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Bioorganic & Medicinal Chemistry Letters 18 (2008) 885–890
Bioorganic & Medicinal Chemistry Letters

Synthesis and SAR of selective muscarinic acetylcholine receptor subtype 1 (M1 mAChR) antagonists

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Received 29 November 2007; revised 18 December 2007; accepted 19 December 2007
Available online 4 January 2008

Abstract—This Letter describes the synthesis and SAR, developed through an iterative analogue library approach, of a novel series of selective M1 mAChR antagonists for the potential treatment of Parkinson's disease, dystonia and other movement disorders. Compounds in this series possess M1 antagonist IC_{50}s in the 441 nM–19 μM range with 8- to >340-fold functional selectivity versus rM2–rM5.

The muscarinic acetylcholine receptors (mACHRs) are members of the G protein-coupled receptor (GPCR) family A that mediate the metabotropic actions of the neurotransmitter acetylcholine.[1,2] To date, five distinct subtypes of mAChRs (M1–M5) have been cloned and sequenced. M1, M3, and M5 activate phospholipase C and calcium through Gq whereas M2 and M4 block the action of adenylyl cyclase through Gi/o.[1,2] The cholinergic system, mediated by mAChRs, plays a critical role in a wide variety of CNS and peripheral functions including memory and attention mechanisms, motor control, nociception, regulation of sleep wake cycles, cardiovascular function, renal and gastrointestinal function, and many others.[1–4] As a result, agents that can selectively modulate the activity of mAChRs have the potential for therapeutic use in multiple pathological states. However, due to high sequence conservation within the orthosteric binding site of the five mAChR subtypes, it has been historically difficult to develop mAChR subtype selective ligands.[1–5]

To date, the majority of reported muscarinic antagonists are unselective, such as a scopolamine, **1**.[6] Recently, pirenzapine, **2**, has emerged as a relatively selective M1 receptor antagonist (20- to 50-fold versus M2–M5) and there are numerous reports of moderately selective M3 antagonists (20- to 50-fold versus M2) such as **3**.[7] Interestingly, the most selective M1 antagonist, MT7, **4**, the 65 amino acid peptide (>1000-fold versus M2–M5) was derived from venom extracts of the green mamba snake (Fig. 1).[8] Based on brain expression and cellular localization, data from mAChR knock-out mice, and clinical trials with muscarinic agents, the M1 mAChR subtype is an attractive molecular target for the treatment of Alzheimer's disease (AD), Parkinson's disease (PD), and dystonia due to its role in cognition and motor control.[9] Indeed, pan-muscarinic agonists, such as the M1/M4 preferring xanomeline, showed efficacy in Phase III clinical trials in AD patients; however, activation of peripheral M2 and M3 receptors led to intolerable adverse side effects.[10] Moreover, anti-cholinergic agents have also demonstrated efficacy in both PD and dystonia patients, and this benefit is believed to be derived from antagonism of the M1 mAChR subtype;

Keywords: Muscarinic acetylcholine receptors; M1; Antagonist; Dystonia; G protein-coupled receptors.
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doi:10.1016/j.bmcl.2007.12.051

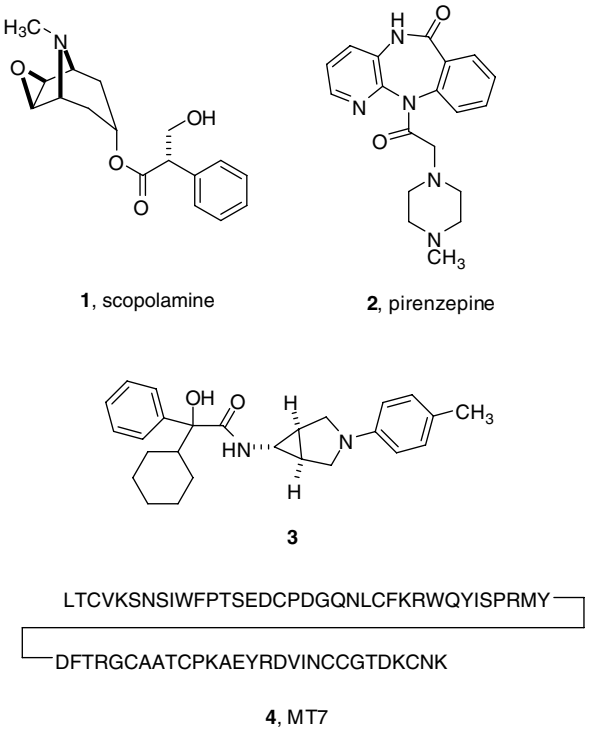


Figure 1. Structures of representative mAChR antagonists.

however, the relative contributions from M4 are unclear.[1–10] In order to probe the role of M1 antagonism as a potential therapeutic approach for Parkinson's disease, dystonia, and other movement disorders, potent small molecule mAChR antagonists are required with a high degree of M1 versus M4 selectivity for study in preclinical models.

The Vanderbilt Screening Center for GPCRs, Ion Channels and Transporters, and the companion Chemistry Center, were established as members of the Molecular Libraries Screening Center Network (MLSCN) initiated and supported by the NIH Molecular Libraries Roadmap.[11,12] The MLSCN is a nationwide consortium of facilities that provide high-throughput small molecule screening and medicinal chemistry expertise for the development of chemical probes for use as tools to explore biological targets/pathways for which small molecule tools are unavailable.[12] One such target which lacks the appropriate small molecule tools are the muscarinic acetylcholine receptors (mAChRs).[1–10]

Based on this unmet need in the scientific community, our MLSCN Center initiated an effort to identify potent small molecule mAChR antagonists with high specificity for M1 for use as a chemical probe and lead for further optimization toward a novel therapeutic. Toward this goal, we optimized a real-time cell-based calcium-mobilization assay employing a rat M1/CHO cell line (Z′ averaged 0.7), screened a 63,656 member MLSCN compound library, and identified 2179 primary M1 antagonist hits.[13] Of these primary hits, 1665 were available from Biofocus-DPI for re-test, and duplicate testing afforded 723 confirmed hits (43%). These compounds were then counter-screened against an mGluR4/CHO cell line which eliminated 9 hits. The remaining compounds were tested in triplicate in 10-point concentration–response curves against both rat M1/CHO and rat M4/CHO cells to identify compounds with ~10-fold selectivity for M1 versus M4, our initial cutoff for a lead. While the vast majority of compounds displayed no selectivity for M1 versus M4, we identified two related structures based on a *N*-(4-(4-ethylpiperazin-1-yl)phenyl amide scaffold, **5** (rM1 $IC_{50} = 0.49\ \mu M$, rM4 $IC_{50} = 7.9\ \mu M$) and **6** (rM1 $IC_{50} = 0.58\ \mu M$, rM4 $IC_{50} = 5.1\ \mu M$), which displayed ~16- to ~9-fold selectivity, respectively, for rM1 versus rM4 and displayed comparable inhibition of human M1 (Fig. 2).

Analogues of **5** and **6** were synthesized in a library format according to Scheme 1. Both requisite anilines **7** and **8**, 3-chloro-(4-(4-ethylpiperazin-1yl)aniline and (4-(4-ethylpiperazin-1yl)aniline, were commercially available and acylated under standard conditions employing polymer-supported reagents and scavengers to afford 24-member libraries of analogues **9** and **10**, respectively.[14] In the initial lead optimization phase, we prepare a 24-member library employing a diversity set of acid chlorides containing aromatic, alphatic, polar, basic, and acidic moieties in order to rapidly probe the breadth and scope of the SAR; subsequent libraries will be more focused. As the chemistry was straightforward, we elected to re-synthesize the parent compounds **5** and **6** within the library. All analogues were purified by mass-guided HPLC to analytical purity.[15] Surprisingly, all analogues **10**, as well as the re-synthesized parent **6**, were found to be inactive on rM1. Moreover, upon re-synthesis in the library, **5** lost considerable efficacy as an M1 antagonist (rM1 $IC_{50} = 13\ \mu M$), but still displayed ~10-fold selectivity versus rM4 ($IC_{50} > 150\ \mu M$).[16] Not surprisingly, analysis of the original screening samples **5** and **6** indicated that there were several impurities in the wells, and we elected not to pursue a complex deconvolution exercise. Despite these findings, the strategy of employing library synthesis and exploding SAR around a primary HTS hit proved advantageous for **5**, as analogues **9** proved to possess intriguing mAChR selectivity profiles.

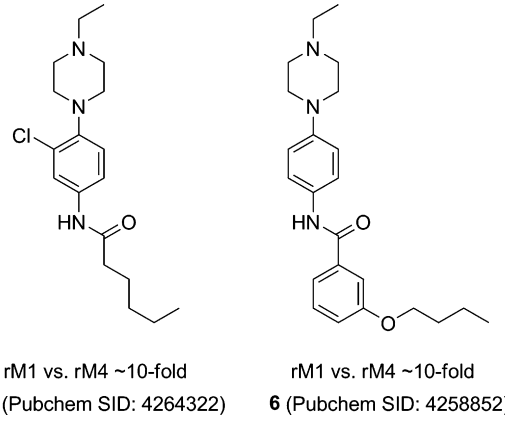
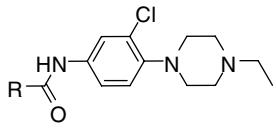
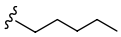
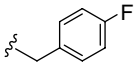
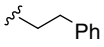
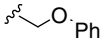
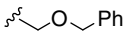
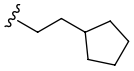


Figure 2. HTS leads **5** and **6**, rM1 antagonists with selectivity versus rM4 of ~10-fold in the primary assays.

Table 1. Structures and mAChR activities of analogues **9**

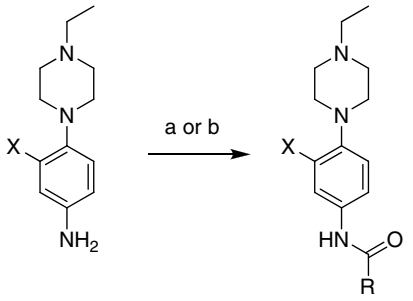


9

Compound	R	M1 IC_{50}[a] (μM)	M2 IC_{50}[a] (μM)	M3 IC_{50}[a] (μM)	M4 IC_{50}[a] (μM)	M5 IC_{50}[a] (μM)
5		13.2	>150	>150	>150	>150
9a		>150	>150	>150	>150	>150
9b		4.6	>150	>150	>150	>150
9c		5.0	>150	>150	66	>150
9d		5.6	>150	>150	>150	>150
9e		1.15	29	24	20	13
9f		1.1	52	70	18	7.6
9g		3.3	>150	>150	>150	>150
9h		18.8	>150	>150	>150	>150
9i		0.44	3.5	3.1	>150	1.1
9j		>150	>150	>150	>150	>150

[a] IC_{50}s are an average of three independent experiments using rat mAChR (CHO) cell lines.

Table 1 highlights SAR and mAChR selectivity for analogues **9** of HTS hit **5**. In general, SAR was rather flat for this series. Truncation of the pentyl side chain of **5** to simpler aliphatic chains, such as *n*-propyl **9a**, led to a total loss of rM1 antagonist activity. Cyclization to form a cyclohexyl ring, as in **9b**, afforded a selective rM1 antagonist (rM1 IC_{50} = 4.6 μM, >32-fold selective versus rM2–rM5), and a 3-fold increase in potency relative to HTS lead **5**. The phenyl analogue **9c** maintained M1 activity relative to **9b**, but mAChR selectivity at rM4 began to erode. However, conversion to a benzyl moiety **9d** once again maintained rM1 activity (rM1 IC_{50} = 5.6 μM) and also displayed >26-fold selectivity for rM2–rM5 (IC_{50}s > 150 μM). Further chain homologation to the phenethyl congener **9f** afforded a low micromolar potency rM1 antagonist (rM1 EC_{50} = 1.1 μM) with high mAChR subtype selectivity (47-fold versus rM2, 63-fold versus rM3, 16-fold versus rM4 and 6.9-fold versus rM5). Introduction of a cyclic constraint in the form of a cyclopropyl moiety in the phenethyl chain as in **9e** provided a compound with an in vitro profile roughly equivalent to **9f**. Incorporation of an oxygen atom in the phenylether as in **9g** provided an M1 antagonist of modest potency (rM1 IC_{50} = 3.3 μM), but with >45-fold selectivity versus rM2–rM5 (Fig. 3). Replacement of the phenyl moiety with a cyclopentyl group afforded compound **9i**, with an rM1 IC_{50} of 441 nM and with >340-fold selectivity versus M4, but modest selectivity versus rM2, rM3, and rM5 (7.9-fold, 7-fold, and 2.4-fold, respectively). Compound **9i** possessed the potentcy requirements for an MLSCN M1 antagonist probe molecule (affinity/activity >500 nM) as well as the required selectivity (>10-fold selectivity) versus rM4 (>340-fold selectiv-



Scheme 1. Library synthesis of analogues **9** and **10**. Reagents and conditions: (a) i—PS-DCC, HOBt, RCOOH, ii—MP-CO_3^{2-}, 62–98% or (b) i—RCOCl, PS-DIEA, ii—PS-trisamine, 79–98%. All library compounds were purified by mass-guided HPLC to >98% purity.[15]

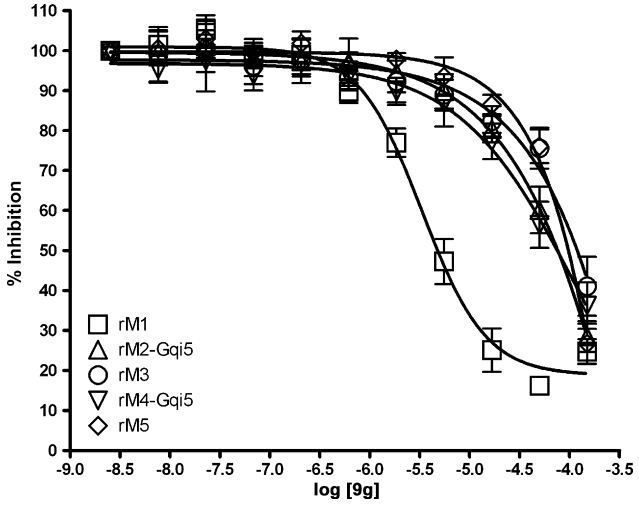


Figure 3. Concentration–response curves for **9g** on rat M1–M5. Compound **9g** displays >45-fold selectivity versus M2–M5. Curves represent the average of three separate experiments.

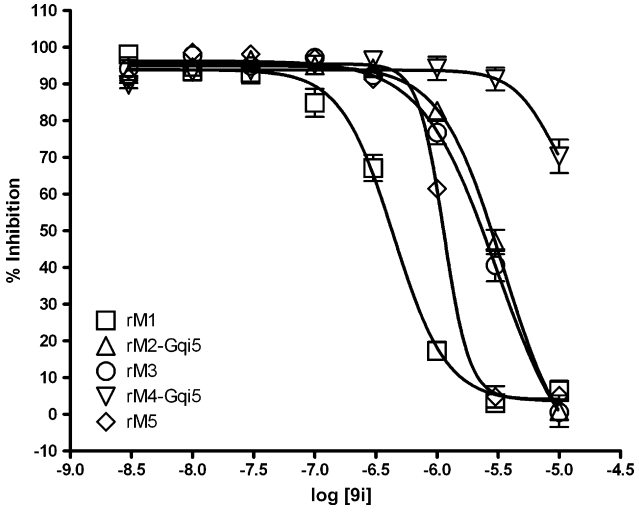


Figure 4. Concentration–response curves for **9i** on rat M1–M5. Compound **9i** displays 7.9-fold selectivity versus M2, 7-fold selectivity versus M3, >340-fold selectivity versus M4, and 2.4-fold selectivity versus M5. Curves represent the average of three separate experiments.

ity).[11,12] When evaluated against other receptors and enzymes, **9i** displayed no significant ancillary pharmacology (see Fig. 4).

Our attention now focused on examining mAChR subtype selectivity in binding assays to determine if the functional selectivity was mirrored in competition radioligand binding experiments and to determine whether **9i** was binding at the orthosteric versus an allosteric binding site. For these experiments, we evaluated the ability of **9i** to displace [^{3}H]-*N*-methylscopolamine ([^{3}H]-NMS), an orthosteric radioligand, versus all five mAChR subtypes with atropine as a positive control (Fig. 5).[17] In the event, **9i** was shown to possess an rM1 K_i of 12.7 nM with selectivity versus rM2–rM5 (6- to 35-fold) and atropine controls demonstrated pan-mAChR antagonism as anticipated (Table 2). Gratifyingly, the functional rM1 versus rM4 selectivity was

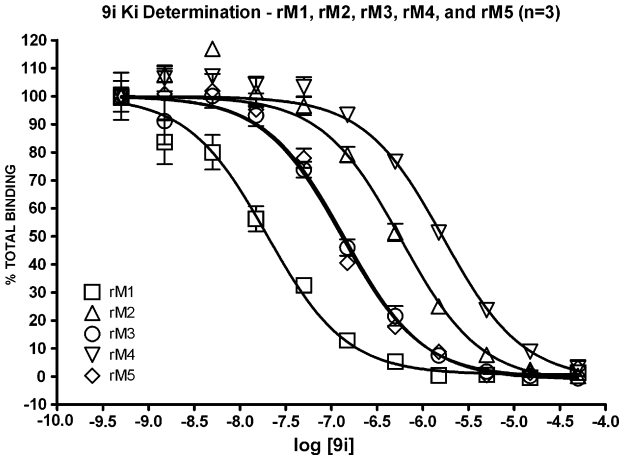
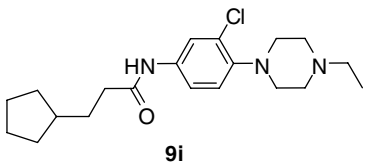


Figure 5. [^{3}H]-NMS competition binding experiments for **9i** on rat M1–M5. Compound **9i** displays 27-fold selectivity versus M2, 6-fold selectivity versus M3, 35-fold selectivity versus M4, and 7-fold selectivity versus M5. Curves represent the average of three separate experiments.

Table 2. K_i determinations and binding fold selectivity for **9i**



9i

mAChR	**9i** K_i[a] (nM)	Fold selectivity (vs M1)	Atropine K_i (nM)[a]
M1	12.7 ± 1.7		0.88 ± 0.04
M2	338.0 ± 13.5	27	2.69 ± 0.20
M3	74.8 ± 4.3	6	0.96 ± 0.03
M4	445.1 ± 23.8	35	0.56 ± 0.01
M5	85.7 ± 15.9	7	1.80 ± 0.11

[a] K_is are an average of three independent experiments using rat mAChR (CHO) cell lines.

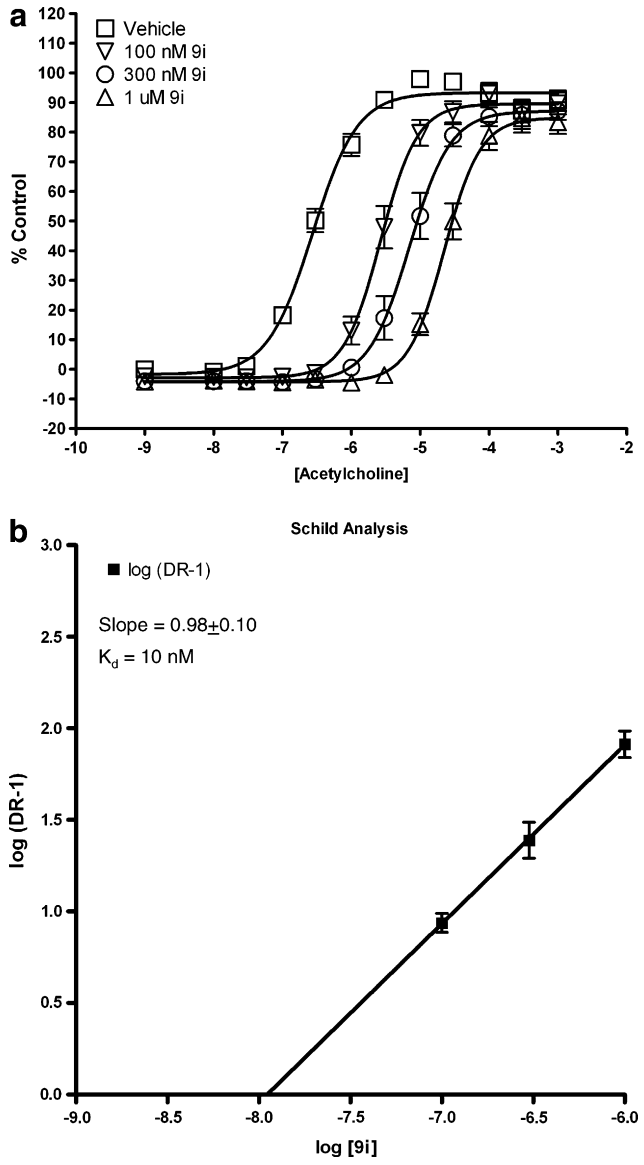


Figure 6. PI hydrolysis studies and Schild analysis for **9i** on rat M1. These data strongly support an orthosteric mode of binding for **9i**. Data represent the average of three separate experiments.

mirrored in the radioligand competition binding experiment, but the fold selectivity had diminished ~10-fold. We often observe shifts in potency and selectivity between binding and functional assays, and we view the functional activity/selectivity as a more important measure of mAChR selectivity as a binding event does not dictate a functional response, that is, mAChR inhibition.

Phosphoinostitide (PI) hydrolysis studies and Schild analysis were performed on **9i** to confirm its activity in an alternate signaling pathway modulated by M1 and to further elucidate its binding mode. As shown in Figure 6, **9i** causes a dose-dependent rightward shift of the ACh concentration–response curve in a PI hydrolysis experiment which translates in a Schild analysis to a K_d of 10 nM and a slope of 0.98 ± 0.10. These data strongly support the [^{3}H]-NMS binding data and indicate that **9i** is an orthosteric M1 antagonist; however, they do not rule out a binding mode wherein **9i** partially overlaps with the orthosteric binding site which could account for the observed competitive binding with [^{3}H]-NMS and high rM1 versus rM4 subtype selectivity.[17] Nor do these data rule out the possibility that **9i** is in fact binding to a non-overlapping allosteric site which causes a conformational exclusion of the orthosteric ligand binding site. Mutagenesis and off-rate experiments are planned to address these possibilities.

In summary, an MLSCN M1 antagonist chemical probe development project afforded **9i**, a selective rM1 versus rM4 orthosteric antagonist which meets the criteria for a small molecule MLSCN chemical probe. Our hit-to-lead strategy of iterative library synthesis to explode SAR and to re-synthesize HTS hits within the first generation libraries proved highly beneficial, as the intial HTS 'hits' lost considerable activity upon re-synthesis and evaluation. Had we employed a more traditional approach wherein HTS 'hits' were first re-synthesized and evaluated prior to generating analogues, these series would not have been pursued further, and **9i** would not have been identified. Clearly, serendipity played a major role in the success of this lead optimization strategy, but this is a high risk approach that must be judiciously employed based on the chemistries involved, the assay capacity, and the overall cost. Further refinements and in vitro/in vivo pharmacology will be reported for this class of M1 antagonists in due course.

Acknowledgments

The authors thank the NIH, NIMH and the MLSCN for funding of the Vanderbilt Screening Center for GPCRs, Ion Channels & Transporters (3U54MH074427), the Chemistry Supplement (3U54MH074427-02S1) and the XO1 (1XO1MH077606-01) M1 antagonist screening application which enabled this work.

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12. For information on the Vanderbilt Molecular Library Screening Center Network Center please refer to: http://www.vanderbilt.edu/mlscn/Templates/index.htm.
13. *Functional assay*. Chinese Hamster Ovary (CHO) cells containing rat M1 (rM1) receptor were plated at 10,000 cells/well and rM2/Gqi5, rM3, rM4/Gqi5, and rM5 were all plated in 384-well plates at 25,000 cells/well in assay media 1 (F12 (Ham), 10% FBS, 2 mM GlutaMAX (Invitrogen), and 20 mM HEPES), except rM4/Gqi5 which used assay media 2 (DMEM, 20 mM HEPES, 10% FBS, 2 mM L-glutamine, 1× non-essential amino acids (NEAA), and 1 mM Na pyruvate). The plates were incubated overnight at 37 °C in 5% CO_2. Media were removed and assay buffer (Hanks' Balanced Salt Solution, 20 mM HEPES, and 2.5 mM probenecid, pH 7.4) containing 4.0 μM Fluo4-AM dye (Invitrogen) was added. Cells were incubated for 45 min (37 °C, 5% CO_2) for dye loading. Cell plates were loaded into the Hamamatsu FDSS equipped with 480 nm excitation and 540 nm emission filters. Test compound in assay buffer + 0.1% DMSO was added at 5 s and simultaneously the plate was kinetically imaged. Subsequently, 8 nM acetylcholine (EC_{80}) in assay buffer was added at 197 s and imaging continued for a total of 4 min acquisition time. DMSO (0.1%), compound vehicle, and 80 nM acetylcholine (EC_{Max}) were added to each plate as controls. Compounds were tested in concentrations ranging from 150 μM to 2.5 nM in triplicate on three different days. Kinetic data were transformed and fit with GraphPad Prism version 4.0 to a 4 parameter logistic equation to determine IC_{50} values.
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16. Typical experimental for the synthesis of **9i**: To a solution of 3-chloro-4-(4-ethylpiperazin-1-yl)aniline **7** (100 mg, 0.417 mmol) in 9:1 DMF:DIEA (2 mL) was added 3-cyclopentyl propanoyl chloride (63.9 μL, 0.417 mmol) all at once. The reaction mixture was stirred at room temperature for 12 h at which time it was determined complete by LC/MS. The reaction mixture was concentrated in situ, redissolved in 1 mL DMSO, and purified on the Agilent 1200 preparative LCMS. Concentration of purified fractions afforded the TFA salt of *N*-(3-chloro-4-(4-ethylpiperazin-1-yl)phenyl)-3-cyclopentylpropanamide **9i** as a white solid (80 mg, 42%). ^{1}H NMR (400 MHz, DMSO-d_6)δ10.03 (br s, 1H), 7.84 (d, J = 2.0 Hz, 1H), 7.44 (dd, J = 2.0, 8.4 Hz, 1H), 7.16 (d, J = 8.4 Hz, 1H), 3.57 (m, 2H), 3.35 (m, 2H), 3.21 (m, 2H), 3.13 (m, 2H), 2.99 (m, 2H), 2.29 (t, J = 7.6 Hz, 2H), 1.73 (m, 3H), 1.58 (m, 4H), 1.47 (m, 2H), 1.25 (t, J = 7.2 Hz, 2H), 1.08 (m, 3H). ^{13}C NMR (100 MHz, DMSO-d_6)δ 171.4, 142.3, 136.2, 127.5, 121.1, 120.6, 118.5, 50.8, 50.7, 48.1, 39.3, 35.7, 32.0, 31.3, 24.7, 8.9; LC–MS, single peak, 2.79 min, *m/e*, 364.2 (M+1).
17. *Phosphoinositide (PI) hydrolysis.* Hamster Ovary (CHO) cells containing rat M1 (rM1) were plated at 120,000 cells per well in standard growth media (F12 (HAM), supplemented with 10% fetal bovine serum, and 20 mM HEPES) in 24-well plates 24 h prior to assay. Cell media were replaced late in the day with standard growth media containing 1 μCi/mL [^{3}H]inositol (Perkin-Elmer LAS) and cells were incubated overnight at 37 °C in 5% CO_2. [^{3}H]inositol-containing media were removed and the rM1 cells were treated with either vehicle or fixed concentrations of antagonist (2×, 500 μL) in a modified Krebs'-bicarbonate buffer (108 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 10 mM Glucose, pH 7.4) equilibrated to 37 °C and 5% CO_2, and supplemented with 30 mM LiCl. Following vehicle or antagonist addition, agonist was added (2×, 500 μL) in modified Krebs'-bicarbonate buffer and cells were incubated for 1 h at 37 °C and 5% CO_2. The accumulation of phosphoinositides was terminated by aspiration, followed by the addition of 1 mL of 10 mM formic acid. Cells were incubated in the 10 mM formic acid for at least 30 min at room temperature to insure extraction of phosphoinositides. Columns packed with a 1 mL bed of AG® 1-X8 Resin 100–200 mesh anion-exchange resin (formate form) (Bio-Rad; Hercules, CA) were washed with 10 mL of water twice. Following the water washes, the entire 1 mL sample volumes were added to columns, avoiding the transfer of any cells. Columns were washed with 2 mL of water, then with 10 mL of water, followed by 10 mL of 5 mM myo-inositol made up in water. Total phosphoinositides (PIs) were eluted with 10 mL of 0.1 M formic acid/0.2 M ammonium formate into vials containing 3a70B liquid scintillation cocktail (Research Products International; Elk Grove Village, IL) and radioactivity was measured by liquid scintillation counting. Following use, columns were regenerated with 10 mL of 0.1 M formic acid/1 M ammonium formate and washed with 30 mL of water. Data were fit with GraphPad Prism version 4.0 to a 4 parameter logistic equation to determine IC_{50} values and Schild Dose-Ratios.