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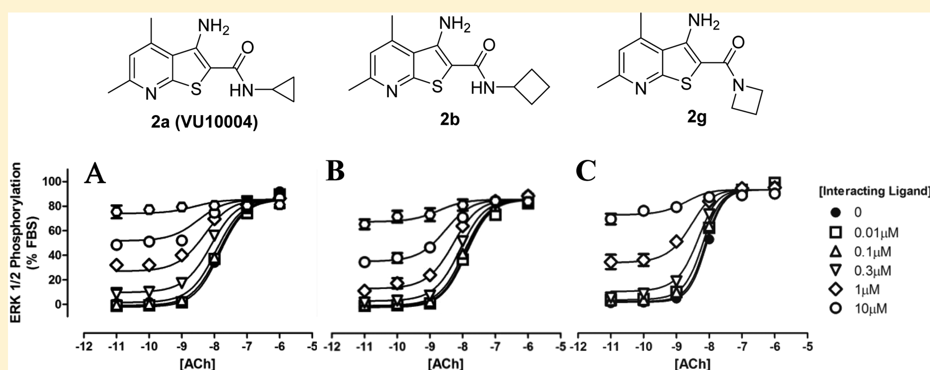
Synthesis and Pharmacological Evaluation of M_4 Muscarinic Receptor Positive Allosteric Modulators Derived from VU10004

Tracey Huynh,† Celine Valant,‡ Ian T. Crosby,† Patrick M. Sexton,‡ Arthur Christopoulos,*,‡ and Ben Capuano*,†

†Medicinal Chemistry and ‡Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Monash University, 381-399 Royal Parade, Parkville VIC 3052, Australia

Supporting Information



ABSTRACT: The M_4 mAChR is implicated in several CNS disorders and possesses an allosteric binding site for which ligands modulating the affinity and/or efficacy of ACh may be exploited for selective receptor targeting. We report the synthesis of a focused library of putative M_4 PAMs derived from VU10004. These compounds investigate the pharmacological effects of target thieno[2,3-*b*]pyridines assembled from primary cycloalkanamines and cyclic secondary amines providing useful estimates of affinity (K_B), cooperativity ($\alpha\beta$), and direct agonist properties (τ_B).

KEYWORDS: *Positive allosteric modulators, PAMs, muscarinic M_4 receptor, LY2033298, VU10004*

The M_4 muscarinic acetylcholine receptor (mAChR), one of the five subtypes of the mAChRs, is a promising target for the alleviation of numerous symptoms associated with schizophrenia.[1] However, due to the high amino acid conservation between each of the mAChR subtypes, orthosteric ligands such as xanomeline[2] (Figure 1), which exhibits promising alleviation of positive and cognitive symptoms but possesses a poor side effect profile, are not actively pursued as treatments for CNS disorders. Allosteric ligands, such as 3-amino-5-chloro-*N*-cyclopropyl-6-methoxy-4-methylthieno[2,3-*b*]pyridine-2-carboxamide (LY2033298, **1**)[3] (Figure 1), have been identified for the M_4 mAChR to overcome the difficulties in the discovery of subtype selective ligands.

These ligands target a topographically distinct site, which possesses a more divergent amino acid sequence between each of the subtypes than the orthosteric site. Positive allosteric modulators (PAMs) act by multiple modes of action from which selectivity at a single receptor subtype can be attained either by modulation of the affinity and/or efficacy of the orthosteric ligand.[4−7] A common approach to the screening and pharmacological assessment for prospective allosteric modulators relies on modulator titration curves, where a fixed concentration (e.g., EC_{20}) of orthosteric agonist is titrated with increasing concentrations of the prospective allosteric ligand to determine modulator potency. All of the VU analogues depicted in Figure 1 were originally identified using this approach at a muscarinic M_4-mediated calcium mobilization assay.[7,8] Despite being useful for structure−activity relationship (SAR) studies, however, a potential limitation of the titration curve design is that it may often yield apparently "flat" SAR profiles if the determination of compound activity relies on a single potency parameter.

The potency of an allosteric modulator and its apparent maximal effect are amalgams of minimally four parameters, specifically, the affinity of the allosteric ligand for the free receptor (K_B); the cooperativity factors that define the magnitude and direction of the allosteric ligand's effect on the orthosteric ligand's affinity (α) and/or downstream efficacy (β); and the intrinsic agonist efficacy of the allosteric ligand (τ_B).[9,10] By implementing an operational model of allosterism,[11] we have previously shown how chemical modification causing changes in one of the parameters may be offset by changes in another parameter in the opposite direction.[12] This potentially accounts for some of the apparent flat SAR based on potency derived from titration curves alone. Derivation of the parameters of allostery can be routinely obtained from a more

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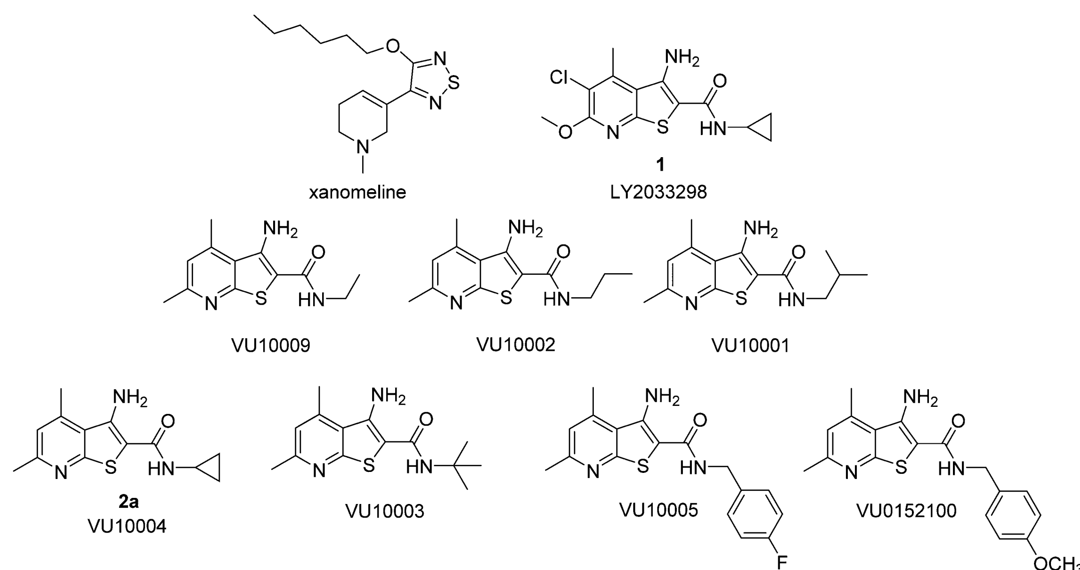
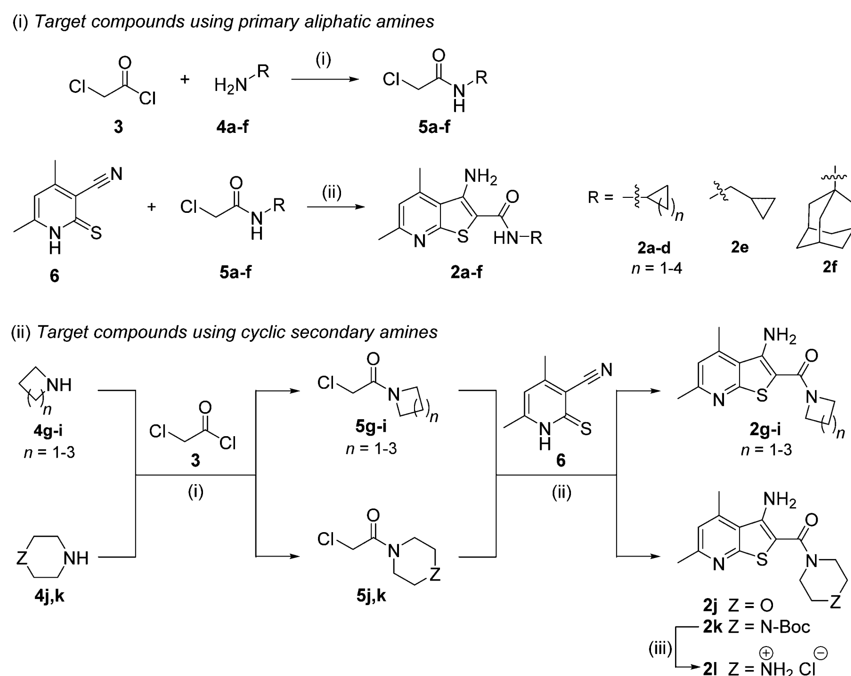


Figure 1. Structures of M_1/M_4-preferring orthosteric agonist, xanomeline; M_4 PAM, LY2033298 (**1**); and structurally related M_4 PAMs developed by Vanderbilt University (VU) including VU10009, VU10002, VU10001, VU10004 (**2a**), VU10003 (*N*-alkyl analogues), and VU10005, VU0152100 (*N*-substituted benzyl analogues).

Scheme 1. Chemical Synthesis of Target Thieno[2,3-*b*]pyridines (2a–l) as Their Hydrochloride Salts from Key Intermediate Core (6) and *N*-Substituted Chloroacetamides (5a–k)[a]



[a]Reagents and conditions: (i) Et_3N, dichloromethane, −5 °C to rt, 49–99%; (ii) KOH (aq), dimethylformamide, rt, 24 h followed by 50 °C, 15 h, 39–81%: (iii) 1 M HCl in dioxane, 0 °C, 77%.

comprehensive functional concentration–response curve analysis, and we propose that this can yield a more "enriched" SAR for describing GPCR allostery. In turn, this can allow more informed decisions to be made in the preclinical workflow associated with drug candidate selection including: (i) whether a given chemical scaffold can only be optimized in terms of a subset of allosteric parameters (e.g., cooperativity but not affinity, or vice versa), (ii) insights into potential mechanisms underlying the allosteric modulation, and (iii) identification of tool compounds with known differences in their allosteric properties that can subsequently be used in vivo to identify an optimal efficacy profile. An example of earlier SAR studies that were performed in this manner enabled the extraction of key pharmacological parameters of M_4 mAChR allosterism, identifying favorable substitution patterns within a series of *N*-benzyl thieno[2,3-*b*]pyridine analogues, in particular the 3-fluoro ($\log \alpha\beta = 1.63 \pm 0.21$, $\log\tau_B = 0.76 \pm 0.13$), 3-fluoro-4-methoxy ($\log \alpha\beta = 1.53 \pm 0.14$, $\log\tau_B = 0.48 \pm 0.07$), the 2,3-difluoro-4-methoxy ($\log \alpha\beta = 0.83 \pm 0.22$, $\log\tau_B = 0.41 \pm 0.09$) derivatives.[12] This method of pharmacological profiling was

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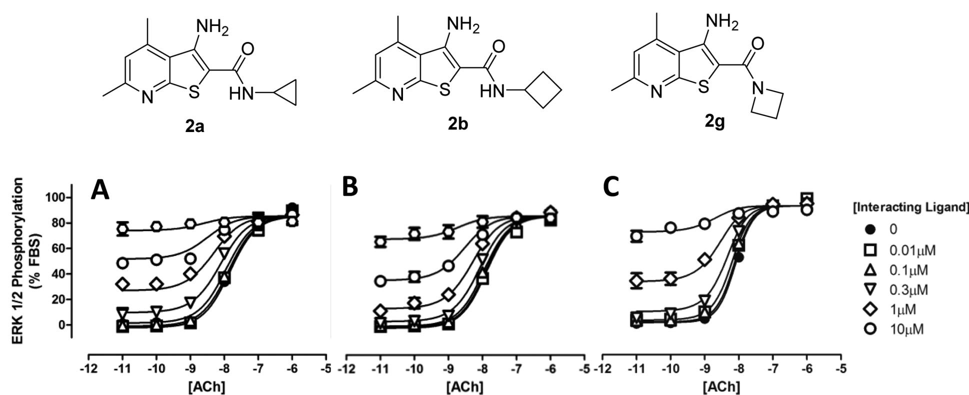

Figure 2. Effect of (A) VU10004 (**2a**) and representative test compounds (B) **2b** and (C) **2g** on ACh-induced ERK1/2 phosphorylation in CHO cells expressing the hM_4 mAChR. Data points are represented as mean percentage of the peak ERK1/2 phosphorylation response elicited by 10% FBS SEM obtained from at least three experiments performed in duplicate.

implemented in the current study for a novel series of prospective PAMs of the M_4 mAChR to better identify allosteric activity. Specifically, research conducted by Shirey et al.[8] described the development of highly selective M_4 PAMs, whereby the top three compounds exhibiting the highest degree of potentiation of the response to an EC_{20} concentration of ACh in a M_4-mediated calcium mobilization assay all possessed an *N*-substituted benzyl moiety. The synthesis of 3-amino-*N*-cyclopropyl-4,6-dimethylthieno[2,3-*b*]pyridine-2-carboxamide (VU10004, **2a**), as part of a series of *N*-alkyl analogues targeted as PAMs of the M_4 mAChR, was also reported, and demonstrated the fourth best activity in this assay. Nevertheless, we viewed **2a** with notable interest on the basis that it shared striking structural similarities to LY2033298 (**1**) (the only difference being the chemical nature of the substitution pattern on the thieno[2,3-*b*]pyridine scaffold) with potential scope for further structural elaboration and refinement from an SAR perspective.

For this series of compounds, we initially chose to synthesize a series of *N*-cycloalkyl analogues with the VU thieno[2,3-*b*]pyridine biyclic scaffold to identify new prospective M_4 PAMs. We investigated the biological impact of increasing steric effects from the cyclopropyl group to the cyclohexyl group (**2a**−**d**) concluding with the adamantyl derivative (**2f**) as a demonstration of the effect of steric bulk. Given the inherent activity of **2a**, we synthesized the analogous cyclopropylmethyl derivative (**2e**) to observe the preliminary effects of incorporating a spacer in this part of the molecule. We also investigated the biological effects of incorporating the nitrogen of the amide functionality in the aforementioned VU compounds into a ring through the application of cyclic secondary amine precursors (azetidine, pyrrolidine and piperidine) with compounds **2g**−**i**, respectively. As an extension to the piperidine analogue **2i**, we synthesized the morpholine analogue (**2j**) to explore the effect of isosteric replacement of CH_2 for O which incorporates further hydrogen-bond acceptor capacity within the molecule. Further isosteric replacement of CH_2 for NH reveals the piperazine analogue (**2l**) that, in addition to exhibiting hydrogen-bond acceptor properties, exemplifies hydrogen-bond donor characteristics with the potential for an electrostatic interaction at physiological pH.

RESULTS AND DISCUSSION

Chemistry. The general synthesis for the target thieno[2,3-*b*]pyridines (**2a**−**l**) is illustrated in Scheme 1. Synthesis of the key pyridinethione core (4,6-dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile, **6**) has been previously described[12,13] and was furnished accordingly in good yield. The chloroacetamide derivatives **5a**−**k** were synthesized in good to excellent yield in accordance with literature,[14] whereby chloroacetyl chloride (**3**) was reacted with the appropriate primary cycloalkanamine (**4a**−**f**) and cyclic secondary amine (**4g**−**k**). The synthesis of bicyclic compounds **2a**−**k** was achieved following the procedure described by Shirey et al.,[8] whereby the chloroacetamide (rather than the described bromoacetamide) was reacted with **6** in the presence of aqueous potassium hydroxide in DMF at elevated temperature. The piperazine analogue (**2l**) was furnished from **2k** via a Boc-deprotection with 1 M ethereal hydrogen chloride and dioxane with overnight stirring, and isolated as the dihydrochloride salt. The target compounds **2a**−**j** were converted to their respective monohydrochloride salts for biological evaluation using 1 M ethereal hydrogen chloride; these compounds afforded bright yellow crystalline solids following recrystallization from methanol.

Biological Activity. To assess the biological activity of the bicyclic (**2a**−**l**) series of compounds, cell-based functional assays of M_4 receptor-mediated ERK1/2 phosphorylation assays were performed using intact FlpIn-CHO cells stably transfected with the human muscarinic M_4 receptor. Time-course assays were initially performed to ascertain the optimal incubation time for maximum ERK1/2 phosphorylation for each compound. Subsequently, concentration−response curves were established at this time point to determine the potency and maximal effect of each compound as a potential direct (allosteric) agonist of the M_4 mAChR. Finally, ACh interaction studies were performed to identify prospective M_4 PAMs from the series **2a**−**l** and to quantify their activity. Although 7 of the 12 compounds synthesized have been previously reported (**2a**, **2d**, **2f**, and **2k** are literature compounds,[7,8] and **2c**, **2h**, and **2i** are part of the Vanderbilt University Specialized Chemistry Center for Accelerated Probe Development database (Vanderbilt University Medical Center)),[15] a detailed pharmacological assessment of the allosteric profiles of these ligands has not been performed to date.

In order to assess the allosteric effects of the series **2a**−**l**, the previously described operational model of allosterism was

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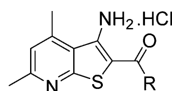
Table 1. Binding Affinity (K_B), Functional Cooperativity ($\alpha\beta$), and Intrinsic Efficacy (τ_B) Estimates of Test Compounds on M_4 mAChR-Mediated Stimulation of ERK1/2 Phosphorylation in Intact CHO FlpIn Cells Expressing the hM_4 mAChR, in the Presence of Increasing Concentrations of ACh



2a-l

Compound	R	pK_B (K_B, μM)	$\log\alpha\beta$ ($\alpha\beta$)	$\log\tau_B$ (τ_B)
1 (LY2033298)		6.13 ± 0.29 (0.74)	1.84 ± 0.28 (68.7)	1.03 ± 0.20 (10.7)
2a	N–H	5.37 ± 0.14 (4.3)	1.72 ± 0.17 (52.2)	0.77 ± 0.11 (5.82)
2b	N–H	5.38 ± 0.17 (4.2)	1.67 ± 0.22 (46.7)	0.90 ± 0.14 (7.93)
2c	N–H	5.28 ± 0.12 (5.2)	1.53 ± 0.15 (34.0)	0.68 ± 0.09 (4.84)
2d	N–H	6.31 ± 0.23 (0.49)	0.36 ± 0.08 (2.29)	-0.67 ± 0.12 (0.21)
2e	N–H	5.17 ± 0.16 (6.8)	1.42 ± 0.20 (26.5)	0.74 ± 0.12 (5.50)
2f	N–H	na[b]	na	na
2g	N	5.52 ± 0.13 (3.0)	0.87 ± 0.18 (7.50)	0.44 ± 0.08 (2.76)
2h	N	5.01 ± 0.18 (9.8)	1.59 ± 0.20 (38.6)	0.94 ± 0.16 (8.62)
2i	N	na	na	na
2j	N, O	na	na	na
2k[a]	N, N–Boc	na	na	na
2l	N, NH_2^+ Cl^-	na	na	na

[a]Isolated as the free base. [b]na: Denotes that the compound does not display quantifiable activity in the pharmacological assay, or possibly possess a pharmacological profile of a neutral allosteric ligand.

implemented to estimate the negative logarithm of the binding affinity of the ligands at the allosteric site (pK_B), their respective intrinsic efficacies (τ_B) as direct allosteric agonists, and the composite cooperativity ($\alpha\beta$) as a global measure of the allosteric ligand's effect on ACh affinity and efficacy.[11] Interaction studies revealed varying degrees of activity in the bicyclic series (**2a–l**). Representative members from this series (**2a**, **2b** and **2g**) are illustrated in Figure 2, demonstrating the effects on ERK1/2 phosphorylation in CHO cells expressing the M_4 mAChR in the presence of increasing concentrations of acetylcholine (ACh). The binding affinity, functional cooperativity and intrinsic efficacy estimates for all test compounds (**2a–l**) on the aforementioned assay are tabulated in Table 1.

As mentioned previously in the study by Shirey et al.,[8] **2a** demonstrated an intermediate degree of allosteric potentiation of M_4-mediated calcium mobilization (ranking fourth of the nine active compounds) and was not further characterized in terms of potency. In our pharmacological assessment, **2a** demonstrates robust potentiation of ACh ($\log\alpha\beta = 1.72 \pm 0.17$) and significant intrinsic efficacy ($\log\tau_B = 0.77 \pm 0.11$) which is comparable to LY2033298 ($\log\alpha\beta = 1.84 \pm 0.28$ and $\log\tau_B = 1.03 \pm 0.20$). Because the cooperativity value of **2a** is comparable to that of LY2033298, this suggests that the presence of the 6-methoxy and 5-chloro does not significantly assist in the modulatory effect of these compounds on ACh. However, the modification did result in reduced affinity of **2a** for the free receptor relative to LY2033298, suggesting that this region may contribute to interaction with the allosteric binding pocket. We illustrated in our earlier study[12] that similar compounds possess a strong correlation (slope = 1.00 ± 0.28) between cooperativity and intrinsic efficacy estimates in their pharmacological profiles. This is further supported by studies of this series of M_4 PAMs. As shown in Figure 3, we found a significant correlation (slope = 1.02 ± 0.15) between

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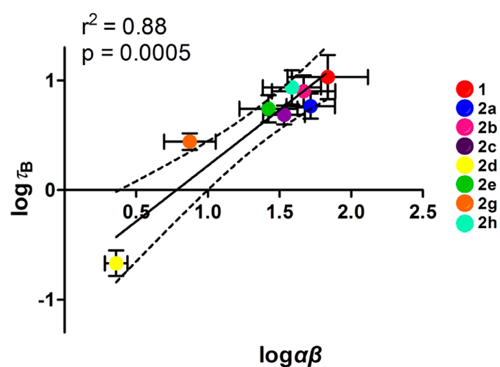

Figure 3. Correlation analysis of efficacy (log τ_B) and cooperativity (log $\alpha\beta$) parameter estimates for **1** and the bicyclic series, **2a**−**e** and **2g**,**h**.

cooperativity and intrinsic efficacy estimates derived from the operational model of allosterism, suggesting that the mechanism of positive modulation involves, at least in part, the ability of these molecules to preferentially stabilize an active state of the M_4 mAChR.

As previously described, a series of *N*-substituted benzyl analogues were synthesized in an earlier study which was pharmacologically evaluated providing a more detailed allosteric profile. These compounds were pursued as they were chosen by Shirey et al. as lead compounds for further studies[8] and their seemingly promising activity in the calcium mobilization assays. The allosteric profiles for the *N*-benzyl analogues varied considerably, as many lacked any activity while a few demonstrated activity comparable to that of LY2033298. Members of the *N*-alkyl series presented herein demonstrated activity similar to that of LY2033298, suggesting that the smaller cycloalkyl moiety is more favorable than the larger substituted benzyl moiety.

The cycloalkyl derivatives (**2a**−**d**) displayed a trend toward loss in activity with increasing size of the cycloalkyl group. The cyclopropyl and cyclobutyl derivatives (**2a** and **2b**) demonstrated very similar relative activities while the cyclopentyl (**2c**) displayed a marginal drop in affinity and allosteric activity. The cyclohexyl derivative (**2d**), however, showed a discernible and unexpected loss of cooperativity and agonism, though the affinity improved, highlighting the biological impact associated with the incorporation or removal of a methylene unit. This result was somewhat surprising as a previously synthesized *N*-benzyl derivative (VU10005 where R = 4-F-$C_6H_4CH_2NH$−) which exhibits a structural motif comparable in size to **2d** possesses a more favorable allosteric profile (pK_B = 5.60 ± 0.15 (K_B = 2.52 μM), log $\alpha\beta$ = 1.12 ± 0.20 ($\alpha\beta$ = 13.2), and log τ_B 0.44 ± 0.09 (τ_B = 2.77)).[12] The greater flexibility of the cyclohexyl ring due to its size and sp^3 character may be less desirable in the binding site while the substitution on the aryl ring and its inherently planar sp^2 nature may be crucial. Alternative, the *N*-alkyl derivatives may bind in a different manner to the *N*-substituted benzyl analogues. This scenario also demonstrates the individual effects of each of the parameters of allosterism, in contrast to the single potency parameter defined by the titration curve that is an amalgam of each of the allosteric parameters. The incorporation of an adamantyl moiety (**2f**) resulted in either a complete loss of allosteric activity and affinity or possibly binding to the allosteric site but exhibiting neutral cooperativity (i.e., a neutral allosteric ligand).

We also investigated the effects of incorporating the nitrogen of the amide functionality within the cycloalkyl ring. The azetidine derivative (**2g**) demonstrated a moderate binding affinity (pK_B = 5.52 ± 0.13) and a 7-fold loss in cooperativity and 2-fold loss in intrinsic agonism compared to **2a**. The pyrrolidine derivative (**2h**) demonstrated a loss in affinity while maintaining cooperativity and intrinsic agonism similar to the literature cyclopropyl ligand (**2a**). An increase in ring size to the 6-membered piperidine analogue (**2i**) resulted in a loss of activity. The effects of **2d**, which still exhibited PAM activity, suggest that the steric tolerance at the amide position is relatively low and substitution or hindrance at this position is unfavorable as the trajectory of the steric bulk differs substantially. We subsequently investigated the role of hydrogen-bond acceptors with the morpholine derivative (**2j**) and H-bond acceptors in the form of the piperazine (**2l**) in addition to the potential for an electrostatic interaction to determine whether activity could be recovered through an additional interaction. Both analogues displayed no allosteric activity as did the precursor Boc-protected piperazine analogue (**2k**) which aligns with the result observed for **2i**, most likely due to the unfavorable steric bulk positioning on the amide.

■ CONCLUSION

A focused library of target thieno[2,3-*b*]pyridines derived from the previously described VU series of M4 PAMs were synthesized and characterized, investigating the effects of incorporating varying primary cycloalkanamines and cyclic secondary amines. The target compounds were pharmacologically profiled in an ERK1/2 phosphorylation assay using intact FlpIn-CHO cells stably transfected with the human muscarinic M_4 receptor to elucidate their binding affinity to the allosteric site (pK_B), intrinsic efficacies (τ_B), and cooperativities ($\alpha\beta$) of the orthosteric ligand ACh. It is noteworthy that all synthesized target ligands that demonstrated positive allosteric modulation of the M_4 mAChR also exhibited intrinsic agonism (τ_B) at the allosteric site in their own right; a pharmacological feature also observed for the M_4 PAM LY2033298. The compounds that exhibited the most comparable affinity, cooperativity and intrinsic efficacy profiles compared to the VU reference compounds **2a** and the previously synthesized 4-methoxybenzyl derivative, VU0152100, (pK_B = 5.95 ± 0.16, log $\alpha\beta$ = 0.68 ± 0.22, and log τ_B = 0.22 ± 0.06), were the cyclopentyl (**2b**), azetidine (**2g**), and pyrrolidine (**2h**) derivatives. Future studies of these compounds will investigate their selectivity for the M_4 mAChR over the other mAChR subtypes, along with metabolic and physiological studies to determine if they demonstrate improvement over LY2033298. **2a** will also be investigated as a lead compound for further SAR studies probing the structure and substitution patterns around the thieno[2,3-*b*]pyridine core to identify the most favorable substitution pattern for M_4 positive allosteric modulators.

■ METHODS

General Procedure for the Synthesis of the Chloroacetamides 5a−k. The appropriate primary cycloalkanamine (**4a**−**f**) (8.25 mmol) or cyclic secondary amine (**4g**−**k**) (8.25 mmol) and triethylamine (1.26 mL, 9.08 mmol) was dissolved in dichloromethane (5−10 mL) under nitrogen and cooled to −2 to −5 °C in an ice/acetone bath. Chloroacetyl chloride (**3**, 656 μL, 8.25 mmol) dissolved in dichloromethane (3 mL) was added dropwise to the mixture and a vigorous reaction occurred with a precipitate forming. The mixture was allowed to stir for 30 min at 0 °C and was then returned to room temperature and stirred for a further 30 min. The precipitate was then

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filtered and washed with dichloromethane. The filtrate was washed with 2 M aqueous hydrochloric acid (2 × 5 mL) and brine (2 × 15 mL). The organic layer was then dried over anhydrous sodium sulfate, filtered, and concentrated in vacuo to afford the desired chloroacetamides **5a**–**k**, which were then recrystallized from dichloromethane/hexane.

*2-Chloro-N-cyclopropylacetamide (**5a**).* Fine colorless needles (1.91 g, 81%); mp 83–84 °C [lit.[16] mp 81–84 °C]. ^{1}H NMR ($CDCl_3$) δ 0.56–0.60 (m, 2H), 0.81–0.86 (m, 2H), 2.72–2.79 (m, 1H), 4.03 (s, 2H), 6.67 (br s, 1H). ^{13}C NMR ($CDCl_3$) δ 6.46 (CH_2), 6.46 (CH_2), 22.8 (CH), 42.5 (CH_2), 167.3 (C).

*1-(Azetidin-1-yl)-2-chloroethanone (**5g**).* Fine white needles (954 mg, 58%); ^{1}H NMR [lit.[17]] ($CDCl_3$) δ 2.14–2.22 (m, 2H), 3.73 (s, 2H), 3.91 (app t, J = 7.7 Hz, 2H), 4.13 (app t, J = 7.7 Hz, 2H). ^{13}C NMR ($CDCl_3$) δ 15.1 (CH_2), 39.0 (CH_2), 48.2 (CH_2), 50.4 (CH_2), 165.4 (C).

General Procedure for the Synthesis of the Target Bicyclic Thieno[2,3-*b*]pyridines 2a–k from 4,6-Dimethyl-2-thioxo-1,2-dihydropyridine-3-carbonitrile (6). Compound **6** (1 equiv) and the appropriately substituted *N*-chloroacetamides **5a**–**k** (1.5 equiv) were dissolved in *N,N*-dimethylformamide (5–10 mL) followed by the addition of solid potassium hydroxide (5 equiv) dissolved in water (1 mL). The mixture was stirred at room temperature for 24 h and then heated to 50 °C for 15 h. The reaction mixture was partitioned between ethyl acetate (30 mL) and water (30 mL). The organic layer was removed and the aqueous layer was further extracted with ethyl acetate (2 × 20 mL). The organic layers were combined, washed with brine (20 mL), dried over anhydrous sodium sulfate, filtered, and then concentrated in vacuo. The resulting residues, except for **2k**, were dissolved in ethyl acetate and converted to their hydrochloride salt products using ethereal hydrogen chloride (1 M); all of which yielded bright yellow crystalline solids following recrystallization from methanol.

*3-Amino-N-cyclopropyl-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide hydrochloride (**2a**).*[8] Bright yellow solid (137 mg, 71%); mp 225–227 °C dec. ^{1}H NMR (DMSO-d_6) δ 0.56–0.62 (m, 2H), 0.63–0.68 (m, 2H), 2.58 (s, 3H), 2.79 (s, 3H), 5.69 (m, 3H), 7.19 (s, 1H), 7.89 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 5.74 (CH_2), 5.74 (CH_2), 20.0 (CH_3), 22.9 (CH_3), 98.2 (C), 122.4 (CH), 124.4 (C), 147.1 (C), 147.1 (C), 156.8 (C), 166.2 (C). HRMS (ESI+): exact mass calcd for $C_{13}H_{16}N_3OS$ (MH^+) 262.1009, found 262.1014. HPLC (λ = 254 nm) t_R (gradient) = 7.15 min, 99.7% purity.

*(3-Amino-4,6-dimethylthieno[2,3-b]pyridin-2-yl)(azetidin-1-yl)methanone hydrochloride (**2g**).* Bright yellow solid (310 mg, 50%); mp 226.5–227.7 °C. ^{1}H NMR (DMSO-d_6) δ 2.23–2.31 (m, 2H), 2.52 (s, 3H), 2.75 (d, J = 0.6 Hz, 3H), 4.08–4.31 (m, 4H), 5.55 (br s, 3H), 7.10 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 15.3 (CH_2), 19.9 (CH_3), 23.3 (CH_3), 32.3 (CH_2), 97.7 (C), 122.0 (CH), 122.6 (C), 143.8 (C), 145.6 (C), 148.4 (C), 158.1 (C), 166.4 (C). HRMS (ESI+): exact mass calcd for $C_{13}H_{16}N_3OS$ (MH^+) 262.1009, found 262.1007. HPLC (λ = 254 nm) t_R (gradient) = 7.21 min, 99.9% purity.

*tert-Butyl-4-(3-amino-4,6-dimethylthieno[2,3-b]pyridine-2-carbonyl)piperazine-1-carboxylate (**2k**).* Bright yellow solid (214 mg, 76%); mp 186.4–187.9 °C. ^{1}H NMR (DMSO-d_6) δ 1.48 (s, 9H), 2.59 (s, 3H), 2.75 (d, J = 0.6 Hz, 3H), 3.50–3.53 (m, 4H), 3.73–3.75 (m, 4H), 5.85 (br s, 2H), 6.87 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 20.3 (CH_3), 24.3 (CH_3), 28.4 (CH_3), 43.5 (CH_2), 45.6 (CH_2), 80.3 (C), 98.8 (C), 122.0 (CH), 123.0 (C), 143.2 (C), 147.6 (C), 154.6 (C), 159.0 (C), 160.2 (C), 167.6 (C). HRMS (ESI^+): exact mass calcd for $C_{19}H_{27}N_4O_3S$ (MH^+) 391.1798, found 391.1802. HPLC (λ = 254 nm) t_R (gradient) = 6.40 min, 98.1% purity.

*(3-Amino-4,6-dimethylthieno[2,3-b]pyridin-2-yl)(piperazin-1-yl)methanone dihydrochloride (**2l**).* Compound **2k** (1 equiv) was dissolved in dioxane (20 mL) and stirred at 0 °C in an ice/water bath. Ethereal HCl (1 M, 3 equiv) was added to the solution and stirred for 24 h. An additional 1 equiv of ethereal HCl was added to ensure complete removal of the protecting group to form the dihydrochloride salt of the desired product. The solvent was removed by rotary evaporation, and the product was suspended in diethyl ether and filtered to give the desired product as a bright yellow solid (97 mg, 77%); mp 273–274.5 °C. ^{1}H NMR (DMSO-d_6) δ 2.55 (s, 3H), 2.79 (s, 3H), 3.15–3.20 (m, 4H), 3.79–3.81 (m, 4H), 6.25 (br s, 2H), 7.16 (s, 1H), 9.58 (br s, 1H). ^{13}C NMR (DMSO-d_6) δ 20.0 (CH_3), 22.8 (CH_3), 41.8 (CH_2), 42.4 (CH_2), 83.0 (C), 122.2 (CH), 123.9 (C), 124.0 (C), 130.7 (C), 143.1 (C), 156.9 (C), 165.1 (C). HRMS (ESI^+): exact mass calcd for $C_{14}H_{19}N_4OS$ (MH^+) 291.1274, found 291.1275. HPLC (λ = 254 nm) t_R (gradient) = 3.71 min, 95.0% purity.

■ ASSOCIATED CONTENT

Supporting Information

Full details of characterization and the pharmacological evaluation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: arthur.christopoulos@monash.edu (A.C.).
*E-mail: ben.capuano@monash.edu (B.C.).

Author Contributions

Tracey Huynh: synthesized and characterized the intermediate and target compounds, writing, performed all pharmacological assays. Celine Valant: expertise, supervision and data analysis for the pharmacological assays; manuscript revision Patrick M. Sexton & Arthur Christopoulos: supervised the pharmacological analysis, experimental design, data analysis, writing and funding Ian T. Crosby & Ben Capuano: supervised the chemistry, experimental design, data analysis, writing and funding. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

mAChR, muscarinic acetylcholine receptor; FlPIn, FlP-In system from Invitrogen; GPCR, G protein-coupled receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2; CHO, Chinese hamster ovary

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