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Discovery and SAR of muscarinic receptor subtype 1 (M₁) allosteric activators from a molecular libraries high throughput screen. Part I: 2,5-dibenzyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-ones as positive allosteric modulators

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Abstract

Results from a 2012 high-throughput screen of the NIH Molecular Libraries Small Molecule Repository (MLSMR) against the human muscarinic receptor subtype 1 (M₁) for positive allosteric modulators is reported. A content-rich screen utilizing an intracellular calcium mobilization triple-addition protocol allowed for assessment of all three modes of pharmacology at M₁, including agonist, positive allosteric modulator, and antagonist activities in a single screening platform. We disclose a dibenzyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one hit (DBPQ, CID 915409) and examine *N*-benzyl pharmacophore/SAR relationships versus previously reported quinolin-3(5*H*)-ones and isatins, including ML137. SAR and consideration of recently reported crystal structures, homology modeling, and structure-function relationships using point mutations suggests a shared binding mode orientation at the putative common allosteric binding site directed by the pendant *N*-benzyl substructure.

Keywords

Muscarinic receptor 1; mAChR; M₁; positive allosteric modulator (PAM)

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The pursuit of positive allosteric modulators (PAMs) of the muscarinic acetylcholine receptor (mAChR) subtype 1 (M_1) as a novel and promising therapeutic approach to treat Alzheimer's disease (AD)¹⁻⁴ and schizophrenia (SZ)^{5,6} continues to be an active area of research. Early studies demonstrating cholinergic loss in the cortex of AD patients^{7,8} and in the striatum of SZ patients⁹ suggested important links from muscarinic receptor function to disease pathology. In addition, ex vivo slice data within underlying circuits as well as preclinical studies in cognition and antipsychotic models provided compelling mechanistic evidence to further support the need for selective M_1 receptor activators. Importantly, clinical studies in AD and SZ patients using the mAChR M_1/M_4 -preferring agonist xanomeline¹⁰⁻¹² have suggested that both M_1 and M_4 activation are promising approaches for the development of novel CNS therapeutics.¹³ Unfortunately, xanomeline's limited subtype selectivity led to unacceptable dose-limiting adverse events including severe GI distress and excessive salivation and sweating, all largely attributed to peripheral activation of M_2 and M_3 receptors. Through focused efforts on M_1 activation via PAMs, receptor subtype selectivity has been readily achieved in a tractable and reliable manner.¹⁴⁻²⁰ Recently reported co-crystal structures²¹ of the M_2 mAChR in complex with orthosteric agonist iperoxo and a ternary complex with both PAM LY2119620 and agonist, reveal insights into a common transmembrane domain allosteric binding site just above the orthosteric ligand and separated by a Tyr₄₂₆ 'floor'. Above this floor a large solvent-accessible cavity contains an upper extracellular vestibule, which includes several non-conserved amino acids thought to provide a potential basis and rationale for selective allosteric ligand receptor-subtype binding and activation.²²

Thus far, structural classes of M_1 PAMs have remained limited. At present, scaffolds are represented by the Merck HTS hit BQCA (**1**, **Fig. 1**)^{14,23} and congeners²⁴⁻³⁶ thereof as well as isatin and indole sulfone chemotypes discovered from a NIH Molecular Libraries Production Center Network (MLPCN) sponsored Vanderbilt 2009 HTS campaign of ~60K compounds, represented by probes ML137 (**2**)³⁷ and the ML169¹⁶ derivative VU0456940¹⁷ (**3**). In an effort to identify additional structurally diverse M_1 PAM chemotypes as well as M_4 and M_5 modulators, a second sponsored M_1 screen was conducted in 2012, encompassing a significantly expanded Molecular Libraries Small Molecule Repository (MLSMR) of ~300K compounds. From this collaborative effort with the Scripps Research Institute Molecular Screening Center, a single hit was found with confirmed potency below 10 μ M, represented by dibenzyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one **4** (DBPQ). This compound exhibited an in vitro EC₅₀ of 473 nM and a relatively low acetylcholine maximum response (ACh max) of 40% in a calcium mobilization assay utilizing a concentration-response curve (CRC) of the PAM and a submaximal concentration of acetylcholine. Structurally, the quinolinone core of DBPQ is found within BQCA and has a strong resemblance to several reported cyclic ketoacid replacements, including the pyrazolinone fragment itself which is also described in the Merck M_1 patent literature.²⁴⁻³⁶ A further in-depth examination of the patent literature encompassing the pyrazolinone ring substructure of DBPQ, as well as related five and six-membered heterocyclic ring systems,³⁸ reveals that the dibenzyl substitution pattern noted within DBPQ was rarely exemplified,³⁹ suggesting this substructure pattern was either non-optimal for M_1 PAM activity and/or potentially presented challenges with respect to DMPK properties (e.g. CYP450-mediated

oxidative metabolism). Unfortunately, with the extensive prior art surrounding the pyrazolo[4,3-*c*]quinolin-3(5*H*)-one system at both M₁ as well as previously reported molecular targets, including PDE4 inhibitors⁴⁰ and CB2 selective agonists,⁴¹ DBPQ fails to provide a viable starting point for further expansion in the development of novel M₁ PAMs. However, the peripheral dibenzyl substitution pattern within DBPQ presented an interesting opportunity to examine more in-depth SAR within the template to investigate if 1) the quinolinone core can engage in multiple binding modes via a preferred *N*-benzyl substituent/subpocket at the allosteric site and 2) if potential SAR overlap exists between DBPQ and the *N*-benzyl M₁ PAM pharmacophore element present within ML137¹⁵ and related non-isatin congeners.^{18,19} In addition, in the context of the recent M₂ mAChR X-ray crystal structures²¹ and mutagenesis data^{14,42} involving Tyr₁₇₇ and Trp₄₂₂ and its central importance for PAM activity and key interaction with the core of LY2119620 (see **Fig. 2** for binding schematic and key interactions), we hypothesized that perhaps the isatin ring of ML137 and the pyrazolo[4,3-*c*]quinolin-3(5*H*)-one scaffold of DBPQ and BQCA might share a common interaction within the core of the allosteric binding site, involving the related M₁ residues Tyr₁₇₉ and Trp₄₀₀. Herein we performed an SAR examination of DBPQ analogs and hybrid analogs of ML137 and DBPQ to gain insight into whether or not a common benzylic sub-pocket at the allosteric site might exist between these well-established M₁ PAM templates and to speculate on potential binding orientations at the M₁ allosteric site.

To begin to deconvolute the overlapping benzylic pharmacophore fragments between DBPQ and BQCA, we prepared and evaluated a series of 2-(2,5-demethylbenzyl) and 5-benzyl analogs within DBPQ using iterative parallel synthesis. Both series were synthesized based on literature procedures (Routes AC, Scheme 1).⁴⁰ *N*-substituted thioquinolinones **7** (Route A) were synthesized via alkylation of commercially available quinolinone **1** and subsequent treatment with phosphorous pentasulfide. The desired DBPQ **4** and analogs **8a-c**, **9a-n**, **12m-n** were obtained via condensation with hydrazine followed by *N*-alkylation with appropriate alkylating agent. Alternatively, (Route B) quinolinone **1** was treated with phosphorous oxychloride to yield chloroquinoline **10**. The final DBPQs **11a-d** and **12a-12l** were obtained by the reaction between chloroquinoline **10** and the corresponding alkylated hydrazine followed by *N*-alkylation. Lastly, isatin analogs **14a-c** were synthesized by *N*-alkylation (Route C).

Initial benzyl deletion studies (**Fig. 3**) of DBPQ (**4**) demonstrated weak PAM activity for the 5-benzyl quinolinone (**8a**), while the 2-(2,5-dimethylbenzyl)-pyrazolone analog **11a** was inactive (>30 μM), suggesting that the presence of both *N*-benzyl moieties is required for activity. With these initial starting points in hand, we conducted a survey of 2-benzyl modifications (**9**) and their impact on functional PAM activity at hM₁ using a calcium mobilization assay as previously described (Table 1).¹⁷ *N*-methyl analog **9a** exhibited similar activity as **8a** with some diminution of the ACh max response (68% versus 48%). Parent benzyl analog **9b**, lacking the 2,6-dimethyl found in the HTS hit, lead to an inactive compound. A ‘mono-fluoro walk’ within the *N*-benzyl phenyl ring (**9c-9e**) afforded a weak PAM for 2-fluoro isomer **9c** (EC₅₀ = 1010 nM); however, the 3 and 4-substituted congeners were ineffective. Similarly, a methyl scan of the *N*-benzyl phenyl ring demonstrated that the

2-position was preferred over the 3 and 4-position, with the 2-tolyl derivative PAM **9f** leading to sub-micromolar functional potency ($EC_{50} = 783$ nM) and comparable ACh max relative to DBPQ (**4**). Examination of pyridyl ring isomers (**9i-9k**) demonstrated a significant loss in potency; however, a notable increase in ACh max within the series (ACh max 54-65%) was observed relative to DBPQ (**4**), with 3-pyridyl **9j** demonstrating weak potency ($EC_{50} = 6310$ nM).

In parallel with the above studies the 2-(2,5-dimethylbenzyl) moiety was held constant and the 5-benzyl substituent surveyed (Table 2, series **12**). Similar to the 2-benzyl series **9**, potency was not improved for series **12** derivatives relative to the original HTS hit DBPQ (**4**). However, with the exception of the tolyl derivatives (**12e-12g**) which were uniformly inactive, fluoro derivatives **12b-12d** and pyridyl derivatives **12h-12j** exhibited potency below 10 μ M, with pyridyl analogs displaying enhanced efficacy (ACh max 75%). Fluoro derivatives **12b-12d** generally had a lower ACh max, from 31-47%. Both fluoro-substitution^{15,38} and *N*-methyl pyridyl derivatives¹⁷ within the *N*-benzyl motif have been utilized with varying success in a number of other M_1 PAM chemotypes (e.g. **2** and **3**) and similarly these modifications proved tolerable, and in some cases resulting in enhanced ACh max.^{15,17,25-28,35}

We next wished to impart additional bias between the *N*-(2) and *N*-(5) benzyl pharmacophores within DBPQ through incorporation of established potency enhancing substituents at the *N*-(5) benzyl ring. These included the 4-methoxy found in BQCA to afford **12k**, a hybrid of ML137 and BQCA to produce the 4-methoxy-2-fluoro derivative **12l**, the ML137 comparator **12m** bearing a 4-(1-methyl-1*H*-pyrazol-4-yl)benzyl moiety reported as beneficial in the BQCA and ML137 series,^{25,37} and analog **12n** bearing the *N*-linked benzyl pyrazole regioisomer reported within the BQCA series.²⁶ Within the context of the 2-(2,5-dimethylbenzyl)-pyrazolone the 4-methoxy substituent resulted in a slight loss (~1.7 fold) in potency (**12k**). Interestingly, the hybrid analog **12l** with an additional 2-fluorine substituent was found to be functionally inactive. Utilizing the 4-(1-methyl-1*H*-pyrazol-4-yl)benzyl substructure from ML137 gave **12m**. Although less potent than BQCA, ML137, and DBPQ (~2-4-fold), **12m** proved to have a ~2.0-fold boost in ACh max response relative to DBPQ (ACh max = 74%). Similarly, *N*-linked benzyl pyrazole **12n** exhibited slightly diminished potency and enhanced apparent potentiation (ACh max = 80%). Thus, although hybrids utilizing the 2-(2,5-dimethylbenzyl)-pyrazolone in the presence of historical *N*-(5) benzyl substituents from prior studies suggest a weak enhancement in cooperativity, these modifications were not inherently beneficial in leading to additive SAR in regard to potency.

In an effort to understand if the pyrazolo[4,3-*c*]quinolin-3(5*H*)-one core might accommodate multiple binding modes at the allosteric binding site through a 'benzylic translocation', we tested a small set of individual analogs incorporating preferred pyrazole biaryl fragment at the *N*-(2) or *N*-(5)-position (**Fig. 4**). As shown in Figure 4, translocation of the benzyl to provide either the 4-(1-methyl-1*H*-pyrazol-4-yl)benzyl analogs **11c** or **12a** or the 4-(1-methyl-1*H*-pyrazol-4-yl)benzyl derivative **11d** proved incompatible with M_1 activity. In addition, within the context of a recently developed³³ and structurally distinct (1*R*,2*R*)-2-

hydroxycyclohexyl moiety at *N*-(2) with potency enhancing properties (**11b**), lack of a benzyl substitution at *N*-(5) continued to be detrimental for activity. In comparison to *N*-(2) analogs **11c** and **12a**, progression of a series of related *N*-(5) analogs from hydrogen to methyl to benzyl (**8c**, **9l-9m**) afforded M₁ PAMs with diminishing potency from 1.2-10 μ M, thus establishing the *N*-(5) position as the preferred site for the benzylic pharmacophore and furthermore indicating limited flexibility at the allosteric site between these benzylic *N*-(2) and *N*-(5) positional isomers. The preference for the *N*-(5) position is also reinforced by the ML137 overlapping 2-fluoro-4-(1-methyl-1*H*-pyrazol-4-yl)benzyl analog **8b** with an EC₅₀ of 610 nM and an ACh max of 89%. Lastly, in an attempt to conversely apply the DBPQ *N*-(2) benzyl SAR to the isatin ring system, the *N*-benzyl isatin congeners **14a-14b** were prepared. In addition, a 4-(1-methyl-1*H*-pyrazol-4-yl)benzyl derivative **14c** was prepared within the isatin series for comparison to **11c** and **8c**, a substructure preferred within the BQCA class and related scaffolds.²⁶ As shown in Figure 4, inactive to weak PAM activity was noted by these modifications (**14a-14c**) and indicated narrower SAR within the isatin *N*-benzyl motif, with 4-(1-methyl-1*H*-pyrazol-4-yl)benzyl being preferred.

Collectively, on the basis of the studies described for DBPQ, *N*-benzyl SAR at the *N*-(2) position is relatively shallow and the *N*-(5) position is the preferred benzylic pharmacophore for M₁ PAM potency. In addition, overall trends suggest partial overlap between the *N*-(5) benzyl and the *N*-benzyl moiety of the ML137 isatin with some subtle differences appreciated to indicate they are not fully overlapping (e.g. above azole preference, C(4) versus *N*-linked).^{15,26} Utilizing the available co-crystal structure²¹ of the M₂ mAChR in complex with LY2119620 and iperoxo, there are 26 residues within six Ångstroms of the allosteric modulator. Based on sequence differences between the M₂ and M₁ human receptors, nine of these residues are unique to M₁ and can be readily mutated and visualized using homology modeling tools. Unlike LY2119620, BQCA and ML137 contain two units of extended aromaticity that could formally interact in a π - π stack interaction with Trp₄₀₀; the respective quinolinone and isatin cores and the common pendant benzyl biaryl 'tail' group as supported by the data presented. Thus, rather than maintaining an interaction with the 'core' of the ligand at the common allosteric site, a conceivable alternative, which is consistent with the overall SAR described, invokes the pendant benzyl biaryl engaging in a three-layered stacking interaction with the M₁ Trp₄₀₀ and Tyr₁₇₉ residues similar to the M₂-LY2119620 bound agonist complex. This alternative hypothesis is supported in part by recent M₁ homology, molecular dynamics, and site-directed mutagenesis studies with BQCA reported at the Monash Institute in which the *p*-methoxybenzyl tail of BQCA is shown to have a direct interaction with Trp₄₀₀ at the common allosteric site.⁴²

Preliminary docking studies using an M₁ model based upon the M₂-LY2119620 crystal structure and the data herein suggest a binding pose for ML137 directing the pendant benzyl biaryl group deep within the core of the common allosteric site (**Fig. 5**). Similar to the quinolinone core pose observed for BQCA,⁴² the isatin ring is pointing outward towards the extracellular domain; however, rather than interacting with residues in transmembrane helix 2 (TM2), the isatin heterocycle projects towards TM5-TM6 and engages residues within extracellular loops 2 and 3 (ECL2-ECL3), a region more closely associated with the position of the acylpiperazine fragment of LY22119620 found in the M₂ receptor. In addition, the

central 2-fluoro-phenyl ring of ML137 is oriented between Trp₄₀₀ and Tyr₁₇₉, and a potential hydrogen bonding interaction with the phenol of Tyr₈₂ and the pyrazole *N*-(2) nitrogen can be found. Overall, the proposed binding orientation is not inconsistent with the pendant benzyl biaryl benzylic pharmacophore and suggests the potential for a shared binding motif for other M₁ chemotypes bearing an *N*-benzyl substructure. Furthermore, proximity of the isatin ring towards ECL2-ECL3 is intriguing as a basis for selective allosteric ligand receptor-subtype activation, as 5 out of the 9 residues within the 6 Ångstrom shell that are unique to M₁ can be found within these regions. Coincidentally, pronounced changes in receptor subtype pharmacology have been achieved through subtle modification on the isatin ring system to afford M₅ selective PAMs (e.g. see ML129 and 5-CF₃O as a M₅ 'molecular switch').³⁷

In summary, we have studied a series of pyrazolo[4,3-*c*]quinolin-3(5*H*)-one derivatives as M₁ PAMs based on HTS hit DBPQ (**4**) in an effort to expand SAR and provide insight into potential overlap with an isatin M₁ chemotype. With this SAR and knowledge we sought to assess potential binding modes at the common allosteric binding site of the M₁ AChR on the basis of recent M₁ homology models and structural information from M₂ and M₃ crystal structures. In contrast to our initial hypothesis that the isatin ring of ML137 and the quinolin-3(5*H*)-one scaffold of DBPQ and BQCA interact at the common core of the allosteric binding site, our analysis and findings indicate that the *N*-benzyl substructures found within isatin ML137 and the quinolinone *N*-(5) benzyl, serve as a common pharmacophore that likely occupies the core pocket deep within the allosteric binding site. Further rigorous modeling, mutagenesis structure-function studies, and x-ray crystallography efforts using the M₁ chemotypes described will be instrumental in providing new insights into the origin of muscarinic receptor selectivity and allosteric modulator action.

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Figure 1.

M₁ PAM BQCA (**1**), HTS-derived isatin ML139 (**2**), indole sulfone VU0456940 (**3**), and 2nd-generation MLPCN HTS hit dibenzyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one M₁ PAM DBQP (**4**, CID9154093).

Figure 2.
Binding site diagram of LY22119620-M₂ crystal structure (PDB code: 4MQT).²¹

Scheme 1.

Reagents and conditions: (a) KI (0.1 equiv.), K₂CO₃ (2.0 equiv.), DMF, rt, 6 h, quantitative; (b) P₄S₁₀ (1.0 equiv.), pyridine, 115 °C, 1 h, quantitative; (c) NH₂NH₂ (1.0 equiv.), EtOH, 75 °C, 1 h, 78%; (d) NaH (3.0 equiv.), Ar₁CH₂X (3.0 equiv.), DMF, rt, 10 min (20-90%); (e) POCl₃, reflux; (f) dioxane, reflux; (g) NaH, Ar₂CH₂X, DMF, rt, 30 min (35-60%); (f) KI (0.1 equiv.), K₂CO₃ (2.0 equiv.), rt, 1 h, 60-86%.

Figure 3.
DBPQ benzyl deletion analogs **8a** and **11a**.

Figure 4. Benzyl translocation (**11c-d**, **12a**), non-benzyl *N*-(5) (**11b**), *N*-linked pyrazole biaryls (**8c**, **9l-m**), and isatin-DBPQ/BQCA hybrids (**14a-c**).

Figure 5. Proposed binding mode of ML137 at the common allosteric binding site of an M_1 receptor model with key residues Tyr₈₂ and Trp₄₀₀.

Table 1Structures and activities of 2-benzyl analogs **9**.^a

Cmpd	Ar ¹	pEC ₅₀ ^b (±SEM)	EC ₅₀ (nM)	ACh _{Max} ^b (±SEM)
4 (DBPQ)	2,5-di Me ₂ Ph	6.32 (0.14)	473	40 (1.2)
9a	H	<5.0	>10,000	48 (5.7)
9b	Ph	Inactive		
9c	2-FPh	6.00 (0.17)	1010	36 (1.2)
9d	3-FPh	Inactive		
9e	4-FPh	Inactive		
9f	2-CH ₃ Ph	6.11 (0.12)	783	47 (1.1)
9g	3-CH ₃ Ph	Inactive		
9h	4-CH ₃ Ph	Inactive		
9i	2-pyridyl	<5.0	>10,000	59 (2.2)
9j	3-pyridyl	5.20 (0.12)	6310	65 (2.7)
9k	4-pyridyl	<5.0	>10,000	54 (0.83)

For hM₁ EC₅₀ values reported as >10,000 nM the corresponding maximal percent ACh response is from the highest tested concentration of compound (30 μM). All compounds ranked as 'inactive' did not induce a >10 % increase in the response at 30 μM.

^a CHO-K1 cells stably expressing the human M₁ receptor (ref. 17).

^b pEC₅₀ and ACh_{Max} are average of three independent determinations performed in triplicate.

Table 2Structures and activities of 5-benzyl analogs **12**.^a

Cmpd	Ar ²	pEC ₅₀ ^b (±SEM)	EC ₅₀ (nM)	ACh _{Max} ^b (±SEM)
4 (DBPQ)	Ph	6.32 (0.14)	473	40 (1.2)
12a	H	Inactive		
12b	2-FPh	6.18 (0.02)	656	46 (4.2)
12c	3-FPh	5.85 (0.32)	1420	31 (5.2)
12d	4-FPh	5.69 (0.24)	2020	47 (5.0)
12e	2-CH ₃ Ph	Inactive		
12f	3-CH ₃ Ph	Inactive		
12g	4-CH ₃ Ph	Inactive		
12h	2-pyridyl	5.42 (0.11)	3790	83 (3.9)
12i	3-pyridyl	5.44 (0.03)	3660	75 (2.2)
12j	4-pyridyl	5.81 (0.02)	1550	82 (2.9)
12k	4-MeOPh	6.09 (0.02)	821	39 (2.2)
12l	4-MeO, 2-FPh	Inactive		
12m		5.71 (0.03)	1940	74 (0.76)
12n		5.94 (0.05)	1150	80 (3.0)

For hM₁ EC₅₀ values reported as >10,000 nM the corresponding maximal percent ACh response is from the highest tested concentration of compound (30 μM). All compounds ranked as 'inactive' did not induce a >10 % increase in the response at 30 μM.

^aCHO-K1 cells stably expressing the human M₁ receptor (ref. 17).

^bpEC₅₀ and ACh_{Max} are average of three independent determinations performed in triplicate.