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Continued optimization of the MLPCN probe ML071 into highly potent agonists of the hM$_1$ muscarinic acetylcholine receptor

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ABSTRACT

This Letter describes the continued optimization of the MLPCN probe molecule ML071. After introducing numerous cyclic constraints and novel substitutions throughout the parent structure, we produced a number of more highly potent agonists of the M$_1$ mAChR receptor. While many novel agonists demonstrated a promising ability to increase soluble APPα release, further characterization indicated they may be functioning as bitopic agonists. These results and the implications of a bitopic mode of action are presented.

Continued efforts to develop potent and highly subtype-selective ligands for each of the five (M$_{1-5}$) muscarinic acetylcholine receptors (mAChRs) remains a challenging, but potentially highly rewarding endeavor. Tremendous clinical promise has been shown for muscarinic activators in the treatment of Alzheimer's disease and schizophrenia with the moderately selective muscarinic agonist xanomeline.[1,2] In the absence of truly subtype-selective activators, it has not been definitively established which mAChRs are primarily responsible for the positive outcomes observed with xanomeline. While a spectrum of mAChR-knock out (KO) mice have strongly implicated the M$_1$ and M$_4$ receptors in mediating beneficial CNS effects,[3] the study of selective mAChR ligands in non-mutant species and under various disease states remains a worthy goal. The ability to develop selective mAChR agonists is complicated by the highly conserved orthosteric binding site shared across all five receptor subtypes. As a result, classic orthosteric agonists, such as xanomeline, do not display the desired levels of receptor subtype selectivity. Recently, the focus of novel mAChR activators has moved away from the orthosteric site in favor of allosteric sites.[4] These allosteric sites have the potential to be less conserved in their amino acid sequences, across the M$_{1-5}$ receptors, thereby giving rise to much higher levels of receptor subtype selectivity. Allosteric ligands can function as purely allosteric agonists, activating the receptor independent of acetylcholine (ACh), positive allosteric modulators (PAMs), which enhance the activity of endogenous ACh, or as a combination of these two modes (an ago-PAM). Considerable progress has been made in the development of highly selective M$_1$ PAMs,[5–7] M$_4$ PAMs,[8] M$_5$ PAMs,[9] and M$_1$ allosteric agonists.[10] Most recently, we have described the

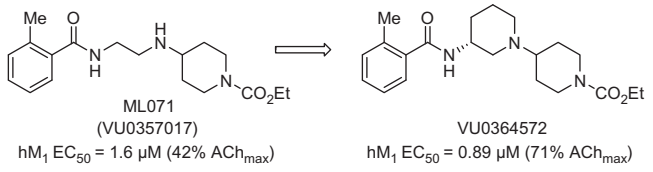

Figure 1. The MLPCN probe molecule ML071 and the next-generation highly selective, orally bioavailable, and CNS penetrant agonist VU0364572.

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development and characterization of VU0364572 (Fig. 1) starting from the MLPCN probe ML071.[11,12] Concurrently, we pursued an alternative optimization of ML071 through the introduction of numerous diverse constraints and now detail those efforts in this Letter.

Continued optimization of ML071 built on the highly modular and versatile nature of the synthesis routes utilized previously. Briefly, all analogs appearing in subsequent tables and figures were prepared according to the general routes appearing in Scheme 1, employing routine methodology as required for elaborating protecting groups into libraries of targets. Starting materials were either commercially available or prepared according to literature procedures. Route A depicts the reductive amination step involved when the Eastern piperidine-like portion of the final target arises from a ketone building block. Route B depicts the corresponding reaction between an aldehyde and the Eastern region already containing a primary or secondary amine.

Given the demonstrated benefits associated with the introduction of the central ring present in VU0364572 (Fig. 1),[11] we first explored other modes of limiting molecular flexibility (Fig. 2). For the compounds appearing in Figure 2, ML071 and **1** were prepared via route A (Scheme 1), while the remaining analogs (**2**–**5**) were prepared according to route B. Assay descriptions and experimental protocols for the functional muscarinic assays have been described previously.[12] Compound **1** shows that a potency enhancement of roughly threefold could be achieved by introducing the tropane scaffold into the Eastern region. This was not surprising since these types of tropane structures are commonly associated with muscarinic ligands (e.g., atropine and scopolamine). However, this was an inseparable mixture of *endo*- and *exo*-isomers (roughly 70:30 *endo*/*exo* based on proton NMR). Although, the four other compounds in Figure 2 were less active than **1**, the diazadecalins **4** and **5**, were viewed as an instructive pair of isomers. Relative to **1**, the *trans*-fused bicycle **4** has its central nitrogen, and the point of attachment to the Western half of the molecule, displayed in an equatorial fashion which might mimic the minor *exo*-isomer of compound **1**. Conversely, the *cis*-fused bicycle **5** has its central nitrogen positioned in an axial sense, thereby mimicking the major, *endo*-isomer component of **1**. While the potencies of **4** and **5** are not drastically different, it was encouraging to see that the axial nitrogen point of attachment in **5**, which more closely mimics the *endo*-tropane of **1**, was preferred over the equatorial sense of attachment in **4**. This implied that the *endo*-isomer component of **1** was likely the major contributor to its improved activity, and that further efforts to improve potency should focus on the production of *endo*-tropane isomers.

One straight forward method for increasing the proportion of the *endo*-isomer relative to the *exo*-isomer when preparing analogs of **1** via route A (Scheme 1), would be to perform the reductive amination step with secondary amines, thereby relying on the increased steric interactions to provide more of the desired *endo*-isomer. When the analogs appearing in Table 1 were prepared, an increased preference for the *endo*-isomer was observed with

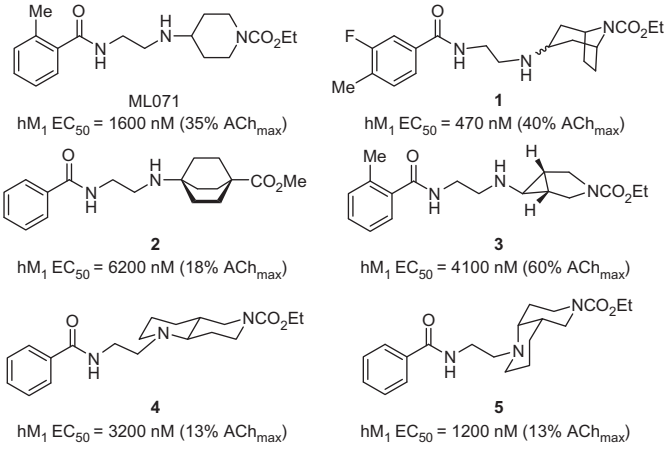
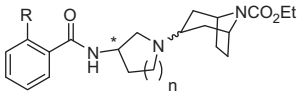


Figure 2. Novel, bicyclic analogs of ML071.

Table 1
Structures and activities of tropane analogs **6a–i**



6a-i (>90:10 endo:exo)

Compd	R	*	*n*	hM_1 EC_{50}[a] (μM)	ACh_{max}[b] (%)
6a	Me	(*S*)	2	2.1	52
6b	H	(*S*)	2	>10	12
6c	Me	(*R*)	2	0.059	75
6d	H	(*R*)	2	0.84	75
6e	Me	(*S*)	1	>10	22
6f	H	(*S*)	1	1.2	39
6g	Me	(*R*)	1	1.2	55
6h	H	(*R*)	1	0.32	62
6i	H	—	0	2.2	34

[a] Average of at least three independent determinations performed in triplicate. Standard deviations ranging from 5% to 18% of the value reported.

[b] When hM_1 EC_{50} is '>10', the reported ACh_{max} equals an average of the %ACh obtained when the compound was tested at the highest concentration (30 μM). Standard deviations ranging from 2% to 11% of the value reported.

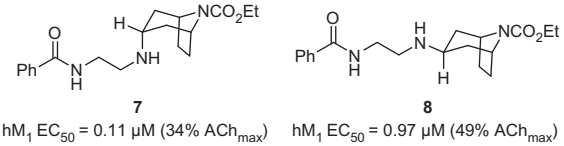
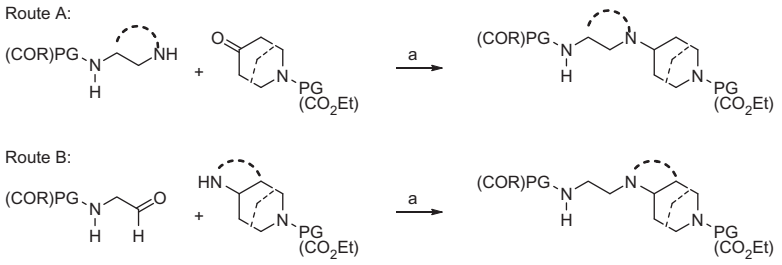
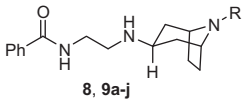


Figure 3. Comparison of the individually prepared *endo*- (**7**) and *exo*- (**8**) tropane isomers.



Scheme 1. General methods for the production of novel mAChR agonists. Reagents: (a) $NaHB(OAc)_3$, DCE or DCM, HOAc (0–4 equiv).

Table 2
Structures and activities of *exo*-substituted tropane analogs **8**, **9a–j**



8, **9a-j**

Compd	R	hM_1 EC_{50}[a] (μM)	ACh_{max}[b] (%)
8	$-CO_2Et$	0.97	49
9a	$-CO_2Me$	>10	21
9b	$-CO_2$allyl	2.0	40
9c	–(CO)Et	—	—
9d	–(CO)Pr	—	—
Be	–(CO)*i*-Pr	5.4	23
9f	$-CO_2Bn$	>10	7
9g	$-CO_2Ph$	—	—
9h	$-(CO)NMe_2$	—	—
91	$-SO_2Me$	—	—
9j	$-SO_2Et$	—	—

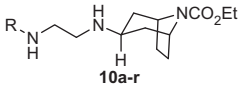
[a] Average of at least three independent determinations performed in triplicate. '—' indicates an inactive compound (<5% ACh_{max}) up to the highest concentration tested (30 μM). Standard deviations ranging from 10% to 15% of the value reported.

[b] When hM_1 EC_{50} is '>10', the reported ACh_{max} equals an average of the %ACh obtained when the compound was tested at the highest concentration (30 μM). Standard deviations ranging from 3% to 21% of the value reported.

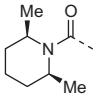
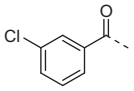
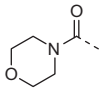
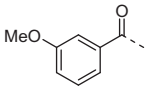
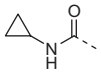
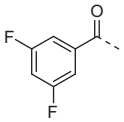
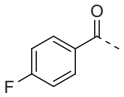
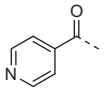
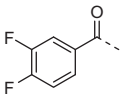
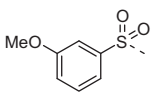
endo:*exo*-isomer ratios now heavily favoring the *endo*-isomer. In the context of a central piperidine (Table 1, $n = 2$), this provided the corresponding tropane analogs of VU0364572. The preference for the (*R*)-stereochemistry, as seen previously for VU0364572,[11] was maintained and was most pronounced for the $n = 2$ analogs (compare **6a** with **6c**). Although the (*S*)-isomers **6a** and **6b** appear to display hM_1 activity, this could also result from contamination with the (*R*)-isomer arising from the commercially available 95% ee starting material. As the ring size decreased ($n = 1$) this stereochemical preference for the (*R*)-isomer decreased beyond what could be explained by trace impurities, while the high *endo*:*exo* ratios were maintained. The achiral azetidine analog **6i** possessed a similar potency and efficacy as ML071.

Compound **6c** represented one of our most potent hM_1 agonists prepared to date and when administered to rats exhibited moderate PK properties. A standard rat IV (1 mg/kg)/PO (10 mg/kg) study found that **6c** possessed a high CL of 189 mL/min/kg, but with a V_{ss} of 11.8 L/kg and a $t_{½}$ of 46 min. Additionally, **6c** was orally bioavailable with a %F of 70 (AUC_{IV} = 92.2 ng h/mL, AUC_{PO} = 639 ng h/mL). Unfortunately, compound **6c** was found to be lacking in its muscarinic subtype selectivity profile, along with **6d**. Table 5 (vide infra) shows that while both **6c** and **6d** were

Table 3
Structures and activities of *exo*-substituted tropane analogs **10a–r**



10a-r

Compd	R	hM_1 EC_{50}[a] (μM)	ACh_{max}[b] (%)	Compd	R	hM_1 EC_{50}[a] (μM)	ACh_{max}[b] (%)
10a		1.1	47	**10j**		2.5	39
10b		2.3	37	**10k**		0.57	53
10c		—	—	**10l**		>10	11
10d		0.44	63	**10m**		2.0	44
10e		3.6	33	**10n**		—	—
10f		1.9	43	**10o**		—	—
10g		0.83	54	**10p**		>10	6
10h		0.81	50	**10q**		1.0	33
10i		1.5	34	**10r**		—	—

[a] Average of at least three independent determinations performed in triplicate. '—' indicates an inactive compound (<5% ACh_{max}) up to the highest concentration tested (30 μM). Standard deviations ranging from 2% to 12% of the value reported.

[b] When hM_1 EC_{50} '>10', the reported ACh_{max} equals an average of the %ACh obtained when the compound was tested at the highest concentration (30 μM). Standard deviations ranging from 1% to 22% of the value reported.

potent hM_1 agonists, they showed activity at various hM_{2-5} receptors.[13] Although **6c** was about 100-fold selective over hM_2, this level of selectivity and the appearance of some level of agonist activity across all muscarinic subtypes was not desirable. Whether the emergence of hM_{2-5} activity resulted from structural modifications that moved these agonists back into the orthosteric binding site at the expense of the high-affinity allosteric site on hM_1 (bitopic agonists) or if these agonists are still allosteric ligands at hM_1 but not allosteric with respect to their binding at hM_{2-5} has not yet been determined.

To enable a more rigorous comparison of *endo*- versus *exo*-tropane isomers, each primary amine was individually prepared according to literature procedures[14] and utilized via synthesis route B (Scheme 1) to prepare pure *endo*-analog **7** and pure *exo*-analog **8** (Fig. 3). As surmised for the isomer components of **1**, the *endo*-isomer **7** was more potent than the *exo*-isomer **8**. Interestingly, the less potent isomer **8** displayed a superior efficacy (%ACh_{max} = 49%), compared to the more potent isomer **7**. When we determined the M_{1-5} receptor subtype selectivity for these two compounds, the more potent *endo*-isomer **7** displayed antagonist activity across hM_{2-5}, while the *exo*-isomer appeared completely selective (Table 5). This sort of agonist/antagonist profile seen with **7** is similar to the profile seen with the M_1 allosteric agonist TBPB[10b] (Table 5, compound **12**). As for the high apparent selectivity demonstrated by **8**, it remained to be seen if further optimization with respect to hM_1 potency would engender activity at the other muscarinic subtypes. Ultimately, we decided to move away from tropane analogs, where the point of attachment was displayed in an axial fashion, and focus on tropane analogs which position the central nitrogen in an equatorial manner, which might be envisioned as better approximating the lowest energy conformation of ML071.

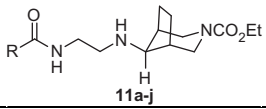
Viewing compound **8** as a new starting point for medicinal chemistry optimization, we began by exploring replacements for the ethyl carbamate terminus (R-groups, Table 2). The structure-activity relationship (SAR) in this region was remarkably steep. The methyl carbamate **9a** lost greater than 10-fold potency, but remained an agonist. The only minor modification which appeared to be somewhat tolerated was the introduction of an allyl carbamate, although this substitution resulted in a twofold decrease in potency. The propyl- and butylamides (**9c** and **9d**, respectively) were not tolerated and speak to the importance of having a carbamate at this location. Ureas (like **9h**) and sulfonamides (**9i** and **9j**) were similarly unproductive. Clearly the ethyl carbamate stood out as the premier moiety at this location.

Concurrently we explored SAR around the Western end of compound **8** (Table 3). Among the substituted benzamides (**10a–h**), *meta*-substitution with a chlorine atom (**10d**) provided the only clear improvement over **8**. Various urea termini were tolerated at this location; however, only **10k** produced an increase in hM_1 potency. The two nitrogen-containing heterocycles **10o** and **10p** were not tolerated, whereas, the thiophene analog **10q** was equipotent with compound **8**. A sulfonamide analog **10r** was relatively inactive, as were a handful of other sulfonamides at this location (data and structures not shown), but showed a trend toward hM_1 antagonist activity. Although potencies were not improved to very low nanomolar levels with the analogs **10**, we continued to explore alternate tropane scaffolds that positioned the nitrogen in an equatorial manner.

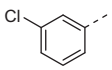
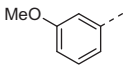
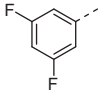
Utilizing synthesis route A (Scheme 1), and an alternate tropanone wherein the bridgehead was moved away from the ethyl carbamate, we easily obtained the analogs appearing in Table 4. Interestingly, the reductive amination step which sets the *endo*/*exo*-connectivity in **11** now afforded only the *endo*-product as a result of the bridgehead being closer to the reaction center thereby enhancing its steric influence. Furthermore, since the bridgehead had moved to the opposite side of the piperidine ring (compare **10** with **11**) we could now directly obtain the desired equatorial orientation of the central nitrogen.

This change in tropane scaffold afforded an almost uniform increase in potency for analogs **11** compared to the corresponding tropanes **10**. Only a handful of analogs were prepared, but even with this small sampling of R-groups, hM_1 potencies were now in the low nanomolar range for compounds **11a** and **11c**. Interestingly, when central piperidines analogous to compounds **6a–d** were introduced into this alternate tropane scaffold, only weakly potent agonists or wholly inactive compounds were obtained (data and structures not shown).

Table 4
Structures and activities of *endo*-substituted tropane analogs **11a–j**



11a-j

Compd	R	hM_1 EC_{50}[a] (μM)	ACh_{max}[b] (%)	Compd	R	hM_1 EC_{50}[a] (μM)	ACh_{max}[b] (%)
11a		0.092	53	**11f**		1.0	37
11b		0.17	51	**11g**		0.69	9
11c		0.047	52	**11h**		3.6	28
11d		0.24	47	**11i**		>10	11
11e		0.20	53	**11j**		0.17	54

[a] Average of at least three independent determinations performed in triplicate. Standard deviations ranging from 3% to 23% of the value reported.
[b] When hM_1 EC_{50} is '>10', the reported ACh_{max} equals an average of the %ACh obtained when the compound was tested at the highest concentration (30 μM). Standard deviations ranging from 2% to 10% of the value reported.

Table 5
hM_{1-5} selectivity profile for selected hM_1 agonists from an assay of calcium mobilization

Compd	hM_1 mode[a]	EC_{50} or IC_{50}[b] (μM)	%ACh[c]	hM_2 mode[a]	EC_{50} or IC_{50}[b] (μM)	%ACh[c]	hM_3 mode[a]	EC_{50} or IC_{50}[b] (μM)	%ACh[c]	hM_4 mode[a]	EC_{50} or IC_{50}[b] (μM)	%ACh[c]	hM_5 mode[a]	EC_{50} or IC_{50}[b] (μM)	%ACh[c]
ML071	E	2.6	34		Inactive			Inactive			Inactive			Inactive	
6c	E	0.017	59	E	1.8	66	E	>10	36	E	>10	28	E	3.7	62
6d	E	0.35	65	E	>10	27		Inactive			Inactive		E	>10	21
7	E	0.21	37	I	>10	15	I	>10	13	I	3.1	2	I	4.9	2
8	E	1.2	57		Inactive			Inactive			Inactive			Inactive	
11a	E	0.018	46		Inactive		I	>10	45	I	>10	48	E	2.8	31
11c	E	0.096	58		Inactive			Inactive		I	>10	40	E	4.2	22
12	E	0.27	40	I	2.7	1	I	2.2	0	I	0.70	3	I	4.6	3
13	E	0.041	81	I	>10	30	E	5.9	19	I	>10	26	E	1.0	28

[a] Mode of action in the calcium flux assay: E-represented an agonist response, I-represented an antagonist response and a blank represented a lack of clearly discernible activity at the receptor subtype up to the top dose of 30 μM. Experiments were single determinations conducted in triplicate, with routine error in the 1–25% range.
[b] When the mode of action was that of an agonist (E) value is an EC_{50}, when an antagonist (I) value is an IC_{50} and when an acceptable curve fit could not be generated, but a clear mode of action could be determined, '>10' is entered.
[c] When the mode of action was that of an agonist %ACh represents the maximal activity observed as a percentage of the maximum response generated from 80% of a saturating dose of ACh. When the mode of action was that of an antagonist %ACh represents the remaining activity observed at the highest dose (30 μM) as a percentage of the maximum response generated from 80% of a saturating dose of ACh. When the IC_{50} or EC_{50} is '>10', the reported %ACh equals an average of the %ACh obtained when the compound was tested at the highest concentration (30 μM).

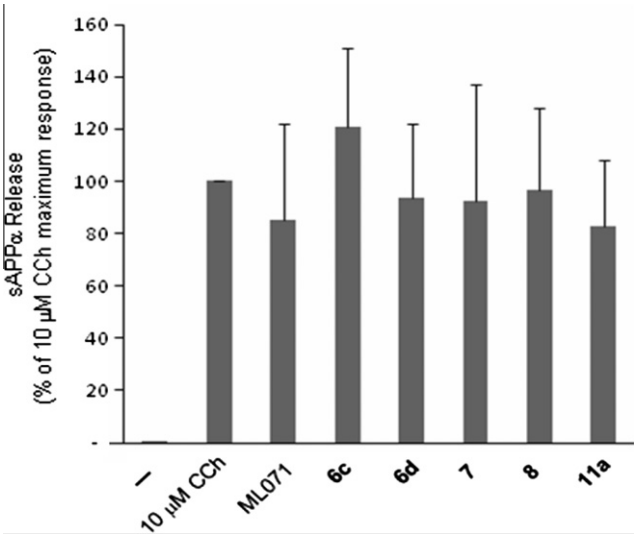


Figure 4. Ability of hM_1 partial agonists to stimulate nonamyloidogenic sAPPα release in TREx293-hM_1 cells. Agonists were tested at 2 μM and the results presented were from three independent experiments.

With another series of highly potent hM_1 partial agonists in hand, the paramount question of receptor subtype selectivity was quickly explored. Unfortunately, as shown in Table 5, both compounds displayed off target activity at subsets of the hM_{3-5} receptors. Compound **11a** had mixed activity as an antagonist at hM_3 and hM_4, but functioned as a partial agonist at hM_5. Similarly, **11c** was an antagonist at hM_4, but a partial agonist at hM_5. We have observed this mixed profile with other hM_1 allosteric agonists in our functional calcium mobilization assays, in particular AC260584 (compound **13**).[15]

Having optimized hM_1 potency in three related series of tropane partial agonists, we tested select compounds for their ability to enhance the release of soluble APPα (sAPPα),[16] as has been demonstrated with previous M_1 agonists[11] and PAMs.[7] Gratifyingly, all five of the tropane agonists tested (**6c**, **6d**, **7**, **8** and **11a**, Fig. 4) were able to stimulate the release of sAPPα in TREx293-hM_1 cells to the same extent as 10 μM carbachol (CCh) and 2 μM ML071. These experiments continue to support the belief that activation of M_1 may have the potential to be disease modifying for the treatment of Alzheimer's disease.

Although, these tropane agonists were very attractive from an M_1 potency aspect, the loss of selectivity accompanied by their increase in M_1 potency brings into focus a potential weakness within this series of hM_1 bitopic agonists. Although the highly selective interaction with the M_1 receptor may be allosteric at low concentrations due to a high-affinity allosteric site, the bitopic nature of these agonists may result in orthosteric interactions at higher concentrations or at the M_{2-5} receptors as potency is improved at the M_1 receptor. As such, a potentially more tractable approach toward the selective activation of each individual muscarinic subtype may be realized with subtype selective PAMs. Detailed in vitro pharmacological studies around this group of bitopic[17] partial agonists are in progress and will be reported shortly.[18]

In summary, we have expanded the SAR surrounding ML071, resulting in the development of highly potent hM_1 partial agonists containing two different tropane scaffolds. However, as potency was increased within each of the three series, off-target activity at the other muscarinic receptors surfaced. This may be related to the bitopic nature of these weak partial agonists.

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13. Absolute potencies for hM_1 appearing in Table 5 are different from the values appearing in earlier tables/figures and may arise from slightly different receptor expression and/or receptor reserve levels between the experiments used in the selectivity profiling experiments and those used for the rest of the manuscript, as well as other forms of experimental variability.
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16. See Ref. 7 for experimental details.
17. Bitopic refers to a ligand which can occupy two sites on a given receptor either individually or concurrently, wherein one site is the orthosteric site and the other is an allosteric site, which may or may not be adjacent to the orthosteric site. See Refs. 12,18 for a more detailed description of the pharmacology.
18. Manuscripts in preparation.
19. For information on the MLPCN and information on how to request probe compounds, such as ML071, see: http://mli.nih.gov/mli/mlpcn/.