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Synthesis and Structure–Activity Relationships of Allosteric Potentiators of the M₄ Muscarinic Acetylcholine Receptor

J. Phillip Kennedy^{[a],+}, Thomas M. Bridges^{[b],+}, Patrick R. Gentry^[b], John T. Brogan^[a], Alexander S. Kane^[b], Carrie K. Jones^{[b],[c]}, Ashley E. Brady^[b], Jana K. Shirey^[b], P. Jeffrey Conn [Prof.]^[b], and Craig W. Lindsley [Prof.]^{*,[a],[b]}

^[a] Department of Chemistry, Vanderbilt University 12475 MRB4, Nashville, TN 37232-6600 (USA)

^[b] Vanderbilt Program in Drug Discovery, Department of Pharmacology Vanderbilt University Medical Center 12475 MRB4, Nashville, TN 37232-6600 (USA)

^[c] U.S. Department of Veterans Affairs, Tennessee Valley Healthcare System (TVHS), 1310 24th Avenue South, Nashville, TN 37212 (USA)

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The five subtypes of the muscarinic acetylcholine receptor (mAChR_{1–5} or M_{1–5}) are differentially expressed G protein-coupled receptors (GPCRs) important to a variety of physiological functions, including attention, learning and memory, pain, sleep, movement, gastrointestinal motility and cardiovascular regulation, among others.[1–5] Based on mounting data, M₁ and M₄ receptors are considered potential therapeutic targets for numerous CNS diseases and disorders such as Alzheimer's disease and schizophrenia.[6–9] However, due to high sequence conservation of the orthosteric binding site across subtypes, discovery of truly subtype-selective compounds has proven historically challenging. Indeed, M₂- and M₃-related side effects (e.g. GI disturbance, salivation, lacrimation and bradycardia) have contributed to failure in the clinical development of muscarinic agonists despite promising therapeutic efficacy.[6,7] Furthermore, deep biological insight into the specific roles of the mAChRs in both basic neurobiology and CNS pathologies has been hindered by the paucity of selective tools. The additional drug metabolism/pharmacokinetic (DMPK)-related challenges inherent to CNS drug discovery have also hampered progress in this area. Despite these hurdles, a number of novel subtype-selective and centrally penetrating muscarinic compounds, including agonists, antagonists, and potentiators, have recently emerged from functional cell-based screening approaches.[10–15] We previously reported the identification of a series of novel M₄-selective potentiators (positive allosteric modulators or PAMs) that enhance receptor activation in response to acetylcholine (ACh) by an allosteric mechanism (Figure 1).[10,12] These compounds increase the potency of ACh at M₄ but lack intrinsic agonist activity on their own. Initial optimization focused on improving the physiochemical properties of lead compound VU10010 (1), which possessed an EC₅₀ value of 400 nM and elicited a 47-fold leftward shift of an ACh concentration–response curve (CRC) by Ca²⁺ mobilization assay in rat M₄/G_{q15}-expressing cells, but suffered from solubility issues and lack of brain penetration.

[10,12] This limited effort produced two analogues, VU0152099 (2) and VU0152100 (3), which had similar potency and comparable efficacy to the parent compound i but were centrally penetrating and displayed in vivo activity in a rodent behavioral model predictive of antipsychotic efficacy.[10] These compounds were also devoid of ancillary pharmacological activity when evaluated against a large number of GPCRs, ion channels, and enzymes.[10] Despite the utility of compounds 2 and 3 for in vitro and in vivo pharmacological studies, we sought to further explore the SAR in this series with a more exhaustive optimization campaign by employing an iterative analogue library approach. The rationale for this effort stemmed in part from an initial limited lead optimization campaign, poor metabolic stability of compounds 2 and 3 (< 10 % parent remaining after 90 min in human and rat liver microsomes) and as a result, suboptimal rodent PK values that were adequate but not ideal for in vivo studies.[10]

Numerous modifications to the thieno[2,3-b]pyridine scaffold, including substitution of the primary amine, deletion or extension at the 4-methyl position and variation of the pyridine to a pyrazine or benzene ring, were all previously found to compromise activity regardless of amide side chain substituent.[10] Metabolite identification experiments indicated that hydroxylation of the 6-methyl group on the pyridine ring was the major oxidative metabolite. Having tentatively designated the p-methoxybenzamide moiety of 3 as a favored substituent based on its in vitro functional activity at rat M₄, we first held this side chain constant while exploring alternative substituents at the 6-position to prevent formation of this major metabolite. The initial synthesis of 20-member alkylamine and 20-member ether libraries, as shown in Scheme 1, began with condensation between 3-aminocrotonitrile (4) and 2-cyanothioacetamide (5) to furnish the aminopyridyl core 6.[16] 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, intermediate 8 was reacted with various alkyl chlorides to generate analogues 9 a–p. The ether library was synthesized in a similar fashion by reacting ethylacetoacetate (i0) with 2-cyanothioacetamide (5) to provide the core pyridine 11,[17] which was then cyclized with compound 7 to give the key intermediate 12. Finally, alkylation of 12 with various alkyl chlorides gave analogues 13 a–s, which are similar, yet distinct from related M₄ PAMs disclosed by Eli Lilly.[18,19]

These second generation libraries were first screened in a single-point Ca²⁺ mobilization assay using a fixed 10 mM concentration added to rat M₄/G_{q15}-expressing cells prior to addition of a submaximal concentration (~ EC₂₀) of ACh. This allowed efficient triage of analogues for further characterization. In general, the alkylamine library 9 a–p showed weak efficacy with elevation of the ACh response ranging from none (inactive) to modest (< 50% ACh max). However, the ether library 13 a–s contained a number of robust potentiators (~ 60–90 % AChMax). EC₅₀ values for compounds selected from both libraries based on their potentiation efficacy and structural characteristics were then obtained from full CRCs in Ca²⁺ assays testing for potentiation of ACh EC₂₀ (Table 1).

More than half of these compounds possessed EC₅₀ values over 10 mM with potentiation effects emerging only at the 10 mM and 30 mM concentrations. Among alkylamines 9 a–p, only the ethyl morpholine congener 9c had an EC₅₀ value just below 10 mM, reflecting the relatively weak activity of this library. In contrast, compounds from the ether library 13 a–s possessed improved potencies. Particularly, picolyl analogues 13 k, 13 l, and 13 m each exhibited an EC₅₀ value of ~2 mM. A full CRC for 13 k in the presence of fixed ACh EC₂₀ is presented in Figure 2 a. Analogue 13 k elicited a robust potentiation of M₄ 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 13 k 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, the six compounds that exhibited EC₅₀ values below 10 mM were examined for their ability to shift

a full ACh CRC to the left when applied at a fixed 30 mM concentration in a similar functional Ca^{2+} assay with $\text{rM}_4/\text{G}_{\text{q}15}$ -expressing cells (i.e. fold-shift assay). In the case of other allosteric potentiators of GPCRs, compound potency often fails to correlate tightly with fold-shift magnitude. For example, a potentiator with high potency but low efficacy can exhibit next to no fold-shift effect, and conversely one with low potency but high efficacy can 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. As shown in Table 1, neither morpholino compound 9c nor ethyl compound 9p caused a leftward 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 analogue 13 a from the ether library. However, the ether linked morpholino 13 c and pyrrolidine 13 d analogues demonstrated strong left-ward fold-shifts (37 x and 25 x , respectively). Interestingly, movement of the nitrogen from the 2-position or 3-position of the picolyl ethers i3 m and i3 l to the 4-position of i3 k (Figure 2 b) progressively increased the fold-shift from 5x to 9x and ultimately 50 x , respectively.

Taken together, these data suggested ether-linked modifications to the 6-position of the scaffold were more tolerated than alkylamine-linked changes. However, despite retention of robust potentiation properties in terms of fold-shift for analogues 13 c, 13 d, and particularly 13 k, the potency of these analogues was moderately diminished relative to the parent compound 3 ($\text{EC}_{50} = 380 \text{ nM}$). Furthermore, the SAR for these two libraries underlines the aforementioned importance of considering both fold-shift and potency when evaluating allo – steric potentiators. Although each of the three picolyl ether analogues had $\sim 2 \text{ mM EC}_{50}$ values, their left-shift effects on the ACh CRC revealed dramatic differences in potentiation efficacy. For the next library iteration, we postulated that with the pi- colyl 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 favored for M_4 potency. Therefore, we opted to rescan the amide with 18 side chain groups, while holding constant each of the three picolyl ether modifications, the morpholino ether, and the dimethylpropylamine ether. The morpholino and 4-picolyl were clear choices based on their degree of 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 13 a (i.e. a matrix-like approach to broaden SAR).

These third generation libraries began with the cyclization between pyridine 11 and ethyl chloroacetate (14) to produce thienopyridone ethyl ester 15. To obtain the five alkyl ethers i6 a–e, compound i5 was reacted with the five selected side chains from our previously mentioned second generation library. These five scaffolds were saponified and immediately coupled with 18 amines to produce five alkyl ether libraries with different amide side chains 17 a–p, 18 a–r, 19 a–o, 20 a–p, 21 a–o (Scheme 2).[18]

As before, these libraries were screened first in a single-point 10 mM potentiation assay that tested their ability to enhance the response of a submaximal ($\sim \text{EC}_{20}$) concentration of ACh in rat $\text{M}_4/\text{G}_{\text{q}15}$ -expressing cells (Figure 3). Potentiation ranged from absent to robust within each of these libraries, revealing generally consistent SAR across all of the five ether-linked modifications held constant on the left-hand side of the structure. From this screen, eleven compounds were selected for CRC and 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 2.

All selected compounds possessed an EC_{50} value below 10 mM except for derivative i9 n, the difluorobenzyl-substituted 2-picolyl analogue. Similar to earlier libraries, the fold-shift magnitude did not track closely with potency, as shown, for example, with i8 h. This tert-butyl-

substituted morpholine analogue had near 9 mM potency but caused a robust 62 x fold-shift of the ACh CRC. Furthermore, the two dimethylpropyl analogues 17 a and 17 o displayed approximately 3 mM potency yet had only a moderate ACh fold-shift. Interestingly, this dimethylpropylamine moiety at R¹ conferred poor potency (>10 mM) in its parent compound 9b that possessed the p-me-thoxybenzyl group at R², but the amide scan producing library 17 a–p discovered side chains that rescued activity for this left- hand side modification.

In general, difluorinated benzylic substitutions at R² were favored, providing analogues with EC₅₀ values in the 2–5 mM range at rat M₄ and broad fold-shift values. The 4-picolyl moieties of R¹ with the 2,3-difluoro and 2,5-difluoro substitutions at R² of compounds 21 n and 21 o proved most desired when seeking a balance of both potency and potentiation efficacy, consistent with previous SAR. However, the morpholines at R¹ of library 18 a–r with bare alkyl and mono-oxygenated side chains at R² possessed strong fold-shift effects despite moderately weaker potency compared to 21 n and 21 o. Figure 4 presents the CRC for elevation of an ACh ~EC₂₀ and fold-shift on a full ACh CRC for analogue 21 n. Interestingly, this 2,3-di-fluorobenzyl substituted analogue did not elevate the maximal response of ACh at the top of the CRC (Figure 4 b), which contrasts with 4-methoxybenzyl analogue 13 k (Figure 2b).

Despite generation of a multidimensional library of analogues varying both sides of the lead scaffold, the approximately 400 nM potency (rat M₄) of the first generation compounds 1–3 could not be maintained despite retention of strong potentiation activity in terms of ACh CRC fold-shift (e.g. > 50 x). Indeed, compounds 13 k, 18 b, and 21 o each caused substantial leftward shift of ACh CRCs when applied at 30 mM, but were approximately an order of magnitude less potent than the first generation compounds at rat M₄ receptor. These SAR suggest the presence of a possible ~2 mM potency floor for this chemotype with 6-position ether or amine modifications, as variation of the amide side chain failed to provide congeners with EC₅₀ values below this level.

In parallel, we evaluated the microsomal stability of i3 k, 21 n, 21 o, and 18 h in both rat and human microsomes.[18] Re-placement of the metabolically labile 6-methyl group with the ether linkage did indeed improve metabolic stability for all four analogues 13 k, 21 n, 21 o, and 18 h (> 90 % parent remaining after 90 min) as compared to 1–3 (< 10 % parent remaining after 90 min). Moreover, incorporation of the basic amine moieties in 13 k, 21 n, 21o, and 18 h also improved solubility providing either homogeneous solutions or uniform microsuspensions, as the HCl salts at 10 mg mL⁻¹, across a panel of pharmaceutically acceptable vehicles (b-cyclodextrin, PEG400/H₂O, etc.) relative to 2 and 3, which were only soluble in 10 % Tween 80. In fact, 21 n afforded a homogeneous solution at 15 mg mL⁻¹ in pH 3 saline.

Despite micromolar potency at rat M₄, we evaluated i3 k, 21 n, and 21 o in our standard reversal of amphetamine-induced hyperlocomotion in vivo model, since a longstanding question in the PAM field has centered on whether EC₅₀ or fold-shift is more relevant to provide in vivo efficacy.[20] Recall, both 2 and 3 (EC₅₀ values ~ 400 nM, fold-shifts of 30x and 70x, respectively) were efficacious in this model. Interestingly, both i3k and 2i o produced modest decreases in amphetamine-induced hyperlocomotion while 2i n had no effect over the time course tested (Figure 5). These findings suggest that the diminished potency of these new compounds may have translated to reduced in vivo efficacy relative to 2 and 3.

Primarily, our efforts reported here were aimed at exploring SAR at rat M₄ and optimizing this series for beneficial DMPK and vehicle formulation properties for in vivo rodent behavioral studies. While stability and physiochemical properties were improved, potency at rat M₄ was diminished to a point where in vivo efficacy was reduced and, in the case of 21 n, in vivo efficacy was lost. However, rat and human mAChRs do diverge and species differences have

been noted for other mAChR PAMs. Therefore, we opted to evaluate representative compounds 13 k, 21 n, 21 o, and 18 h in analogous functional cell-based Ca^{2+} assays using cells expressing the human M_4 receptor (and promiscuous G_{q15} for Ca^{2+} mobilization readout). To this end, these four compounds were submitted to Millipore Corp. (St. Charles, USA) and assayed by 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 (Figure 6 a), more than an order of magnitude greater potency than at the rat M_4 receptor. Each compound also elicited large leftward shifts of the control ACh CRC in human M_4 cells (Figure 6 b) similar to their respective fold-shifts at rat M_4 . In contrast, the prototypical M_4 PAMs 1–3 and about 20 other first generation analogues, displayed near equivalent EC_{50} values at rat and human M_4 , suggesting the basic residues in these newer analogue contact divergent residues in human M_4 . While receptor expression levels in the two cell lines is not known, ACh EC_{50} values in the two cell lines are equivalent (rat M_4 ACh, EC_{50} = 154 nM; human M_4 ACh, EC_{50} = 100 nM), and all first generation analogues were also equipotent. These human M_4 data exemplify the differences that may exist between species in terms of compound potency, efficacy, and other pharmacological parameters, despite relatively high structural similarity between rat and human mAChRs.

In addition, 13 k, 21 n, 21 o, and 18 h and related second generation analogues remained highly M_4 selective at both human and rat mAChR cell lines (Figure 7). Whereas a 30 mM concentration of 13 k, 21 n, 21 o, and 18 h afforded large left-ward shifts (44–63 x) of the ACh CRCs of M_4 , these same concentrations of compound had no effect on the ACh CRCs of M_1 , M_2 , M_3 or M_5 (data shown is for rat mAChRs).

Finally, while M_1 PAMs and allosteric agonists have the potential to effect both the positive and cognitive symptom clusters of schizophrenia, M_4 PAMs should only treat the positive symptoms.[9-13] Upon recognition that compounds 13 k, 21 n, 21 o, and 18 h possess the basic features of the refined H_3 pharmacophore model 22 (Figure 8), we evaluated these compounds for their ability to function as H_3 antagonists and provide procognitive attributes.[21,22] Compounds 13k, 21 o and 18 h were found to inhibit human H_3 with IC_{50} values of ~ 10 mM, while 21 n afforded an IC_{50} value of 6.3 mM. While weak, this result suggests that it is possible to “dial in” H_3 antagonist activity into this new series of M_4 PAMs, and future efforts will focus on optimizing compounds with comparable M_4 PAM and H_3 antagonist activity for the treatment of the positive and cognitive symptom clusters of schizophrenia.

In summary, a lead optimization campaign around M_4 PAMs 1–3 provided novel analogues with improved metabolic stability and physiochemical properties, but diminished efficacy at rat M_4 (EC_{50} values ~ 2 mM) while retaining comparable fold- shift (14–67 x) of the ACh CRC. Moreover, though weak at rat M_4 , several analogues displayed modest in vivo efficacy in reversing amphetamine-induced hyperlocomotion, a classic preclinical antipsychotic model. Surprisingly, we noted significant species differences within this new series of M_4 PAMs, where analogues such as 21 n 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 mM) with comparable fold-shifts (human, 60 x; rat, 44 x) and high M_4 mAChR subtype selectivity. To further expand the therapeutic relevance of these new M_4 PAMs for the treatment of schizophrenia beyond the positive symptom cluster, we evaluated analogues against the H_3 receptor as they align well with the refined H_3 pharmacophore model. M_4 PAM 21 n was found to provide modest inhibition of H_3 with an IC_{50} value of 6.3 mM, suggesting that it might be possible to develop analogues with dual M_4 PAM and H_3 antagonist activity to effectively treat both the positive and cognitive symptom clusters of schizophrenia. Efforts in this area are in progress and will be reported in due course.

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Figure 1.

A series of M₄-selective potentiators based on a dimethylamino thienopyridine scaffold. VU10010 (1; EC₅₀ = 400 ± 100 nM, ACh CRC fold-shift = 47 x) served as the lead compound for previous physicochemical property optimization producing VU0152099 (2; EC₅₀ = 403 ± 117 nM, ACh CRC fold-shift = 30 x) and VU0152100 (3; EC₅₀ = 380 ± 93 nM, ACh CRC fold-shift = 70 x). These compounds represent the previously reported first generation analogues associated with this series.

Scheme 1.

Synthesis of analogue libraries 9a–p and 13a–s. Reagents and conditions: a) piperidine, EtOH, MW, 160 8C, 12 min, 42%; b) K₂CO₃, MW, 160 8C, 10 min, 75%; c) R¹Cl, KI, Cs₂CO₃, DMF, MW 160 8C, 30 min, 15–80%; d) morpholine, EtOH, MW 160 8C, 10 min, 62%; e) K₂CO₃, DMF, 0 8C- >RT, 24 h 65%; f) R¹Cl, KI, Cs₂CO₃, DMF, MW 160 8C, 30 min, 15–80%.

Figure 2. Potentiation effects of i3 k in rat M₄/G_{q15}-expressing CHO cells by functional Ca²⁺ mobilization assay. a) Concentration response curve for i3 k (~; EC₅₀ = 2.0 mM) in the presence of a fixed submaximal concentration of ACh (~ EC₂₀). b) Full concentration response curve for ACh in the presence (*) and absence (&) of a fixed 30 mM concentration of i3 k (fold-shift = 50x).

Scheme 2.

Synthesis of analogue libraries i7–2i. Reagents and conditions : a) TEA, DMF, 08C !RT, 62 %; b) R^1Cl , KI, Cs_2CO_3 , DMF, MW, 160 8C, 30 min, 75–95 %; c) 1. 1 M NaOH/EtOH (3 : 1), MW 120 8C, 30 min ; 2. R^2NH_2 , DIC, HOBT, DMF/DIEA (9:1), 20–80%.

Figure 3.

Single-point potentiator screen of libraries i7–2i in rat M4/G_{q15}-expressing cells. Test compound (fixed 10 mM) is added prior to addition of a submaximal (~ EC₂₀) concentration of ACh. Intracellular Ca²⁺ mobilization is used as a functional readout for M₄ activation.

Figure 4.

Potentiation effects of 2i n in rat M₄/qi5-expressing CHO cells by functional Ca²⁺ mobilization assay. a) Concentration response curve for 2i n (& EC₅₀ = 2.4 mM) in the presence of a fixed submaximal concentration of ACh (~ EC₂₀). b) Full concentration response curve for ACh in the presence (*) and absence (&) of a fixed 30 mM concentration 2i n (fold-shift =44 x).

Figure 5.

Modest reversal by i3 k and 2i o of amphetamine-induced hyperlocomotor activity in rats. Rats were pretreated for 30 min with vehicle (10 % Tween 80 i.p., n =9; * and *) or a 56.6 mg kg⁻¹ dose of either i3 k (!), 2in (~), or 2io (&) i.p. (n =4), then given an injection of 1 mg kg⁻¹ s.c. amphetamine (indicated by ! at t=0 min)—except vehicle control *, which was dosed with H₂O instead—and locomotor activity was measured for an additional 60 min. The error bars represent the mean value ± SEM. * denotes P < 0.05 versus vehicle plus amphetamine control group (i.e. *), Dunnett's test.

Figure 6.

Potentiation effects of i3 k, i8 h, 2i n, and 2i o in human M_4/G_{q15-2+} expressing cells by functional Ca mobilization assay. a) Concentration response curves for potentiators (&, i3k, $EC_{50} = 100$ nM; ~, i8 h, $EC_{50} = 192$ nM; !, 2in, $EC_{50} = 95$ nM; ^, 2i o, $EC_{50} = 183$ nM) in the presence of a fixed submaximal concentration of ACh ($\sim EC_{20}$). b) Full concentration response curves for ACh in the absence (*) and presence of a fixed 30 mM concentration of potentiator (&, i3k, fold-shift = 63 x ; ~, i8h, fold-shift = 44x ; !, 2in, fold-shift = 60x ; ^, 2io, fold-shift = 49x). DRFU is the change in relative fluorescence units (% of ACh max response).

Figure 7.

Full concentration response curves for ACh in the absence (&) and presence of a fixed 30 mM concentration of potentiator (*, i3 k; ^, i8h ; ~, 2in ; !, 2i o) on rat a) M₁-expressing CHO cells, b) M₂/G_qi5-expressing CHO cells, c) M₃-expressing CHO cells and d) M₅-expressing CHO cells.

22, Refined H₃ Pharmacophore Model

Figure 8.

Refined H₃ pharmacophore model (22) and alignment with M₄ PAM 2i n.

Table 1

SAR data for select analogues from the alkylamine library (**9 a–p**) and the ether library (**13 a–s**) chosen based on an initial single-point triage screen at rat M₄.^[a]

Compd	R ¹	EC ₅₀ ^[b] [μM]
9 a		> 10
9 b		> 10

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Compd	R ¹	EC ₅₀ ^[b] [μM]
9 p		> 10
13 a		> 10
13 b		> 10

Compd	R ¹	EC ₅₀ ^[b] [μl]
13 c		6.5
13 d		8.2
13 e		> 10

Compd	R ¹	EC ₅₀ ^[b] [μl]
13 f		> 10
13 g		> 10
13 k		2.0
13 l		2.2

Compd	R ¹	EC ₅₀ ^[b] [μM]
13 m		1.8
13 p		> 10

^[a]These data represent the mean values from at least three experiments with similar results

^[b]Concentration of compound eliciting an effect 50 % of its own maximal potentiation of an ACh EC₂₀.

^[c]Leftward shift of an ACh CRC in the presence of 30 μM compound relative to ACh CRC control.

Table 2

SAR results for select analogues from libraries **17–21** chosen based on an initial single-point potentiation screen at rat M₄.^[a]

Compd	R ¹	R ²
17 a
17 o
18 a

Compd	R ¹	R ²
18 b

Compd	R ¹	R ²
18 c
18 g

Compd	R ¹	R ²
18 h
19 m

Compd	R ¹	R ²
19 n
21 n
21 o

^[a]These data represent the mean values from at least three experiments with similar results.

^[b]Concentration of compound eliciting an effect 50 % of its own maximal potentiation of an ACh EC₂₀.

^[c]Leftward shift of an ACh CRC in the presence of 30 μ M compound relative to ACh CRC control.