 10 | - |
| Br | F | H | H | H | 5.37 | 103 |
| A | F | H | H | H | 1.85 | 102 |
| Cl | H | F | H | H | 5.10 | 56 |
| Br | H | F | H | H | > 10 | - |
| A | H | F | H | H | 4.40 | 103 |
| A | H | H | H | F | 3.07 | 96 |
| Br | H | H | H | F | 1.38 | 84 |

This effort proved fruitful as the 3-bromo,6-fluorobenzyl analog exhibited the highest potency of any compound in this series (VU0405652, $EC_{50}$ = 1.4 µM, **Table 3**). 4,6-difluorination of the indole core in combination with the des-methylpyrazole in the 3-position of the benzyl ring provided a similarly potent analog (VU0405645, $EC_{50}$ = 1.9 µM, **Table 3**). With such potencies, these two new leads had reached the general criteria for an $M_1$ PAM probe compound and were therefore carried into further characterization with a concurrent halt in medicinal chemistry. At 30 µM, both compounds exhibited large (~>45-100x) ACh CRC fold-shifts at rat $M_1$ by $Ca^{2+}$ mobilization assay (**Fig. 19**), which is comparable to the magnitude of potentiation induced by BQCA at human $M_1$.
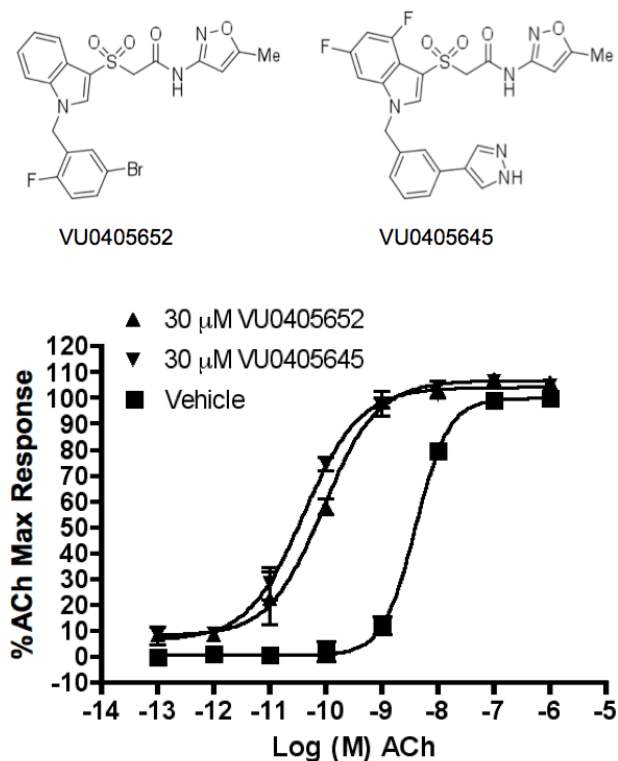


**Figure 19**. Structure and ACh CRC fold-shifts for $M_1$ PAM leads VU0405652 and VU0405645. $Ca^{2+}$ mobilization was used to obtain CRCs for ACh in the absence and presence of 30 µM test compound using $M_1$ cells (VU0405652 = 45x left-shift, VU0405645 = 98x left-shift). Data represent Mean +/- SEM from at least three independent determinations.

Although we had thus obtained potent and efficacious $M_1$ PAMs of a novel HTS-identified drug-like chemotype from this medicinal chemistry effort, subtype-selectivity remained a question for these final lead/probe compounds. Throughout the optimization campaign, both of the parent HTS hits (VU0165202 and VU0108370) as well as various intermediate lead compounds from the project were evaluated in CRCs in the presence of an ACh $\sim EC_{20}$ at $M_3$ and $M_5$ by $Ca^{2+}$ mobilization assays (**Fig. 20**). This was performed due to the higher likelihood that any loss of subtype-selectivity would first manifest at one or both these two off-target mAChRs given the higher structural homology amongst $M_1/M_3/M_5$ and based on the SAR from the other $M_1$ PAM series. None of these compounds exhibited any potentiation at either subtype (**Fig. 20**).
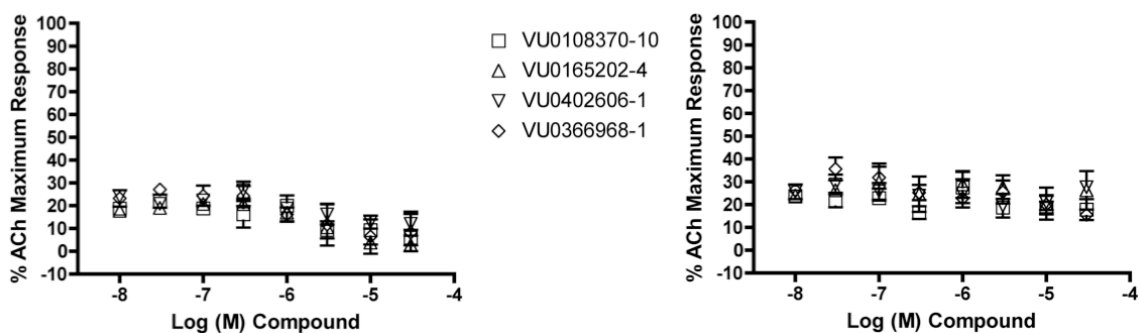


**Figure 20**. $M_3$ and $M_5$ subtype-selectivity CRCs for original $M_1$ PAM HTS hits and select intermediate lead analogs. $Ca^{2+}$ mobilization was used to obtain CRCs for each compound in the presence of an ACh $\sim EC_{20}$ using $M_3$ (left panel) or $M_5$ (right panel) cells. Data represent Mean +/-SEM from at least three independent determinations.

However, to definitively assess full subtype-selectivity for the final probe compound VU0405652, CRC assays at $M_2$-$M_5$ were performed in the presence of an ACh $\sim EC_{20}$ by $Ca^{2+}$ mobilization assay. These experiments revealed complete selectivity for $M_1$ at up to 30 μM, further supporting the conclusion that this chemical series represents a third known chemotype of $M_1$-selective PAMs in addition to the VU0119498- and

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BQCA-derived series (**Fig. 21**). Furthermore, this particular probe (VU0405652) possessed favorable physiochemical and drug-like properties in the form of relatively high aqueous solubility (cLogP = 3.9) and a general lack of obvious DMPK liabilities. Despite their high selectivity for $M_1$ in functional PAM assays, it remains unknown if these novel compounds exhibit true binding specificity for $M_1$ or if they indeed bind to one or more of the other mAChRs but exhibit neutral or negative modulatory effects. Further experiments to address this question as well as a number of *in vitro* and *in vivo* studies aimed at studying $M_1$ neurobiology and therapeutic relevance have been planned and are presently underway.
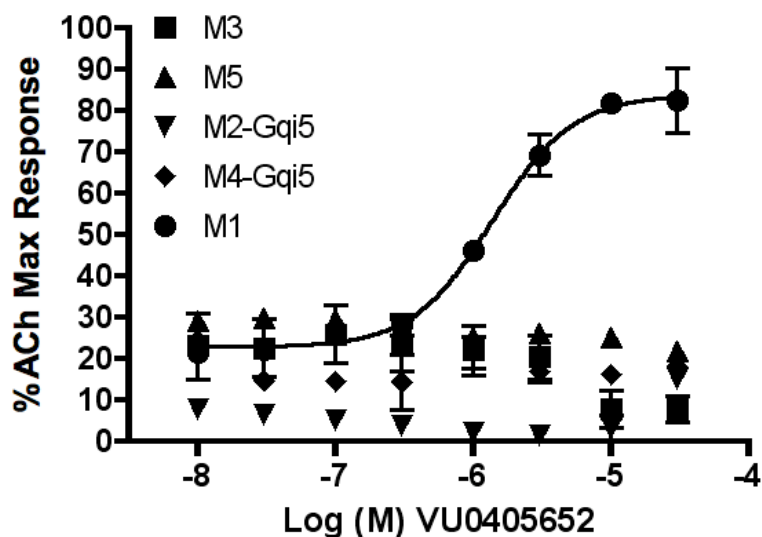


**Figure 21**. mAChR subtype-selectivity for probe compound VU0405652. $Ca^{2+}$ mobilization was used to obtain CRCs in the presence of an ACh $\sim EC_{20}$ in $M_2$-$M_5$ cells ($EC_{50}$ values all > 30 μM). Data represent Mean +/-SEM from at least three independent determinations.

As a final effort to mine the VHTSC compound collection for additional SAR and/or indentify new related scaffolds around this series of novel $M_1$ PAMs, a structure similarity search was performed using via a cheminformatic approach. This virtual

screen produced a large number of compounds within the VU collection, which possessed varying degrees of similarity to the basic **VU0108370** scaffold. To assess $M_1$ PAM activity, these compounds were screened at 10 μM in singlepoint $Ca^{2+}$ mobilization assays at $M_1$ in the presence of an ACh ~$EC_{20}$ (**Fig. 22**). Remarkably, only direct congeners of the **VU0108370** scaffold appeared as active hits, which further confirmed the shallow SAR observed among this series of $M_1$ allosteric modulators.
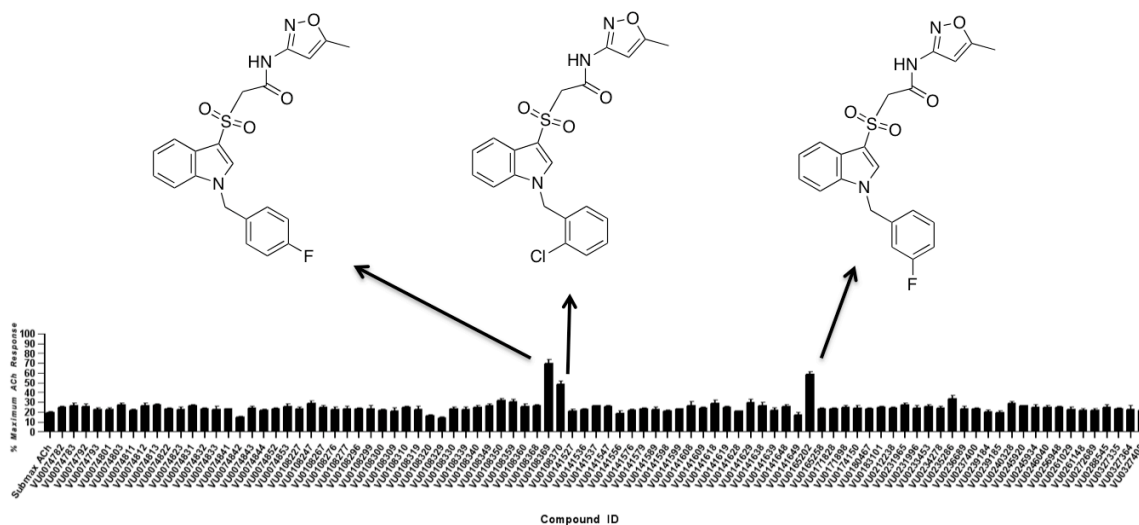


**Figure 22**. Singlepoint (10 μM) screen of a large panel of compounds from the VU HTS collection identified from a VU0108370-based structure similarity virtual screen. $Ca^{2+}$ mobilization was used to obtain singlepoint (10 μM) % ACh max responses for each compound in the presence of an ACh ~$EC_{20}$ using $M_1$ cells. Data represent Mean +/-SEM from at least three independent determinations.

## $M_1$ agonist VU0357017 and analogs

Although a number of useful $M_1$ PAM small molecule tools had been identified from the medicinal chemistry projects, no $M_1$ agonists possessing optimal potency,

selectivity, and DMPK characteristics required for use as *in vivo* tools existed. As discussed in the Introduction (Chapter I), a variety of compounds have been reported to activate the $M_1$ receptor in the absence of ACh with high subtype-selectivity. However, these compounds (such as AC-42, 77-LH-28-1, NDMC, and TBPB) all suffered from one or more flaws that precluded universal suitability as both *in vitro* and *in vivo* tools for studying $M_1$ neurobiology and the therapeutic potential of $M_1$ activation. Such defects include, for example, functional antagonism of one or more of the other mAChRs, significant off-target ancillary pharmacological activities, and/or poor systemic PK profiles. Therefore, it would be highly advantageous to discover novel drug-like $M_1$ agonist chemotype(s) that don't suffer from these and related limitations. Direct activation of $M_1$ in the absence of ACh is likely to reveal deeper neurobiological insight than reliance on $M_1$ PAMs alone, and $M_1$ agonists may offer therapeutic advantages over PAMs in treatment of certain CNS disorders. Furthermore, a growing body of evidence suggests that ligand-directed bias/signaling downstream of a given GPCR target may confer substantially different utilities/effects unique to each small molecule chemotype[117]. In light of these considerations, identification of a novel class of $M_1$ agonists became a focus of our research and of the MLSCN/MLPCN probe discovery enterprise. A comprehensive presentation and discussion of this project, which ultimately identified a number of novel allosteric $M_1$ agonists, including VU0357017, lies beyond the scope of this Chapter[142]. However, a brief overview highlighting this author's contribution is shown in the remainder of this section.

Similar to many of the mAChR allosteric ligand discovery projects, a HTS was performed of the then 65,000-member MLSCN/MLPCN compound collection aimed at

discovering novel $M_1$ agonists via functional cell-based fluorescent $Ca^{2+}$ mobilization

assay. This screen provided approximately 2,000 putative agonist primary hits. A subset

of these compounds were tested in CRCs with $M_1$-CHO cells, and in parallel, counter

screened against $M_4/G_{qi5}$ CHO cells using $Ca^{2+}$ mobilization assays. Two highly

structurally-similar hits, resynthesized as VU0207811 and VU0177548, were found to be

initially selective (>50-fold) for $M_1$ vs. $M_4$ with $M_1$ $EC_{50}$ values of 804 nM (69% ACh

max) and 1.74 μM (52% ACh max), respectively, and both later displayed high

selectivity for $M_1$ versus all other mAChRs (**Fig. 23**). These compounds provided an

excellent starting point to initiate a chemical optimization effort aimed at increasing

potency and developing properties suitable for studies of selective $M_1$ activation *in vivo*.
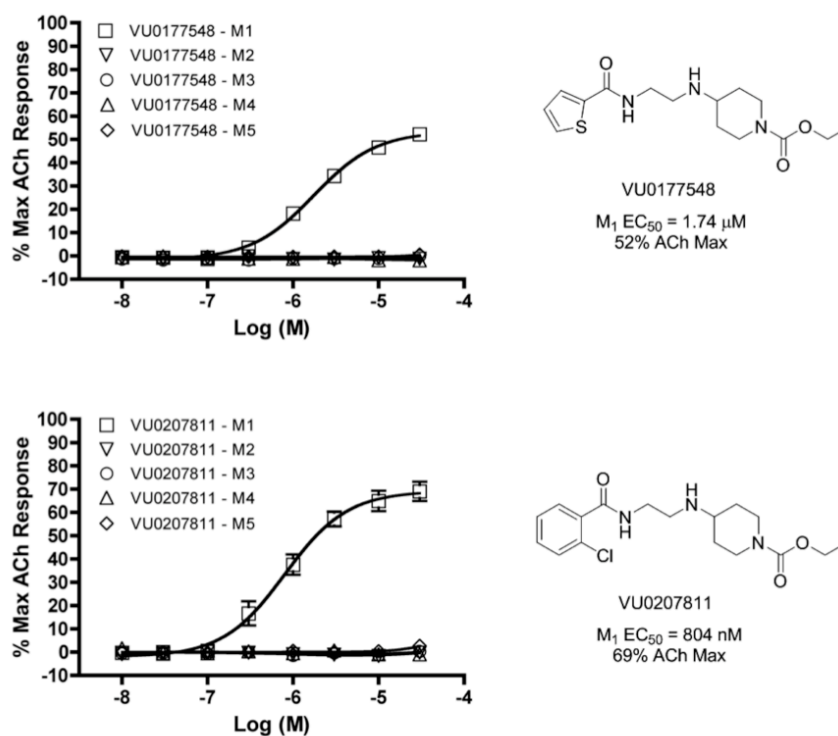


**Figure 23**. Structure and subtype-selectivity profiles of novel $M_1$ agonist HTS hits. $Ca^{2+}$ mobilization was used to obtain CRCs of each compound in $M_1$-$M_5$ cells. Data represent Mean +/-SEM from at least three independent determinations. Syntheses performed by P. Kennedy.

Subsequently, medicinal chemistry began with synthesis of a 25-member matrix library, which simultaneously varied the western aryl, central linker, and eastern carbamate of the scaffold (data not shown). From singlepoint screening of this library using $Ca^{2+}$ mobilization with $M_1$ cells (data not shown), it became clear that SAR was steepest at the western aryl region and progressively shallower throughout the remainder of the scaffold. A variety of substituted phenyl moieties were well tolerated (**Fig 24, top panel**); however, a wide range of further modifications to the central linker and eastern carbamate resulted in complete loss of $M_1$ activity (**Fig. 24, bottom panel**).



**Figure 24.** $M_1$ agonist SAR revealed by initial matrix library and singleton syntheses. $Ca^{2+}$ mobilization was used to obtain CRCs of each compound with $M_1$ cells. Data represent Mean +/-SEM from at least three independent determinations. Syntheses and experiments performed by E. Lebois.

Of these early analogs, the naked phenyl-bearing VU0184670 and its 2-methyl congener VU0357017 exhibited the greatest balance of $M_1$ potency and efficacy (**Fig. 24, top panel**). Therefore, they were selected for further *in vitro* characterization in subtype-selectivity (**Fig. 25**), $M_2$-$M_5$ functional antagonism (**Fig. 26**), and $[^3H]$-NMS competition binding (**Fig. 27**) assays. Similar to the parent HTS hits, neither VU0184670 nor VU0357017 exhibited any activation of $M_2$-$M_5$ at up to 30 μM (**Fig. 25**). Furthermore, they possessed little to no functional antagonism of an ACh ~$EC_{80}$ at these other mAChRs, in contrast to the $M_1$ agonist TBPB control (**Fig. 26**). Consistent with an allosteric mode of binding, both compounds failed to compete with the orthosteric radioligand $[^3H]$-NMS for binding to $M_1$-$M_5$ membranes at up to 10 μM; however, moderate inhibition was observed at 30 μM (**Fig. 27**). Whether or not this represents orthosteric competition or negative cooperativity via an allosteric site remains to be determined.
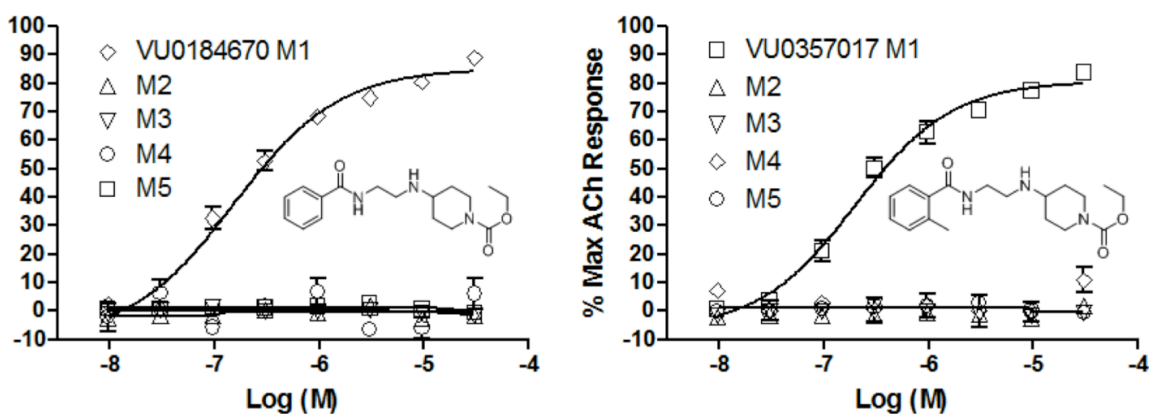


**Figure 25**. mAChR subtype-selectivity profiles of $M_1$ agonist leads VU0184670 and VU0357017. $Ca^{2+}$ mobilization was used to obtain CRCs of each compound with $M_1$-$M_5$ cells. Data represent Mean +/-SEM from at least three independent determinations.
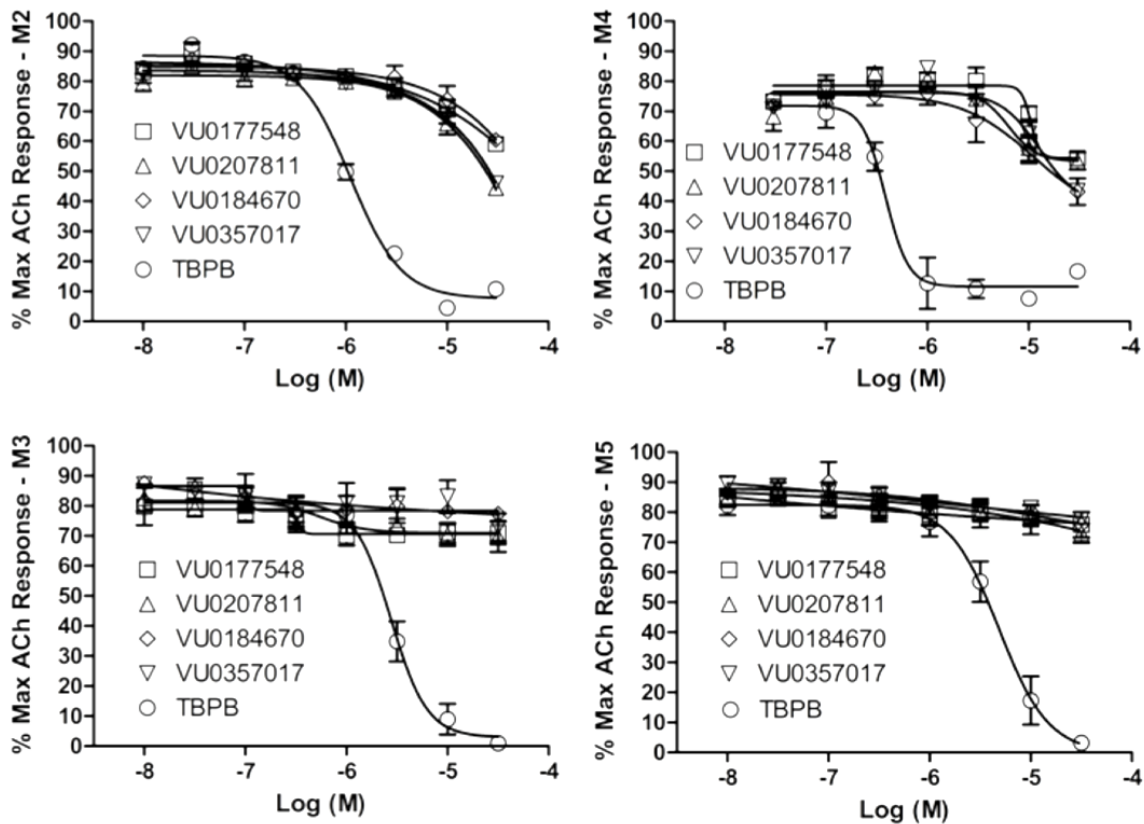
**Figure 26**. $M_2$-$M_5$ functional antagonism profiles of $M_1$ agonist leads VU0184670 and VU0357017 as well as parent hits VU0177548 and VU0207811. $Ca^{2+}$ mobilization was used to obtain CRCs of each compound in the presence of an ACh ~$EC_{80}$ with $M_2$-$M_5$ cells. TBPB included as positive control. Data represent Mean +/-SEM from at least three independent determinations. Experiments performed in part by E. Lebois.
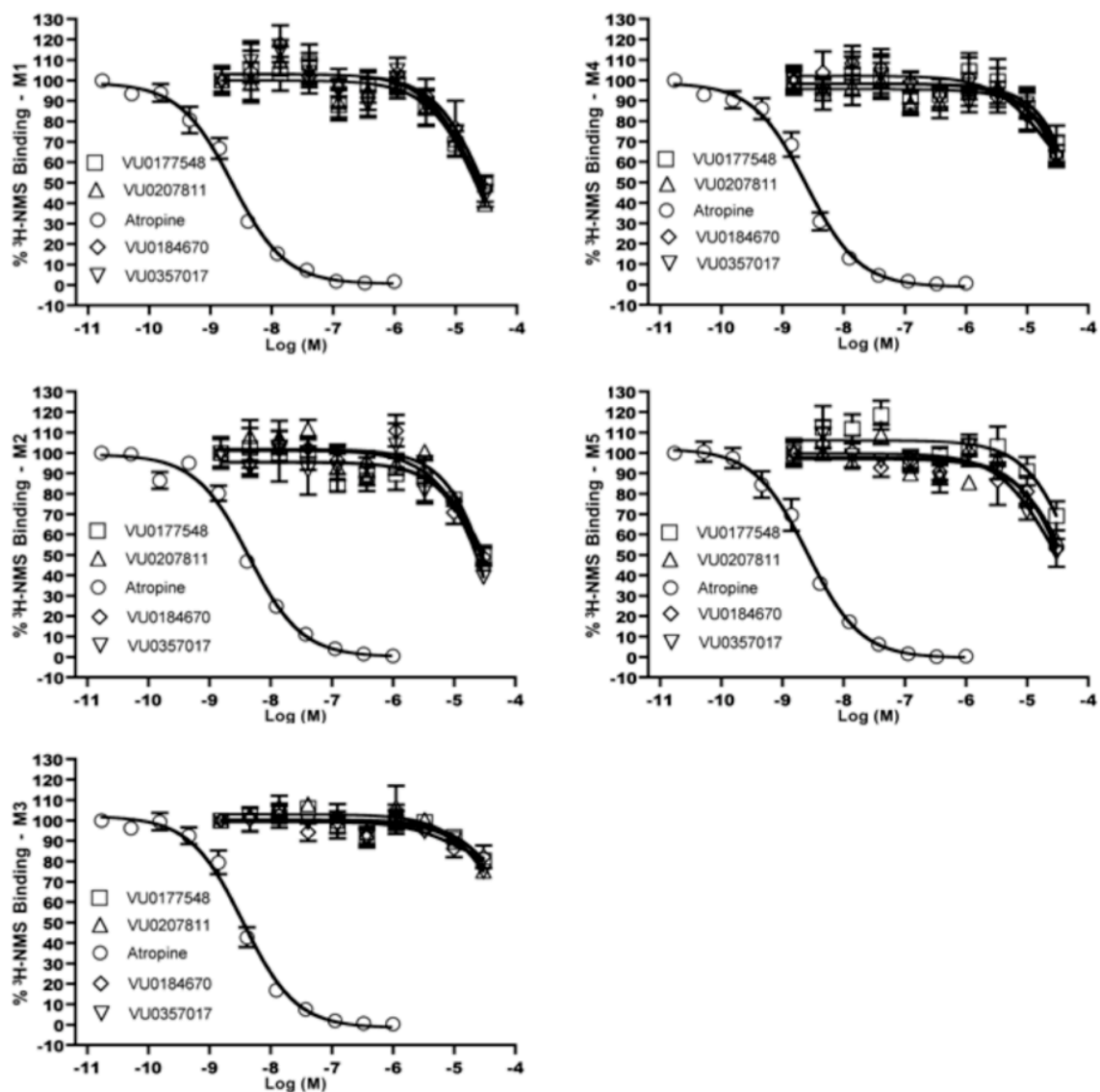
**Figure 27**. [$^{3}$H-NMS] competition binding CRCs with parent M$_1$ agonist HTS hits VU0177548 and VU0207811 as well as lead compounds VU0184670 and VU0357017 (all test compound IC$_{50}$ values = >30 µM). Atropine used as positive control. M$_1$ atropine IC$_{50}$ = 2.22 nM ± 0.60 nM; $K_i$ of 1.27 nM ± 0.36 nM. M$_2$ atropine IC$_{50}$ = 4.32 nM ± 1.63 nM; $K_i$ = 3.24 nM ± 1.16 nM. M$_3$ atropine IC$_{50}$ = 4.16 nM ± 1.04 nM; $K_i$ = 2.21 nM ± 0.53 nM. M$_4$ atropine IC$_{50}$ = 2.38 nM ± 1.07 nM; $K_i$ = 0.77 nM ± 0.34 nM. M$_5$ atropine IC$_{50}$ = 3.39 nM ± 1.16 nM; $K_i$ = 2.84 nM ± 0.84 nM. Results represent Mean ± SEM from at least three independent experiments.

Additional evidence that this novel series of compounds activates M$_1$ via an allosteric mechanism was obtained from Ca$^{2+}$ mobilization assays at the orthosteric-site Y381A mutant M$_1$ receptor, which exhibits a substantial loss in ACh potency versus the

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WT $M_1$ cell line via disruption of a crucial ACh-receptor interaction mediated by the native tyrosine residue. The $M_1$ allosteric agonist NDMC potently and fully activates this Y381A mutant $M_1$ receptor, thereby providing a means for normalization of responses to other test compounds. CRCs of both parent HTS hits as well as the two lead compounds in this cell line revealed retention of agonist potency and partial efficacy (**Fig. 28**), consistent with an allosteric receptor activation and in dramatic contrast to TBPB (data not shown).
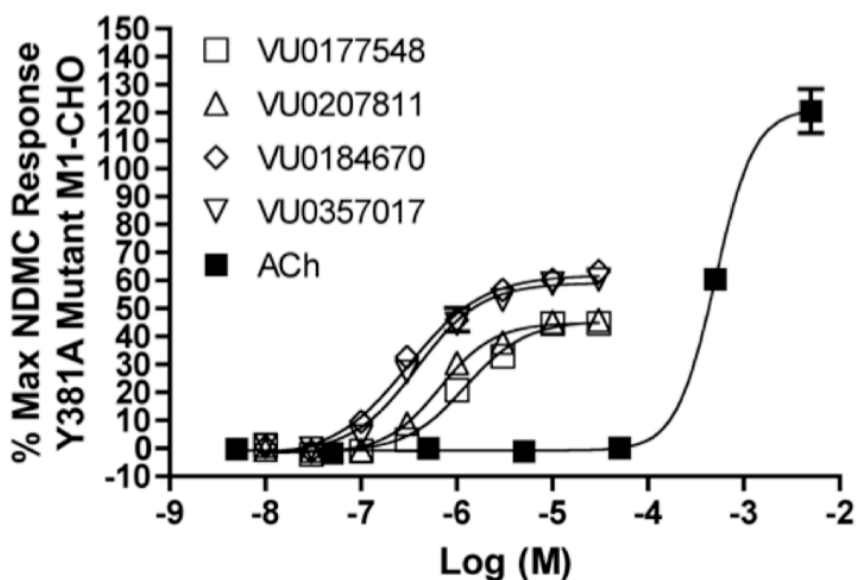


**Figure 28**. CRCs of $M_1$ agonist leads VU0184670 and VU0357017 as well as parent hits VU0177548 and VU0207811 in Y381A mutant $M_1$ cells. $Ca^{2+}$ mobilization was used to obtain CRCs of each compound (and ACh itself as positive control). $EC_{50}$ values: VU017758 and VU0207811 = 1.2 µM and 0.66 µM, respectively; VU0184670 and VU0357017 = 0.30 µM and 0.38 µM, respectively. Data represent Mean +/- SEM from at least three independent determinations, normalized as % max response to a high concentration of NDMC.

Together, these data provided compelling evidence that this novel class of $M_1$ agonists activates the receptor via an allosteric site that is topographically distinct from

the orthosteric site and with a high degree of subtype-selectivity (>100x). Further experiments, including Schild analysis of functional assays with orthosteric antagonist atropine that exhibited a non-competitive interaction (data not shown) and extensive $M_1$ receptor site-direct mutagenesis guided by computer GPCR homology models (data not shown), provided additional insight to the allosteric mechanism of action. In particular, specific residues of extracellular loop 3 and the first turn of transmembrane-spanning helix 7 are believed to comprise the unique allosteric site at which these $M_1$ agonists bind to the receptor (data not shown).

With these *in vitro* mAChR pharmacological and structural biochemical data in hand, we next evaluated the lead compounds VU0357017 and VU0184670 in ancillary/off-target pharmacology binding assays (at MDS Pharma/Ricerca) and in standard single-dose rat PK/PBL studies in order to assess their suitability as *in vivo* research tools (data not shown). Gratifyingly, both compounds possessed remarkably clean ancillary pharmacology profiles at 10 μM across a large panel of 67 different GPCR, ion channel, enzyme, nuclear hormone receptor, and transporter off-targets (only significant activity for either compound was a modest ~65% inhibition of binding to the $Sigma_1$ receptor). Furthermore, they exhibited highly desirable *in vivo* rat PK, with high CNS penetration (brain/plasma ratios: both >>1.0). Ultimately, these novel compounds served as the basis for a large number of *ex-vivo* brain slice electrophysiology and *in vivo* behavioral studies, which have provided crucial insight to the role of $M_1$ activation in discrete brain regions and in various animal models of CNS function/disease. Further chemical optimization of this series has uncovered comprehensive SAR for multiple sub-

series, which possess even greater potency and efficacy as $M_1$ allosteric agonists and are presently serving as potential drug discovery leads.

In order to pharmacologically investigate the role of $M_1$ activation in hippocampal-depending learning and memory, a series of brain slice electrophysiology and *in vivo* behavioral experiments were performed using lead compound VU0184670[142]. $M_1$-mediated regulation of NMDAR currents in CA1 hippocampal pyramidal neurons is believed to play an important role in certain aspects of learning and memory, and some of the pro-cognitive effects of traditional mAChR agonists seen in clinical trials have been attributed to this cholinergic mechanism. We utilized whole-cell patch clamp techniques with rat brain slice preparations to determine the effect of VU0357017 on CA1 hippocampal pyramidal NMDAR currents. At 30 μM (bath application), VU0357017 significantly increased the peak amplitude of NMDA-evoked inward currents to 137% of baseline after approximately 2 minutes (data not shown). In a related rat behavioral study *in vivo* systemic dosing of VU0357017 fully reversed (at highest dose of 10 mg/kg, IP) scopolamine-induced disruption of contextual fear conditioning and in a dose-dependent manner (data not shown). Under the specific test conditions used, contextual fear-conditioning represents a traditional behavioral model of hippocampal-dependent learning and memory function, which is severely disrupted by mAChR antagonists such as scopolamine. Therefore, these findings are consistent with the hypothesis that $M_1$ plays an essential role in ACh-mediated mechanisms of learning and memory in the hippocampus. However, based on data from other pharmacological experiments and on studies using mAChR-KO mice, it is known that other subtypes in addition to $M_1$ have important roles in cholinergic mechanisms of cognitive function (Chapter I).

# M$_1$ agonist TBPB and analogs

Prior to our discovery of the novel M$_1$ allosteric agonist series represented by lead compound VU0357017, we reported on a structurally unrelated compound termed TBPB as a potent mAChR agonist possessing high M$_1$-selectivity in functional cell-based assays (**Fig. 29**)[143]. We performed extensive *in vitro* and *in vivo* characterization of TBPB and found via M$_1$ mutagenesis and other molecular pharmacology studies that this ligand activates M$_1$ in the absence of ACh with high subtype selectivity and via an allosteric mechanism. We also found that TBPB potentiates NMDAR currents in rodent hippocampal pyramidal cells without altering excitatory or inhibitory synaptic transmission using *ex-vivo* electrophysiology techniques, and that TBPB-mediated M$_1$ activation drives non-amyloidogenic processing of APP and decreases amyloid-β *in vitro*. Furthermore, TBPB was efficacious in rodent behavioral models predictive of anti-psychotic activity *in vivo* without inducing M$_2$/M$_3$-related side effects.
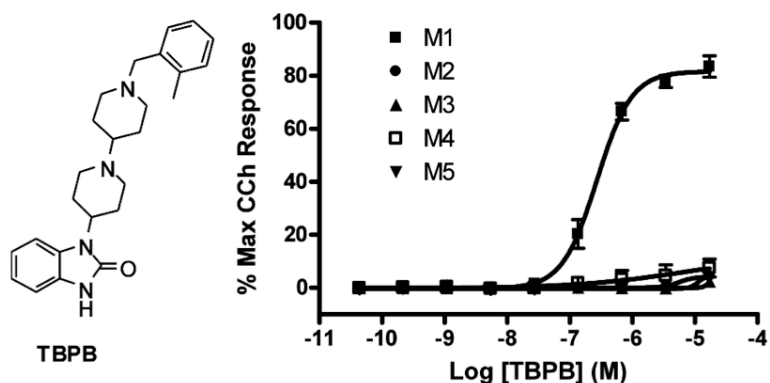


**Figure 29**. Structure and mAChR subtype-selectivity of M$_1$ agonist TBPB. Ca$^{2+}$ mobilization was used to obtain CRCs of TBPB in M$_1$-M$_5$ cells. Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.

However, TBPB was an unoptimized screening hit with moderate antagonist activity at dopamine $D_2$ receptor ($D_2$) ($IC_{50}$ = 2.6 $\mu$M). Despite an [$^{18}$F]-fallypride micro-positron emission tomography (PET) study that confirmed the in vivo efficacy in rodent models of psychosis observed with TBPB was the result of selective $M_1$ activation and not due to $D_2$, we hoped to diminish $D_2$ activity through a lead optimization campaign using an iterative parallel synthesis approach[144,145]. For our initial library, analogs were prepared by a reductive amination sequence employing a functionalized piperidone and 4-(2-keto-1- benzoimidazolinyl)piperidine or the 5-chloro congener and polymer-supported triacetoxyborohydride (**Fig. 30**).
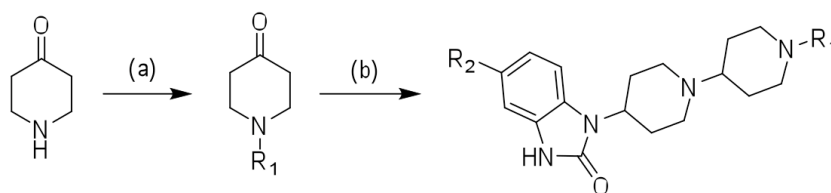


**Figure 30**. First round of synthesis of TBPB analogs. Reagents and conditions: (a) $K_2CO_3$, $ArCH_2Br$, DCM, 70-88%; (b) MP-B(OAc)3H, 4-(2-keto-1-benzoimidazolinyl)piperidine or 5-chloro-1-(4-piperidinyl)-2-benzimidazolone, DCM, rt, 24 hr, 85-95%.

In the first round of library synthesis, efforts focused on commercially available piperidinones (wherein $R_1$ is either an alkyl or carbamate moiety). Subsequent rounds of library synthesis utilized the same scheme but with substituted benzylic $R_1$ moieties generated by standard alkylation chemistry on the piperidone starting material. These analogs were then tested in CRCs at both the $M_1$ and dopamine $D_2$ receptors in cell-based $Ca^{2+}$ mobilization assays. Out of an initial 24-member library with simple aliphatic and carbamate derivatives of TBPB, only an ethyl carbamate analog (compound 1b, **Table 4**) retained $M_1$ agonist activity.

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**Table 4**. $M_1$ and $D_2$ potency/efficacy SAR for TBPB analogs synthesized according to **Figure 30**. $Ca^{2+}$ mobilization was used to obtain compound CRCs with $M_1$ and $D_2$ cells. Data represent Mean +/-SEM of at least three independent determinations. Experiments performed by A. Brady.

| Cmpd | $R^1$ | $R^2$ | M1 $EC_{50}{}^a$ (nM) | %CCh Max[a] | D2 $IC_{50}{}^a$ ($\mu$M) |
|------|-------|-------|------------------------|-------------|----------------------------|
| 1a | 2-MeBn | H | 289 | 82 | 2.65 |
| 1b | $CO_2Et$ | H | 2.1 | 65 | >10 |
| 1c | 2-MeBn | Cl | 1030 | 84 | 35% at 10 $\mu$M |
| 1d | Bn | Cl | 356 | 74 | 35% at 10 $\mu$M |
| 1e | 2-$CF_3$Bn | H | 410 | 82 | 30% at 10 $\mu$M |
| 1f | 2-$CF_3$Bn | Cl | 1500 | 83 | ND |
| 1g | 2-ClBn | H | 260 | 44 | 2.06 |
| 1h | 2-ClBn | Cl | 1800 | 49 | ND |
| 1i | 2-$NO_2$Bn | H | 120 | 48 | 1.13 |
| 1j | 2-$NO_2$Bn | Cl | 1100 | 42 | ND |
| 1k | 2-CNBn | H | 490 | 23 | 40% at 10 $\mu$M |
| 1l | 2-CNBn | Cl | 1600 | 27 | ND |

Compound **1b** displayed substantially improved $M_1$ potency ($EC_{50} = 2.1$ nM) with moderate efficacy (65% CCh max), but lost selectivity versus $M_2$-$M_5$, thereby degenerating into a pan-mAChR partial agonist (**Fig. 31**).
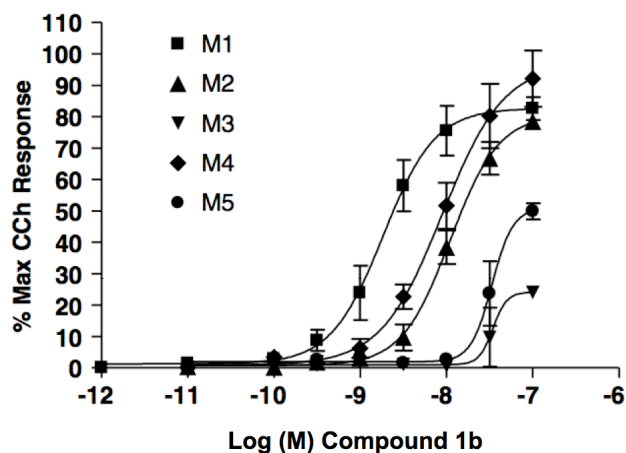


**Figure 31**. mAChR subtype-selectivity profile for TBPB analog **1b**. $Ca^{2+}$ mobilization was used to obtain CRCs with $M_1$-$M_5$ cells. Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by A. Brady.

The subsequent round of parallel synthesis focused on diverse distal benzylic substitutions at $R_1$ with the 4-(2-keto-1-benzoimidazolinyl)piperidine TBPB headpiece bearing either hydrogen or a chlorine atom in the 5-position, the latter of which was introduced to block a site of oxidative metabolism we identified (data not shown). This effort generated over 60 analogs; however, the SAR proved to be rather 'flat'. If the benzyl group possessed substituents in the 3- or 4-positions, all $M_1$ agonism was lost ($EC_{50}$ >20 μM). As shown in **Table 4**, it was possible to synthesize other analogs with selective $M_1$ partial agonism ($M_1$ $EC_{50}$ values of 120 nM to 1.8 μM, >50 μM vs. $M_2$-$M_5$), but the degree of efficacy varied widely (23-84%). Incorporation of a 5-Cl atom into TBPB to block metabolism resulted in compound 1c, an analog that maintained the same efficacy as TBPB, but lost ~5-fold potency (**Table 4**). Removal of the 2-Me group resulted in an analog comparable to TBPB, compound 1d (**Table 4**). Attempts to replace the metabolically labile 2-methyl group with alternative chemical moieties (compounds **1e-1l**) generally led to a significant diminution in $M_1$ efficacy (<50%), $M_1$ potency, or both (**Table 4**). However, the 2-nitrobenzyl analogue (compound **1i**) proved more potent than TBPB ($M_1$ $EC_{50}$ = 120 nM), but efficacy fell to 42% CCh max response (**Table 4**). One exception to this was the 2-$CF_3$ group, which provided an analog with an $M_1$ $EC_{50}$ of 410 nM and 82% max efficacy (**Table 4**).

With respect to $D_2$ inhibition, compound **1e** maintained a mAChR profile comparable to TBPB, but $D_2$ inhibition was greatly diminished (~30% @ 10 μM), indicating that this compound might prove to be a more useful biological tool to probe $M_1$ than TBPB. The moderately more potent than TBPB compound **1i**, also displayed increased $D_2$ inhibition ($IC_{50}$ = 1.13 μM); however, a dual $M_1$ agonist/$D_2$ antagonist may

prove to be an acceptable profile for a novel schizophrenia treatment and therefore this SAR may provide a starting point for a dual-pharmacology optimization project.

We then decided to evaluate the effect of a small structural modification and targeted fluorine substituted 4-(2-keto-1-benzoimidazolinyl)piperidines, prepared by modification of the reported Merck route (**Figure 32**). Commercial fluorine-substituted *ortho*-fluoro-nitroaromatic starting material (compounds **2**) were heated under microwave irradiation with ethyl 4-amino-1-piperdinecarboxylate to deliver the $S_NAr$ products (compounds **3**) in yields ranging from 70-90%. A zinc-mediated nitro reduction afforded intermediates (compounds **4**), which were then treated with triphosgene to provide the benzimidazolones (compounds **5**). Removal of the ethyl carbamate was accomplished under another microwave-assisted protocol to deliver the 4-, 5- and 6-fluoro-1-(piperdin-4-yl)-1-*H*-benzo[*d*]imidazol-2(3*H*)-ones (compounds **6**).



**Figure 32.** Synthesis of fluorine-substituted TBPB analogs. Reagents and conditions: (a) Ethyl 4-aminopiperidine, $Na_2CO_3$, KI, cyclohexanol, mw, 180 $^o$C, 10 min, 70-90%; (b) Zn, 1N HCl, MeOH; (c) tripshosgene, $Et_3N$, THF, rt, 2 h; (d) 10% NaOH, mw, 130 $^o$C, 30 min, 50-60% from intermediate **3**.

**Figure 33**. Synthesis of additional fluorinated TBPB analogs. Reagents and conditions: (a) MP-B(OAc)$_3$H, 4-, 5- and 6-fluoro-1-(piperdin-4-yl)-1-*H*-benzo[*d*]imidazol-2(3*H*)-ones **17**, 50-90%.

The final fluorine-substituted analogs were prepared by a reductive amination sequence employing either 2-methly benzyl piperidone (compounds **7**) or 2-trifluoromethly benzyl piperidone (compounds **8**) and the 4-, 5- or 6-fluoro-1-(piperdin-4-yl)-1-*H*-benzo[*d*]imidazol-2(3*H*)-ones (compounds 9) (**Fig. 33**). As shown in **Table 5**, TBPB analog compounds **9** containing a fluorine atom were uniformly less active than the un-substituted parents, TBPB and compound **1e**. However, analogs such as **9b** and **9c** possessed sub-micromolar EC$_{50}$s and were equally efficacious (850 nM, 85% CCh max and 760 nM, 84% CCh max, respectively); moreover, they carried no significant D$_2$ antagonism. Based on these SAR data, we prepared additional 4-(2-keto-1-benzoimidazolinyl)piperidines and TBPB analogues with alternative substitutions (Br, CN, CF$_3$), as shown in **Table 5.**

**Table 5**. SAR for fluorine-substituted TBPB analogs. $Ca^{2+}$ mobilization was used to obtain compound CRCs with $M_1$ and $D_2$ cells. Data represent Mean +/-SEM of at least three independent determinations. Experiments performed by A. Brady.
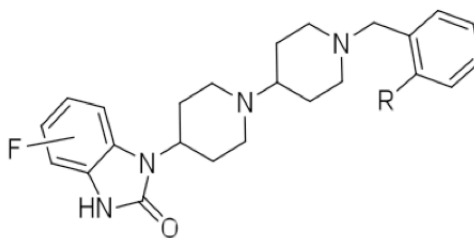


| Cmpd | R | F | M1 $EC_{50}$ $(\mu M)$[a] | %CCh Max[a] | D2 $IC_{50}$ $(\mu M)$[a] |
|---|---|---|---|---|---|
| 9a | $CH_3$ | 4-F | 1.06 | 73 | >10 |
| 9b | $CH_3$ | 5-F | 0.85 | 85 | 40%@10μM |
| 9c | $CH_3$ | 6-F | 0.76 | 84 | >10 |
| 9d | $CF_3$ | 4-F | 1.71 | 75 | >10 |
| 9e | $CF_3$ | 5-F | 1.04 | 80 | 2.69 |
| 9f | $CF_3$ | 6-F | >10 | ND | >10 |

Most fluoro-TBPB analogs were found to be either inactive or partial agonists with <20% maximum efficacy, or they were no longer selective for $M_1$. Ultimately, after numerous sets of analogs had been synthesized and evaluated, we found that this was a rare instance, from our experience, where the original screening hit, TBPB, could not be significantly improved upon using these types of structural modifications. Further SAR was later explored by examining the effect of capping the TBPB scaffold's distal basic piperidine nitrogen with amides, sulfonamides, and ureas (data not shown)[144]. These efforts revealed similarly flat SAR; however, a number of such analogs retained moderate $M_1$ potency, efficacy, and subtype-selectivity (data not shown).

In summary, we identified a novel series of allosteric partial agonists with high selectivity for $M_1$ versus $M_2$-$M_5$ and with various degrees of $D_2$ antagonism based on the

general TBPB scaffold. SAR was essentially 'flat' within this series, with subtle changes resulting in pan-mAChR agonism, loss of $M_1$ potency, significantly decreased efficacy, and/or ancillary $D_2$ antagonism. This was a rare example in which the screening lead proved very difficult to optimize, yet still possessed a profile that enabled both *in vitro* and *in vivo* studies to be conducted. Moreover, this work further exemplifies the numerous challenges inherent to optimization of most allosteric GPCR ligands.

## $M_1$ antagonist VU0255035 and analogs

In addition to the discovery and optimization of numerous new $M_1$-targeted small molecule agonists and PAMs, we hoped to obtain novel $M_1$-selective antagonists for use as research tools and to investigate the therapeutic potential of selective $M_1$ blockade in movement-related and other CNS disorders. Small molecule agonists and potentiators selective for a specific mAChR subtype have clear utility in neurobiology research and in therapeutic target validation contexts wherein activation of the receptor is required; for example, $M_1$ activation in Alzheimer's disease or schizophrenia. Conversely, subtype-selective mAChR antagonism provides a complementary but distinct utility in contexts that require diminution of receptor signaling, such as in the treatment of dystonia and in basic research on the role of $M_1$ signaling under normal disease-free physiological conditions. Furthermore, discovery of a truly selective $M_1$ antagonist small molecule could provide a highly useful control in experiments with mAChR agonists or PAMs.

Therefore, our MLSCN/MLPCN center initiated an effort to identify a potent small molecule antagonist with high specificity for $M_1$ versus $M_4$ for use as a chemical

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probe and lead for further optimization towards a novel therapeutic[140,141]. Our functional HTS (performed as described previously in this Chapter for the MLSCN/MLPCN $M_1$ PAM project) identified a number of hits, and a limited optimization effort produced the $M_1$ versus $M_4$ selective antagonist VU0359517 ($M_1$ $IC_{50}$ = 440 nM, $M_4$ $IC_{50}$ >150 µM); however, this orthosteric mAChR antagonist displayed only 2- to 9-fold selectivity versus $M_2$, $M_3$ and $M_5$ (data not shown). This early effort also produced a series of weak ($M_1$ $IC_{50}$ >3.5 µM) but selective ($M_2$-$M_5$ $IC_{50}$ >50 µM) antagonists based on a 3,6-disubstituted-[1,2,4]-triazolo[4,3-*b*]pyridazine scaffold (data not shown)[139].

While the vast majority of our HTS hits displayed no selectivity for $M_1$ versus $M_4$, compound **1**, based on a *N*-(3-piperazin-1-yl)-3-oxopropyl)benzo[*c*][1,2,5] thiadiazole-4-sulfonamide scaffold, demonstrated a surprising >58-fold selectivity for rat $M_1$ ($IC_{50}$ = 2.55 µM) over rat $M_4$ ($IC_{50}$ >100 µM) with a similar $IC_{50}$ (2.72 µM) for human $M_1$ in $Ca^{2+}$ mobilization assays examining the functional antagonism of an ACh ~$EC_{80}$. Encouraged by this result, we explored the selectivity of compound **1** versus $M_2$-$M_5$. Gratifyingly, it was >58-fold selective for M1 versus M2- M5 (**Fig. 34**).
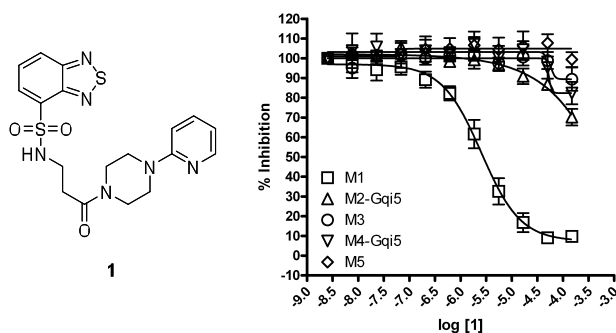


**Figure 34.** $M_1$-$M_5$ CRCs for compound **1**. $Ca^{2+}$ mobilization was used to obtain CRCs of test compound in $M_1$-$M_5$ cells in the presence of an ACh ~$EC_{80}$. $M_1$ $IC_{50}$ = 2.6 µM. Data represent Mean +/- SEM from at least three independent determinations. Experiments performed by D. Sheffler.

While this level of selectivity was unprecedented for a small molecule $M_1$ antagonist, **1** did not meet the potency criteria for an MLSCN small molecule probe ($IC_{50}$ <500 nM).  Therefore, we launched a chemical lead optimization campaign to improve potency for M1 while maintaining high selectivity versus $M_2$-$M_5$.  Analogs of compound **1** were synthesized in a library format according to the scheme presented in **Figure 35**. For the first round of library synthesis, our strategy was to explore alternative amides in place of the 2-pyridylpiperizine moiety.  Commercially available benzo[*c*]thiadiazole-4-sulfonyl chloride (compound **2**) was treated with β-alanine methyl ester to afford, after saponification, acid (compound **3**).  Subsequent coupling with a diverse collection of 24 primary and secondary amines under standard solution phase parallel synthesis conditions employing polymer-supported reagents and scavengers provided analogs **4**.    All compounds were purified by mass-guided HPLC to analytical purity.
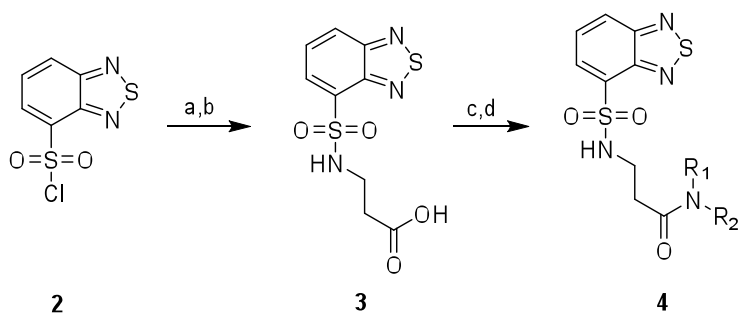


**Figure 35**. Synthesis of compound **1** analogs.  Reagents and conditions: (a) β-alanine methyl ester, DCM, DIEA, rt; (b) LiOH, THF/H$_2$O/MeOH, rt; (c) R$_1$R$_2$NH, PS-DCC, HOBt, DCM, DIEA; (d) MP-CO$_3^{2-}$. Syntheses performed by C. Lindsley.

Evaluation of this library against $M_1$-$M_5$ cells by $Ca^{2+}$ mobilization assay in the presence of an ACh ~$EC_{80}$ demonstrated that SAR for this series was relatively flat, with regiosiomeric pyridylpiperazine amides **5** being the only active analogs of **4** (**Table 6**). The activity of the HTS lead compound **1** was confirmed by re-synthesis ($M_1$ $IC_{50}$ = 2.37 μM, $M_2$-$M_5$ $IC_{50}$ >10 μM). With respect to SAR for this series, functionalized phenyl piperazine analogs, aliphatic piperazines, and other heterocyclic analogs were all devoid of $M_1$ antagonist activity. Relative to the 2-pyridyl analog (compound **1**), the 3-pyridyl congener (compound **5a**) possessed roughly equivalent activity at $M_1$ ($IC_{50}$ = 3.19 μM), while also maintaining selectivity versus $M_2$-$M_5$. The 4-pyridyl variant (compound **5b**) provided a ~17-fold increase in $M_1$ activity ($IC_{50}$ = 133 nM) relative to the HTS hit.

**Table 6.** $M_1$-$M_5$ SAR for active $M_1$ antagonist analogs **5**. $Ca^{2+}$ mobilization was used to obtain compound CRCs in $M_1$-$M_5$ cells in the presence of an ACh ~$EC_{80}$. Data represent Mean +/-SEM of at least three independent determinations. Experiments performed by D. Sheffler.
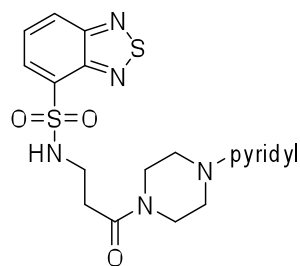


**5**

| | R | M1 $IC_{50}$ (μM)[a] | M2 $IC_{50}$ (μM)[a] | M3 $IC_{50}$ (μM)[a] | M4 $IC_{50}$ (μM)[a] | M5 $IC_{50}$ (μM)[a] |
|---|---|---|---|---|---|---|
| **1** | 2-pyridyl | 2.37±1.6 | >10 | >10 | >10 | >10 |
| **5a** | 3-pyridyl | 3.19±1.5 | >10 | >10 | >10 | >10 |
| **5b** | 4-pyridyl | 0.13±0.03 | >10 | >10 | >10 | >10 |

A high degree of subtype-selectivity (>75x for $M_1$ versus $M_2$-$M_5$) was also maintained ($M_2$-$M_5$ IC$_{50}$s >10 μM) for compound 5b as shown in **Figure 36**. Compound concentrations were solubility limited, but little inhibition of an ACh ~EC$_{80}$ at $M_2$-$M_5$ was observed for compound **5b** at up to 100 μM in some experiments. Thus, this analog represented the most functionally subtype-selective small molecule $M_1$ antagonist ever reported; however, its binding subtype-selectivity remained a question. Due to this unprecedented mAChR subtype-selectivity in functional cell-based assays (**Fig. 36**), we hypothesized that this compound binds to an allosteric site on the M1 receptor.
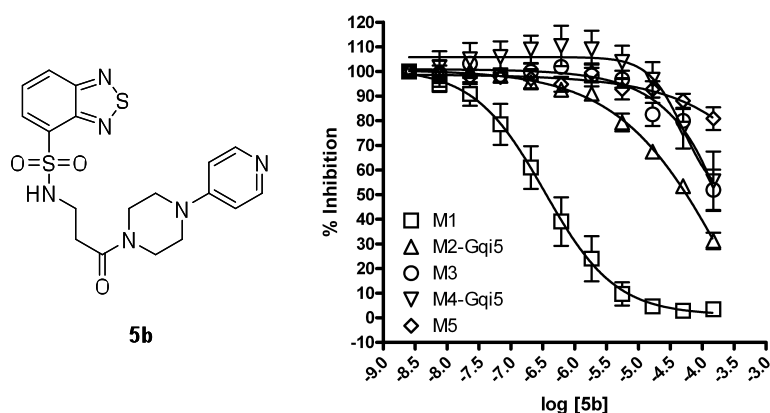


**Figure 36**. $M_1$-$M_5$ CRCs for lead compound **5b**. $Ca^{2+}$ mobilization was used to obtain CRCs of test compound in $M_1$-$M_5$ cells in the presence of an ACh ~EC$_{80}$. $M_1$ IC$_{50}$ = 133 +/- 28 nM. Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by D. Sheffler.

To test this hypothesis, [3H]-NMS competition binding experiments were conducted under equilibrium conditions to obtain $K_i$ values for compound **5b** as well as atropine controls at $M_1$-$M_5$ cell membranes. As shown in **Table 7**, compound **5b** exhibited potent inhibition of orthosteric radioligand binding with an $M_1$ $K_i$ of 14.9 nM with 45- to 159-fold $K_i$ selectivity versus $M_2$-$M_5$.

**Table 7**. Summary of functional and binding mAChR subtype-selectivity for $M_1$ antagonist (compound **5b**). $Ca^{2+}$ mobilization was used to obtain CRCs of test compound in $M_1$-$M_5$ cells in the presence of an ACh ~$EC_{80}$. [$^3$H]-NMS competition binding was used to obtain CRCs of test compound and atropine positive control under equilibrium with $M_1$-$M_5$ cell membranes. Atropine $K_i$ values: $M_1$ = 0.8 nM, $M_2$ = 2.6 nM, $M_3$ = 1.1 nM, $M_4$ = 0.6 nM, $M_5$ = 1.6 nM. Data represent Mean +/-SEM from at least three independent determinations. Experiments performed by D. Sheffler.



**5b**
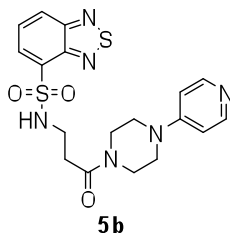
| mAChR | $IC_{50}$ (nM)[a] | Fold selectivity | Ki (nM)[a] | Fold selectivity |
|-------|-------------------|------------------|------------|------------------|
| M1 | 133 | | 14.9 | |
| M2 | >10,000 | >75 | 661.3 | 45 |
| M3 | >10,000 | >75 | 876.9 | 59 |
| M4 | >10,000 | >75 | 1177.6 | 79 |
| M5 | >10,000 | >75 | 2362.3 | 159 |

These results were surprising and led us to further explore the mechanism of antagonism via Schild analysis of PI hydrolysis experiments. In these experiments, compound **5b** caused parallel concentration-dependent rightward shifts of an ACh CRC, which translated in a Schild analysis to a $K_d$ of 16 nM (slope = 0.90$\pm$0.08, data not shown). These data support the previous results from the [$^3$H]-NMS binding experiments in suggesting that compound **5b** is an orthosteric M1 antagonist. Therefore, we rejected our original allosteric binding hypothesis pending further testing in the form of kinetic binding experiments.

Prior to conducting *in vivo* experiments, the selectivity of compound **5b** was evaluated against larger panels of molecular targets. In binding assays at both the UNC Psychoactive Drug Screen and against a large panel of GPCRs, ion channels,

transporters, nuclear hormone receptors, and kinases (MDS Pharma/Ricerca), compound **5b** was devoid of significant ancillary pharmacology (data not shown). Next, we performed a rat single-dose (10 mg/kg) PK/PBL study that revealed a brainAUC/plasmaAUC ratio of 0.48 and other similarly acceptable PK parameters for use of this compound *in vivo* following systemic dosing (data not shown).

In a subsequent acute rodent behavioral study, our $M_1$ antagonist (compound **5b**) exhibited *in vivo* efficacy following systemic (I.P.) administration in a pilocarpine-induced mouse seizure model by reducing $M_1$-mediated seizure activity and increasing survival at doses that did not disrupt acquisition of contextual fear conditioning (data not shown). These results suggested that $M_1$ antagonism in a therapeutic context may not result in learning and memory related side effects, although further investigation is certainly required. Based on these exciting data, we chose to further explore SAR around this chemical series by evaluating the six parameters shown in **Figure 37**. However, SAR was again 'flat' with no active analogs arising from these modifications.
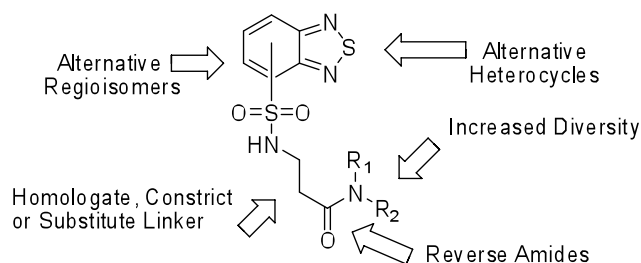


**Figure 37**. Additional SAR exploration around the $M_1$ antagonist scaffold resulting in inactive analogs.

In summary, an MLSCN $M_1$ antagonist chemical probe discovery project provided a selective $M_1$ antagonist lead (compound **1**) after a functional HTS campaign. One round of parallel synthesis afforded compound **5b**, a highly functionally selective

($>$75-fold vs $M_2$-$M_5$), potent ($IC_{50}$ = 133 nM), and high-affinity ($K_i$ = 14.9 nM) $M_1$ antagonist. Moreover, this compound possessed no significant ancillary pharmacology and exhibited a pharmacokinetic profile acceptable for *in vivo* systemic dosing in behavioral and neurochemical studies. Based on these and the preliminary *in vivo* efficacy data, compound **5b** was declared an MLSCN/MLPCN small molecule *in vitro/in vivo* probe and assigned the identifier VU0255035[140]. Further experiments utilizing this novel compound and aimed at investigating the therapeutic potential of selective $M_1$ antagonism as well as the role of $M_1$ in basic neurobiology are currently underway.

## Summary

In summary, numerous novel small molecule ligands selectively-targeting the $M_1$ receptor and suitable for *in vitro* and *in vivo* use have been discovered, including three classes of PAMs, two classes of agonists, and a single class of antagonists. Prior to this work, the mAChR field was largely devoid of truly $M_1$-selective ligands that possessed the drug-like pharmacological characteristics necessary for use in rodent *in vivo* neurobehavioral and neurochemical studies. Utilizing functional cell-based screening and hypothesis-driven medicinal chemistry techniques, highly-selective $M_1$ tools have successfully been discovered, which have provided crucial insight to the role of $M_1$ in basic neurobiology and within the context of therapeutic target validation. Specifically, studies with these compounds have revealed and confirmed the role of $M_1$ activation in mediating ACh-induced potentiation of NMDAR currents in CA1 hippocampal pyramidal neurons, in regulation of APP processing, and in a variety of anti-psychotic

and pro-cognitive behavioral effects. Furthermore, the diversity of chemotypes across the different series of $M_1$ PAMs (e.g. BQCA, VU0366369, and VU0405652) and $M_1$ agonists (e.g. VU0357017 and TBPB) provides a rich set of tools for studying ligand-directed/biased signaling downstream of $M_1$, which represents a growing area of research important to both basic and translational neurobiology.

# Chapter VI

## SUMMARY AND FUTURE DIRECTIONS

Muscarinic acetylcholine receptors are important to numerous physiological processes in both the CNS and periphery[16,18]. In light of their distinct localizations and putative functional roles as suggested by genetic KO mice studies, multiple mAChR subtypes may represent attractive drug targets for treatment of a variety of CNS diseases and disorders[44,111]. However, since the initial cloning of the genes encoding the individual rodent and human mAChR subtypes more than two decades ago, relatively little pharmacological progress has been made towards obtaining a deep understanding of the role(s) of each subtype in mammalian neurobiology. This has been due largely to a failure of efforts aimed at discovering highly subtype-selective mAChR ligands. Indeed, few drug-like muscarinic compounds exist that are suitable for use as research tools or as drug discovery leads. Like other GPCR subfamilies, the orthosteric binding site is highly conserved across the mAChRs, which has contributed heavily to this longstanding paucity of highly subtype-selective ligands. This is especially apparent in the case of $M_5$, as no small molecules possessing even moderate selectivity for $M_5$ have been reported in the international scientific or patent literature. Similarly, until recently no ligands existed for studying the effects of selective $M_4$ modulation. A number of chemotypes have been discovered to possess high functional and/or binding selectivity for $M_1$, yet many of these compounds suffer from sub-optimal pharmacological properties and off-target activities that limit their utility in basic and clinical research (Chapter I).

Here we described a variety of medicinal chemistry and neuropharmacology research projects aimed at discovering, optimizing, and characterizing novel highly subtype-selective mAChR ligands for $M_1$, $M_4$, and $M_5$[27,124,126,131,132,135,138-146]. Using functional HTS and cheminformatic techniques, screening hits were identified that, through subsequent chemical optimization, lead to discovery of novel series of $M_4$ PAMs and $M_5$ PAMs as well as multiple series of $M_1$ PAMs, allosteric $M_1$ partial agonists, and $M_1$ antagonists, each possessing high subtype-selectivity (>30-100x) in functional cell-based assays. Lead compounds from the $M_4$ PAM project and multiple $M_1$-related projects were then used in a variety of electrophysiological and neurobehavioral experiments to investigate the precise role(s) of these subtypes in the CNS. In the case of $M_5$, the first and currently only series of highly subtype-selective ligands, $M_5$ PAMs derived from an initial pan-$M_1$, -$M_3$, -$M_5$ PAM screening hit, were obtained and characterized. Abbreviated summaries for the $M_4$ PAM and $M_5$ PAM projects, associated potential further optimization efforts, as well as future *in vitro* and *in vivo* studies are presented below.

## $M_4$ PAMs based on VU010010

The first series of small molecule mAChR PAMs possessing high potency and subtype-selectivity for rat $M_4$ were obtained using a virtual cheminformatic database mining approach based on the structure of LY2033298, the first potent and selective human $M_4$ PAM (**Fig. 1**)[124,125,127]. This involved the initial discovery of VU010010, which possessed an approximately 400 nM $rM_4$ potency and induced a robust 47x left-

shift of an rM$_4$ ACh CRC at 30 µM in Ca$^{2+}$ mobilization assays (**Fig. 1**). This compound, although useful in limited electrophysiological studies, suffered from poor physiochemical properties (logP ~ 4.5) and lacked CNS penetration *in vivo* following systemic dosing in a rat PK/PBL study. Subsequent optimization efforts employing a DOS strategy revealed flat SAR around most regions of the VU010010 scaffold; however, multiple benzamide substitutions on the eastern side were well tolerated. This initial optimization campaign identified VU0152099 and VU0152100 as analogs of VU010010 possessing similar rM$_4$ PAM potency and efficacy but with improved solubility and ultimately acceptable *in vivo* rat PK profiles (**Fig. 1**). After a series of *in vitro* characterization experiments, which revealed that these compounds potentiated both ACh binding affinity and efficiency of G-protein coupling through an allosteric mechanism, these lead M$_4$ PAMs were used in rat behavioral studies to investigate the antipsychotic potential of selective M$_4$ activation *in vivo*. Both VU0152099 and VU0152100 exhibited robust and dose-dependent reversal of amphetamine-induced hyperlocomotion after I.P. injection with an absence of sedative effects, which provided the first reported behavioral evidence suggesting M$_4$ activation (or PAM) may be efficacious in the treatment of psychosis.
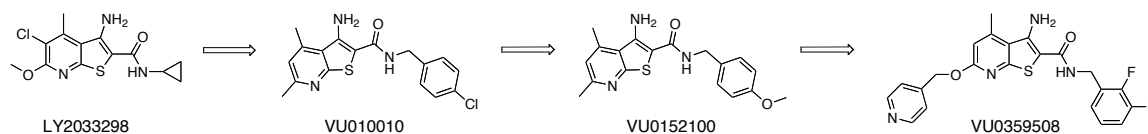


**Figure 1**. Optimization progression summary for M$_4$ PAMs based on a substituted thienopyridine scaffold.

A more comprehensive optimization campaign was then executed in order to further explore SAR and improve the metabolic stability of the VU010010-based $M_4$ PAM scaffold through an iterative analog library approach[126]. This involved introduction of diverse western amino or ether substitutions at the 6-position of the thienopyridine core, although only ether-linked basic amine moieties were well tolerated. Subsequent matrix-like derivatization of the eastern amide side chain led to the discovery of multiple lead compounds possessing high ACh CRC fold-shift (>40-60x at 30 μM) but relatively modest $EC_{50}$ values (~2 μM) at $rM_4$. Importantly, these newer analogs, exemplified by VU0359508 (**Fig. 1**), maintained complete subtype-selectivity, possessed improved metabolic stability *in vitro* (>90% parent remaining after 90 min incubation with rat liver microsomes), and displayed superior vehicle solubility in *in vivo* formulation studies (>10 mg/mL across multiple non-toxic carriers). However, these compounds exhibited poor/absent efficacy in reversal of amphetamine-induced hyperlocomotion following 56.6 mg/kg (I.P.) dosing in rat behavioral studies, which may have been due to their relatively lower potency compared to VU0152099 and VU0152100. Upon *in vitro* testing at the human $M_4$ receptor, these analogs exhibited more than 10x greater potency (~100-200 nM range), contributing to evidence that they could serve as future drug leads. Additionally, alignment with the refined $H_3$ antagonist pharmacophore model and subsequent competition binding experiments at $H_3$ suggest that this latest generation $M_4$ PAM series may also be used as a starting point for discovery and optimization of dual $M_4$ PAM/$H_3$ antagonist compounds, which could potentially confer more comprehensive antipsychotic utility in addressing both positive and cognitive system clusters of schizophrenia.

Although not discussed in Chapter III, preliminary *in vitro* data from experiments with a compound we discovered that is structurally related to the $M_4$ PAM scaffold suggests that certain modifications to the thienopyridine core confer a switch in mode of pharmacology from PAM to NAM/antagonist for this series. However, subtype-selectivity, mode of binding, and tractability in further optimization has not yet been determined. Discovery of a potent and selective $M_4$ NAM from our series of $M_4$ PAMs would provide a highly useful research tool that could be used to probe the effects of decreasing $M_4$ signaling within numerous neurobiology and therapeutic discovery contexts. Additional medicinal chemistry and *in vitro* characterization for such putative $M_4$ NAMs is required.

Beyond these chemical optimization possibilities, a number of *in vitro* and *in vivo* pharmacological studies may be pursued with the highly-selective $M_4$ PAMs described in Chapter III. Each of the lead compounds from this series lacked any detectable PAM activity at $M_1$, $M_2$, $M_3$, or $M_5$ in $Ca^{2+}$ mobilization assays; however, it is not known if this high selectivity is due to binding specificity for $M_4$ or if these compounds in fact bind to one or more of the other mAChRs but exhibit neutral cooperativity (i.e. pharmacological silence) with ACh. To address this question and to assess the potential for probe-dependent PAM activity at other mAChRs, a series of *in vitro* experiments may be performed. These could involve measurement of radioligand binding kinetics and/or functional activity of multiple orthosteric ligands in the absence and presence of a high concentration of $M_4$ PAM test compound at each of the other mAChR subtypes. If differences are seen in the presence of the test compound, such data would provide evidence that the compound does bind to other mAChR subtypes in addition to $M_4$.

Similar assays could be performed using lead compounds from many of the mAChR projects described in Chapter V as well, as the question of binding versus functional selectivity remains unanswered for most mAChR allosteric chemotypes.

In electrophysiology experiments using rodent hippocampal slices, the early $M_4$ PAM lead VU010010 was found to increase CCh-mediated depression of synaptic transmission at excitatory but not inhibitory synapses. Given the high expression of $M_4$ in the striatum and other components of the basal ganglia, additional electrophysiological investigation into the precise role(s) of $M_4$ in these brain regions would advance the understanding of $M_4$ basic neurobiology. Furthermore, more comprehensive evaluation of the effects of selective $M_4$ modulation within the therapeutic context of psychosis would shed additional light on the validity of this target. Although reversal of amphetamine-induced hyperlocomotion is a highly useful acute model for predicting DA-related positive symptom antipsychotic efficacy, additional behavioral studies including conditioned avoidance responding (CAR), reversal of PCP-induced disruption of PPI, and various models of cognition could be performed with our $M_4$ PAMs. For studies with the more recent lead compounds (e.g. VU0359508), which possess improved physiochemical properties and metabolic stability but only modest $M_4$ potency, co-administration of a low dose of mAChR agonist may provide a solution to the lack of *in vivo* effect observed with these compounds. This strategy was used in the case of the original $hM_4$ PAM LY2033298, which lacked efficacy in a rodent CAR behavioral study when dosed alone but exhibited robust effects in the presence of a low dose of oxotremorine (that itself had no effect either)[127]. Alternatively, the intermediate lead compounds VU0152099 or VU0152100 could be used. Such experiments would provide valuable insight to the

question(s) about which mAChR(s) likely mediated the antipsychotic effects observed in clinical trials of the $M_1/M_4$-preferring agonist xanomeline.

## $M_5$ PAMs based on VU0238429

Following discovery of VU0119498 as a small molecule mAChR PAM with similar *in vitro* potency, efficacy, and selectivity for $M_1$, $M_3$, and $M_5$, SAR from a large matrix-analog library synthesis revealed that –$OCF_3$ substitution at the 5-position of the isatin headpiece conferred preference for $M_5$ activity. This was exemplified by the early lead VU0238429, the first reported $M_5$-preferring PAM, which possessed approximately 1 μM potency, induced a 14x left-shift of an $M_5$ ACh CRC at 30 μM, and exhibited >30x selectivity versus $M_1$-$M_4$ in functional $Ca^{2+}$ mobilization assays. Subsequent radioligand binding experiments indicated that VU0238429 modulates $M_5$ by enhancement of ACh affinity through an allosteric mechanism. This compound was then used as the lead structure for an exhaustive chemical optimization campaign aimed at further exploring SAR and obtaining analogs with superior drug-like pharmacological properties. Guided by specific hypothesis-driven strategies, dozens of iterative parallel libraries were generated to systematically probe the effect of various structural modifications to the $M_5$ PAM scaffold on potency, efficacy, and subtype-selectivity. These efforts led to the discovery of intermediate leads such as VU0365114, which possessed modestly lower potency (2.7 μM) but induced a pronounced >50x left-shift of the ACh CRC at 30 μM with even greater subtype-selectivity than VU0238429. Other SAR revealed even more $M_5$ subtype-selectivity could be engendered by introduction of biaryl/heterobiaryl ethers

to the southern region of the scaffold, as exemplified by VU0400265, which possessed a 1.9 μM PAM $EC_{50}$ at $M_5$ and lacked any detectable activity at $M_1$-$M_5$ at up to 30 μM.

Comprehensive survey of SAR around the isatin headpiece confirmed the vital importance of the 5-$OCF_3$ group for $M_5$ PAM activity, as more than 40 different ether substitutions (including trifluoroethyl and trifluoropropyl) were inactive. However, migration of this moiety to the 6-position was tolerated and actually improved efficacy, but simultaneously compromised subtype-selectivity. Efforts to replace the 3-keto component of the isatin core successfully identified the gem-difluoro but not the spirocyclopropyl or tertiary alcohol isosteres as an optimal replacement, which conferred a moderate increase in potency, efficacy, and subtype-selectivity while removing the DMPK-related liabilities of the isatin structure. In libraries containing *ortho*-fluoro, *meta* biaryl/heterobiaryl, or *meta* biaryl/heterobiaryl ether substitutions on the southern benzyl ring, SAR was flat and $M_5$ PAM activity was essentially lost. These and other SAR findings from this optimization campaign are summarized in **Figure 2**.
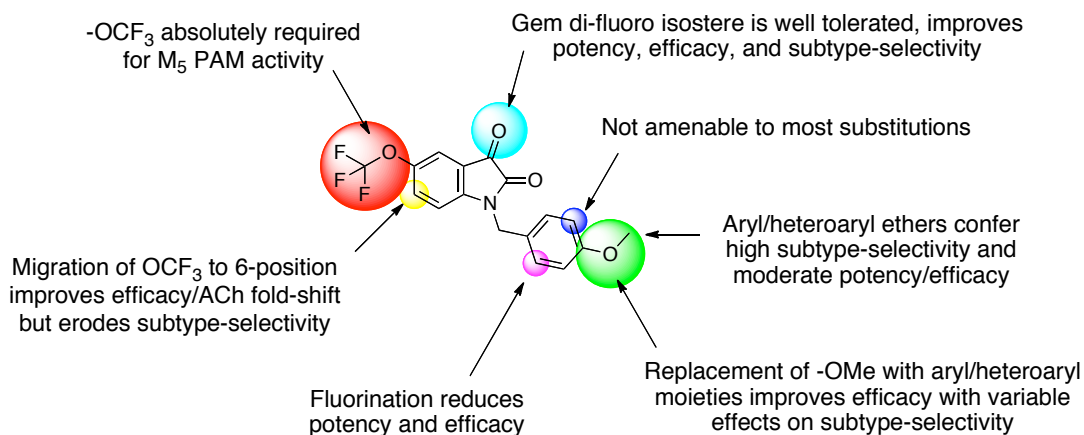


**Figure 2**. Abbreviated SAR summary for $M_5$ PAM optimization based on early lead VU0238429.

In general, the *para* position on the southern benzyl ring proved most amenable to derivatization, and therefore efforts to improve the physiochemical properties and reduce identified ancillary/off-target pharmacology liabilities focused on this region. Analogs bearing linear heterobiaryl southern motifs containing one or more nitrogens in the proximal and/or distal aromatic ring(s), such as the *p*-phenyl substituted 3-picolyl compound VU0415478, were synthesized and found to possess improved aqueous solubility and also maintained $M_5$ PAM activity with moderate potency and subtype-selectivity, but with slightly diminished efficacy. Similar analogs containing heterobiaryl ethers, such as the fluoro-pyridine analog VU0414747, were found to possess solubility, potency, and subtype-selectivity comparable to or greater than that of the straight non-ether heterobiaryl analogs, but with generally lower PAM efficacy.

However, despite their improved physiochemical properties, both of these later sub-series still suffered from numerous ancillary pharmacological activities revealed by a 10 μM competition binding screen across a large panel of off-targets. Ultimately, after hundreds of compounds were synthesized and evaluated, it proved difficult to obtain analogs with the ideal combination of each important parameter such as potency, efficacy, subtype-selectivity, physiochemical properties (i.e. logP), lack of ancillary pharmacology, and drug-like structural aspects favorable for DMPK. The best analogs for each of these attributes were often from different libraries and chemical sub-series; however, a number of lead compounds containing relatively balanced pharmacological profiles, such as VU0402734, represent highly suitable $M_5$ PAM research tools. An overview of the optimization progression for this project, which highlights key early, intermediate, and advanced lead compounds, is presented in **Figure 3**.

VU0415478

Advanced M$_5$ PAM Lead
EC$_{50}$ = 3.8 uM, 76% ACh max
ACh fold-shift @ 30 uM = 14x
Subtype-selectivity: >15x

VU011948

M$_1$, M$_3$, M$_5$ PAM HTS Hit
EC$_{50}$ = 4.1 uM, 74% ACh max
ACh fold-shift @ 30 uM = n.d.
Subtype-selectivity: poor

VU0238429

Early M$_5$ PAM Lead
EC$_{50}$ = 1.1 uM, 91% ACh max
ACh fold-shift @ 30 uM = 14x
Subtype-selectivity: >30x

VU0365114

Intermediate M$_5$ PAM Lead
EC$_{50}$ = 2.7 uM, 85% ACh max
ACh fold-shift @ 30 uM = >50x
Subtype-selectivity: >>30x

VU0402734

Intermediate M$_5$ PAM Lead
EC$_{50}$ = 0.87 uM, 90% ACh max
ACh fold-shift @ 30 uM = n.d.
Subtype-selectivity: >>30x

VU0400265

Intermediate M$_5$ PAM Lead
EC$_{50}$ = 1.9 uM, 75% ACh max
ACh fold-shift @ 30 uM = n.d.
Subtype-selectivity: >>>30x

VU0414747

Advanced M$_5$ PAM Lead
EC$_{50}$ = 1.5 uM, 62% ACh max
ACh fold-shift @ 30 uM = 6x
Subtype-selectivity: >30x

**Figure 3**. Overview of key lead compounds identified during M$_5$ PAM optimization project.

Prior to *in vivo* use, additional PK/PBL studies will be necessary to determine which compound(s) may be suitable for systemic dosing in rodent neurobehavioral and neurochemical investigations. The careful use of experimental controls and thoughtful data interpretation will be required for future studies utilizing these novel compounds given the off-target activities exhibited by many of the lead analogs. However, further optimization could be directed at this scaffold to address these activities, prior to initiation of neurobiology studies. Such efforts could be focused on heterocyclic replacement of the southern benzyl ring with isosteres such as thiophene or *m*-fluoropyridines. Likewise, introduction of one or more heteroatom(s) to the western benzo ring of the isatin core has yet to be performed. These modifications are likely to prove tolerable for $M_5$ PAM activity and may reveal highly useful SAR.

Due to the discrete neuronal localization of $M_5$, which is essentially limited to expression on mesolimbic and nigrostriatal DA projection neurons of the VTA and SNc, respectively, pharmacological investigation into the effects of selective $M_5$ activation will likely focus on these midbrain DA pathways and on their neurobehavioral correlates[34,37]. As discussed in Chapter I, murine experiments measuring stimulation of forebrain DA efflux in the NAc following administration of a muscarinic agonist directly into the VTA or after electrical stimulation of hindbrain cholinergic neurons of the pons that project to the VTA (i.e. Ch.V-VI) found that genetic deletion of $M_5$ abolished the increase in forebrain DA efflux that was observed in WT mice[63]. Thus, under the hypothesis that post-synaptic $M_5$ receptors in the VTA located on DA cell bodies and/or dendrites mediates ACh-induced neuronal excitation leading to increased DA neurotransmassion in the NAc, pharmacological $M_5$ activation with a selective PAM would likely potentiate

DA efflux in the NAc under either of the conditions used in the two $M_5$-KO mice experiments. However, it is important to note that *in vivo* microdialysis studies have determined that extracellular/synaptic ACh levels in the VTA are near or below detection thresholds under resting conditions when no pharmacological or electrical stimulus of mesolimbic signaling is present, and often the measurement of changes in extracellular ACh in this region requires infusion of a low dose of an AChE inhibitor through the dialysis probe[147,148]. It is therefore unlikely that administration of an $M_5$ PAM by itself would increase forebrain DA levels, unless it is co-administered with a submaximal dose of muscarinic agonist or following electrical stimulation of hindbrain cholinergic neurons to increase ACh concentration in the VTA. Analogous experiments focused on the parallel nigrostriatal DA pathway could be also be performed. These would provide useful evidence for or against the putative functional role of $M_5$ on midbrain DA neurons that has been hypothesized from results of $M_5$-KO mice studies.

In animal models of drug reward, $M_5$ PAM administration (be it directly to the VTA or SNc, or via systemic dosing with a suitable compound) would provide behavioral insight complementary to the neurochemical studies. For instance, an $M_5$ PAM would be hypothesized to exacerbate drug reward behavior in cocaine self-administration and CPP models as well as worsen withdrawal-induced anxiety behaviors. Similarly, amphetamine-induced hyperlocomotion would presumably be increased by dosing of an $M_5$ PAM; though, ideally a dose of amphetamine low enough to cause only a subtle elevation in locomotor activity would be used to provide a window for the $M_5$ PAM effect. Additional behavioral paradigms (e.g. CAR) traditionally used to predict antipsychotic efficacy could be used to further characterize an $M_5$ PAM *in vivo*.

Beyond these experiments focused primarily on the functions of neuronal $M_5$, additional studies could be performed to assess the putative role of $M_5$ in mediating ACh-induced cerebrovasodilation. $M_5$-KO mice lack ACh-induced dilation of multiple cerebral blood vessels including capillary beds, arterioles, and larger arteries of the brain[62]. Furthermore, absence of $M_5$ but not $M_1$ or $M_3$ results in a constitutive constriction of the cerebrovasculature with a corresponding decrease in CBF[66]. Thus, measurement of cerebral blood vessel diameter using cranial window techniques, *in vivo* magnetic resonance angiography, and/or *ex-vivo* tissue preparations following administration of an $M_5$ PAM could provide pharmacological evidence to support the hypothesized vasodilatory role of $M_5$ in the brain. Control experiments examining the effect of an $M_5$ PAM on ACh-induced vasodilation of peripheral blood vessels (where $M_3$ is highly-likely the mediating receptor subtype) could be used to indicate whether the $M_5$ PAM compound maintains subtype-selectivity in native tissues. Hemodynamic measures of CBF following $M_5$ PAM dosing could be obtained using laser doppler flowometry methods *in vivo* as well.

The question of whether or not the neuroanatomical and behavioral cognitive defects observed in $M_5$-KO mice are due to absence of neuronal or cerebrovascular $M_5$ (or perhaps both) could be potentially addressed by use of an $M_5$ PAM lacking BBB penetration and thereby acting primarily on non-neuronal $M_5$ receptors of the brain's blood vessels. If the neuronal atrophy and cognitive defects arising from genetic $M_5$ deletion are due solely to decreased CBF arising from cerebrovasoconstriction, use of a non CNS-penetrant compound would be hypothesized to exert pro-cognitive effects, presumably by enhancement of CBF alone. Conversely, if such effects were absent with

a non CNS-penetrant compound but were then found following dosing of a compound that provides high brain exposure, such results would provide evidence that lack of neuronal $M_5$ in the $M_5$-KO mice studies contributes to the cellular and behavioral defects in cognitive function. However, each of these potential studies would require important controls, such as the use of muscarinic antagonists (e.g. atropine) to reverse or compete with any observed positive $M_5$ PAM effects on cerebral hemodynamics or on pro-cognitive behavioral efficacy, as well as the use of $M_5$-KO mice. Unfortunately, these latter studies aimed at exploring the possible role of $M_5$ in learning and memory may be confounded by a lack of 'window' for which to observe such effects. Put differently, enhancement of cognition beyond a normal physiological state in WT animals is generally more challenging to detect. Conversely, neuroanatomical, electrophysiological, and behavioral measures of learning and memory are generally more sensitive to detection of impairments induced by experimental or pharmacological agents. Therefore, discovery of subtype-selective and drug-like $M_5$ NAMs or antagonists could potentially provide more useful and straightforward insight to this particular question.

As presented in Chapter IV, preliminary data suggests that incorporation of basic alkylamine moieties tethered by an ether at the 5-position of the isatin headpiece on the $M_5$ PAM scaffold may engender $M_5$ NAM activity. SAR for this sub-series has revealed additional modifications that improve inhibitory potency of these putative $M_5$ NAMs in functional assays. However, the subtype-selectivity and mode of binding for these compounds has yet to be determined. Indeed, the first series of $M_5$ NAMs may arise from our novel series of $M_5$ PAMs, which would provide a richer set of available pharmacological tools for use in obtaining a thorough understanding $M_5$ neurobiology.

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