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THE ANTIPSYCHOTIC POTENTIAL OF MUSCARINIC ALLOSTERIC MODULATION

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SUMMARY

The cholinergic hypothesis of schizophrenia emerged over 50 years ago based on clinical observations with both anticholinergics and pan-muscarinic agonists. Not until the 1990s did the cholinergic hypothesis of schizophrenia receive renewed enthusiasm based on clinical data with xanomeline, a muscarinic acetylcholine receptor M_1/M_4-preferring orthosteric agonist. In a clinical trial with Alzheimer's patients, xanomeline not only improved cognitive performance, but also reduced psychotic behaviors. This encouraging data spurred a second clinical trial in schizophrenic patients, wherein xanomeline significantly improved the positive, negative and

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cognitive symptom clusters. However, the question remained: Was the antipsychotic efficacy due to activation of M_1, M_4 or both M_1/M_4? Classical orthosteric ligands lacked the muscarinic receptor subtype selectivity required to address this key question. More recently, functional assays have allowed for the discovery of ligands that bind at allosteric sites, binding sites distinct from the orthosteric (acetylcholine) site, which are structurally less conserved and thereby afford high levels of receptor subtype selectivity. Recently, allosteric ligands, with unprecedented selectivity for either M_1 or M_4, have been discovered and have demonstrated comparable efficacy to xanomeline in preclinical antipsychotic and cognition models. These data suggest that selective allosteric activation of either M_1 or M_4 has antipsychotic potential through distinct, yet complimentary mechanisms.

Muscarinic allosteric modulation will clearly be a theme driving antipsychotic drug discovery efforts for decades to come.

With an onset in late adolescence, schizophrenia, a complex psychiatric disorder characterized by a combination of negative (social withdrawal, blunting of emotional responses, anhedonia) and positive (hallucinations, delusions, paranoia, disorganized behavior) symptoms along with significant cognitive dysfunction, is a debilitating disease that affects 1% of the world population regardless of gender, race or socioeconomic status (1–6). Inclusion of schizoaffective disorder increases the incidence rate to ~6% of the world population (7, 8). Significantly, schizophrenia and related disorders require lifelong, daily maintenance therapy at a cost to society of more than USD 65 billion a year (1–6). The etiology of schizophrenia is believed to be based upon dysregulation of dopamine and glutamate neurotransmission in mesolimbic and mesocortical brain regions, and the prevailing dogma by which schizophrenia has been managed for decades states that excessive dopaminergic transmission in the forebrain underlies the disorder—the so called “dopamine hypothesis” or “dopamine hyperfunction hypothesis” (1–6, 9–12). Support for this hypothesis stems in part from neuroimaging studies using positron emission tomography to examine alterations in central dopamine levels in schizophrenic patients, which include the finding that patients given amphetamine exhibit greater increases in dopamine release than nonschizophrenic controls (13). Furthermore, rationale for this hypothesis is based on the fact that all clinically relevant antipsychotic agents, both typical (haloperidol) and atypical (clozapine, olanzapine), possess significant antagonist activity at the dopamine D_2 receptor; unfortunately, these agents have a slow onset of action and mainly treat the positive symptoms of schizophrenia, with limited to no effect on the negative and cognitive symptoms, thereby representing a substantial unmet medical need (14–17). Thus, the dopamine hypothesis fails to account for all dimensions of this complex disorder, and other theories to account for the pathology of schizophrenia have been advanced.

The NMDA receptor antagonist phencyclidine has been shown to induce the positive, negative and cognitive symptoms of schizophrenia in healthy patients and elicit a resurgence of symptoms in stable schizophrenics (18, 19). In the clinic, the observation that administration of the NMDA receptor co-agonist glycine provides a modest improvement in schizophrenic patients suggests that increasing NMDA receptor activation may provide a therapeutic benefit. These observations led to the NMDA receptor hypofunction hypothesis as an alternative theory for the underlying cause of schizophrenia (18–22). According to this

hypothesis, any agent that can potentiate NMDA receptor currents, either directly by action on modulatory sites on the NMDA receptor (i.e., the glycine co-agonist binding site) or indirectly by activation of G protein-coupled receptors (GPCRs) known to potentiate NMDA receptor function (i.e., muscarinic acetylcholine M_1 receptor and metabotropic glutamate receptor $mGlu_5$) has the potential to ameliorate the symptoms of schizophrenia (5). Preclinically, agents acting at three targets that can increase NMDA receptor function can have antipsychotic-like effects. These include $mGlu_5$ positive allosteric modulators (23, 24), glycine transporter GlyT1 inhibitors (25, 26) and an M_1 allosteric agonist (27). Additionally, encouraging clinical data with GlyT1 inhibitors are beginning to surface (28). While these two hypotheses have clearly dominated the development of therapeutic agents for the treatment of schizophrenia, this review will focus on the antipsychotic potential of the selective activation of muscarinic acetylcholine receptors (mAChRs) by allosteric modulation.

MUSCARINIC RECEPTORS AND THE CHOLINERGIC HYPOTHESIS

Acetylcholine (ACh) is a critical neurotransmitter in both the CNS and peripheral nervous system acting through muscarinic acetylcholine and nicotinic acetylcholine receptors. Evidence suggests that cholinergic neurotransmission in the forebrain regions and cholinergic involvement in learning and memory are mediated primarily by mAChRs (29–31). The mAChRs are members of the GPCR family A that mediate the metabotropic actions of the neurotransmitter ACh. To date, five distinct subtypes of mAChRs, M_1–M_5, have been cloned and sequenced. M_1, M_3 and M_5 activate phospholipase C and calcium mobilization through G_q, whereas M_2 and M_4 block the action of adenylyl cyclase through $G_{i/o}$ (Fig. 1) (29–31). mAChR-regulated cholinergic signaling plays a critical role in a wide variety of CNS and peripheral functions including memory and attention mechanisms, motor control, nociception, regulation of sleep-wake cycles, cardiovascular function, renal and gastrointestinal function and many others. As a result, agents that can selectively modulate the activity of specific mAChRs should have therapeutic potential in multiple pathological states (32–41). However, due to high sequence conservation within the orthosteric binding site for all five mAChR subtypes (M_1–M_5), it has been historically difficult to develop mAChR subtype-selective ligands that bind at the orthosteric (ACh) site (42). As a result, all historical clinical outcomes observed with orthosteric mAChR agonists 1–6 or antagonists 7–11 (Fig. 2) have proven difficult to attribute to a single receptor subtype, as these ligands activate or inhibit all five mAChRs to varying degrees.

A third hypothesis for the etiology of schizophrenia, based on mAChRs, surfaced from clinical observations that both compliments, and yet remains distinct, from the aforementioned dominating hypotheses (5). Over 50 years ago, anticholinergic agents, such as scopolamine, were shown to induce a psychotic state similar to schizophrenia and exacerbate symptoms in schizophrenic patients. Around that same time, clinical trials provided evidence that pan-muscarinic receptor agonists were moderately effective as neuroleptic agents (43–46). These findings led to a cholinergic hypothesis of schizophrenia, decades before the dopamine hyperfunction hypothesis or the NMDA hypofunction hypothesis were proposed. In lieu of subtype-selective small-molecule ligands to probe the cholinergic hypothesis, knockout studies and other clinical, genetic, post mortem and

imaging approaches have been employed to further link mAChR expression and function in the pathology of schizophrenia (8, 47).

The M_1, M_2 , M_3 and M_4 subtypes are located in the CNS, distinct regions throughout while M_5 is present at low levels primarily in the midbrain, suggesting these subtypes have potential roles in the pathology of schizophrenia (48–50). From studies in knockout mice, the M_1-muscarinic receptor subtype has been viewed as the most likely candidate for mediating the effects on cognition, attention mechanisms and sensory processing. Interestingly, the M_1-muscarinic receptor also potentiates NMDA receptor firing (51); however, addressing NMDA hypofunction is not the only mechanism by which a cholinergic hypothesis of schizophrenia has merit. The M_4 receptor is localized in dopamine-rich brain regions (the mesolimbic and nigrostriatal dopaminergic pathways), and studies in knockout mice suggest M_4 regulates dopaminergic neurons involved in motor control, cognition and psychosis (52–54). Thus, activation of M_1 may lead to potentiation of NMDA receptor currents and provide antipsychotic efficacy via the NMDA receptor hypofunction hypothesis, while activation of M_4 regulates dopamine levels and falls under the dopamine hyperfunction hypothesis. A 2009 study found that the attenuation of amphetamine-induced activity by xanomeline was absent in M_4 knockout mice and attenuated in M_1 knockout mice (55). The authors conclude that the efficacy of xanomeline in amphetamine-induced hyperlocomotion is predominantly driven by M_4 activation with a contribution from M_1.

Data from studies using M_5 knockout mice suggest that M_5 is the sole mediator of ACh-induced vasodilation in the cerebral vasculature and thereby may have therapeutic relevance for cerebrovascular diseases. However, other work suggests that M_5 may be relevant to schizophrenia as it is believed to regulate dopamine release in the striatum and nucleus accumbens via its midbrain localization (56). The role of M_3 in the CNS is unclear from the knockout mouse with the major observable phenotype being that of hypophagy and lean (47). Again, selective small-molecule tools are required to elucidate the potential role of M_3 as an antipsychotic.

The importance of the cholinergic system in schizophrenia has been further validated clinically by clozapine 12 (Fig. 3), one of the most clinically useful atypical antipsychotics (57–59). Numerous studies suggest the unique efficacy of clozapine is due to the concentration of its major circulating metabolite, *N*-desmethylclozapine (NDMC) 13, an M_1 receptor allosteric agonist (60–65). By no means is the efficacy purely driven by allosteric agonism of M_1, but the combination of dopamine D_2 receptor inhibition, coupled with selective M_1 receptor agonism, may be important for the unique efficacy profile of clozapine in the treatment of recalcitrant schizophrenic patients (64, 65). mAChRs are also genetically linked to schizophrenia. De Luca and coworkers recently reported on a linkage of the M_5 muscarinic gene (*CHRM5*) and 15q13, a gene associated with schizophrenia (66). In 2003, Liao reported on the association of M_1 genetic polymorphisms with psychiatric symptoms and cognitive function in schizophrenia (67).

A role of mAChRs in schizophrenia is also suggested from post mortem CNS tissue studies. Neuropathological studies by Dean and Crook have demonstrated that levels of both M_1 and

M_4 are significantly reduced in the prefrontal cortex, hippocampus, caudate and putamen in post mortem schizophrenic patients (68–73). Moreover, the reduction in M_1 and M_4 receptor expression may be specific to schizophrenia, as similar decreases were not observed in similar studies with patients suffering from bipolar disorder and major depression (74). In addition, an in vivo imaging study employing a SPECT agent, [^{123}I]-IQNB (a pan-mAChR ligand), was performed in 12 unmedicated schizophrenic patients and findings compared to a control group of healthy subjects. mAChR receptor occupancy was diminished 20–35% in the schizophrenic patients relative to the control group (74). While clearly indicating that mAChRs are decreased in schizophrenics, the [^{123}I]-IQNB study does not provide information on which mAChR subtype(s) are decreased. Still, this is consistent with the post mortem studies in schizophrenics.

PRECLINICAL AND CLINICAL FINDINGS WITH XANOMELINE

In numerous phase II and III clinical trials, pan-mAChR agonists were shown to improve cognitive performance in Alzheimer's disease (AD) patients, but the gastrointestinal and/or cardiovascular side effects resulting from activation of peripheral mAChRs were deemed intolerable and the trials were discontinued (38). Importantly, several orthosteric pan-mAChR agonists demonstrated decline of $A\beta_{42}$ in the cerebral spinal fluid of AD patients, suggesting that mAChR activation has the potential to be disease modifying as well as providing palliative cognitive therapy (41, 75–77). More recent studies in 3xTg-AD mice further support a disease-modifying role for mAChR activation (78). Interestingly, the M_1/M_4-preferring agonist xanomeline 5 (Fig. 2), in addition to improving cognitive performance, had robust therapeutic effects on the psychotic symptoms and behavioral disturbances associated with AD including hallucinations, delusions and vocal outbursts (77, 79, 80). Based on the ability of xanomeline to reduce the antipsychotic-like behaviors in AD patients, scientists at Lilly initiated an effort to evaluate xanomeline as an antipsychotic agent.

Typical and atypical antipsychotics increase dopamine release in the prefrontal cortex (PFC) and induce c-Fos expression in the PFC and nucleus accumbens. Xanomeline was found to mirror the standard antipsychotic agents clozapine and olanzapine by increasing extracellular levels of dopamine in the PFC by inducing c-Fos expression in regions in a manner comparable to clozapine (81, 82). These data suggested that xanomeline, by virtue of its M_1 and M_4 activation, may be a novel antipsychotic agent and led to further studies. Electrophysiologically, xanomeline, after either acute or chronic dosing, was found to inhibit A10 but not A9 dopamine cells; moreover, this effect was blocked by the muscarinic antagonist scopolamine (82). Behavioral studies (decrease in apomorphine-induced climbing, decrease in contralateral rotations in 6-OHDA-lesioned rats, inhibition of conditioned avoidance responding, reversal of apomorphine-induced deficits in prepulse inhibition, reversal of amphetamine-induced hyperlocomotion) in mice and rats followed in which xanomeline displayed comparable efficacy to clozapine without causing catalepsy contrary to the typical antipsychotic haloperidol (82, 83). In 2003, similar studies were extended to nonhuman primates (*Cebus paella* monkeys) with similar positive results (84). Then in 2008, the results of a phase II clinical trial in schizophrenic patients were released (85). The study was a 4-week, double-blind, placebo-controlled outcome trial in subjects

with schizophrenia (N = 20) measuring the Positive and Negative Syndrome Scale (PANSS) for schizophrenia, the Brief Psychiatric Rating Scale (BPRS) and the Clinical Global Impression (CGI) scale. Impressively, subjects receiving xanomeline performed significantly better than the placebo group on both BPRS and PANSS scores (85). Cognition was also improved, with the xanomeline group displaying robust improvements in measures of vocal learning and short-term memory function. Moreover, efficacy was observed within 1 week, as opposed to the long onset of action with dopamine D_2 antagonists (85). However, some adverse events were noted due to activation of peripheral mAChRs. Importantly, xanomeline afforded improvement in all three symptom clusters of schizophrenia (positive, negative and cognitive symptom clusters) with a rapid onset of action (85). One key question remained: Is the efficacy of xanomeline mediated by activation of M_1, M_4 or a synergy of M_1 and M_4 activation?

ALLOSTERIC MODULATION OF MUSCARINIC RECEPTORS

Previous attempts to develop agonists that are highly selective for individual mAChR subtypes have failed because of the high conservation of the ACh binding site, which increases the difficulty in developing compounds that are truly subtype-specific (41). To circumvent problems associated with targeting the highly conserved orthosteric ACh site, an alternative approach has focused on developing compounds that act at less highly conserved allosteric (Greek, “other site”) binding sites on the mAChRs that are spatially and often functionally distinct from the orthosteric (ACh) site. In recent years, this approach is proving to be highly successful in developing subtype-selective ligands for multiple GPCRs (e.g., mGlu, mAChR) (41, 75, 86, 87). Allosteric ligands can possess multiple modes of pharmacology. An *allosteric agonist* is a ligand that is capable of receptor activation in the absence of the orthosteric ligand (i.e., ACh) at a site distinct from the orthosteric (i.e., ACh) site. An allosteric modulator is a ligand that increases (positive, PAM) or decreases (negative, NAM) the action of an orthosteric agonist (i.e., ACh) by binding at an allosteric site that leads to a change in receptor conformation; however, such modulators lack intrinsic pharmacological activity at the receptor in the absence of an orthosteric ligand. A PAM may enhance the affinity of the orthosteric ligand and/or facilitate coupling to G proteins while exerting no effects alone. As opposed to a classical agonist, PAMs have three major advantages: 1) they mimic physiological signaling conditions, 2) they have greater subtype and receptor selectivity and 3) they have less risk of target-mediated toxicity due to a “ceiling effect” whereby progressively increasing doses of a PAM beyond a certain point will fail to elicit a further pharmacological response due to the limiting effect of the endogenous orthosteric agonist concentration (41, 75, 86, 87). Also, it is possible for a single molecule to have both allosteric potentiator and allosteric agonist activity (usually at high concentrations), and these ligands are referred to as *ago-potentiators*. Discovery of allosteric modulators typically proceeds from functional high-throughput screening using cell-based assays, which involve preincubation of the cells with test compound followed by addition of a submaximal concentration of orthosteric agonist to identify compounds that enhance the agonist response.

Early proof-of-concept efforts by several laboratories were successful in identifying ligands that possess PAM activity at either M_1 or M_4; however, these first-generation mAChR

PAMs lacked efficacy and physiochemical properties to advance into in vivo studies to dissect the contribution of selective M_1 and M_4 activation for the efficacy of xanomeline (Fig. 4) (88–92). Brucine 14 was the first reported mAChR PAM, and it was a selective, but weak M_1 PAM increasing ACh affinity only 2-fold. Surprisingly, the *N*-oxide of brucine 15 increased ACh affinity ~1.5-fold for M_4 (90). Thiochrome 16 followed as the second reported M_4 PAM and SCH-202676 17 was identified as a PAM of multiple mAChR subtypes (90, 92). For the M_1 subtype, pioneering work also led to the discovery of selective allosteric agonists; however, they too suffered from ancillary pharmacology and poor physiochemical properties. AC-42 18 was the first in a novel class of M_1 agonists that act by binding to an allosteric site rather than the orthosteric ACh site. AC-42 fully activates M_1 and is highly selective for M_1 relative to other mAChR subtypes (93). As with PAMs, this selectivity is accomplished by targeting a site distinct from the ACh binding site. Thus, mutations that render M_1 insensitive to ACh do not alter activity of AC-42; however, the activity of AC-42 can be eliminated by mutations in transmembrane domains 1 and 7 that do not alter activation by ACh. NDMC 13 is another M_1 agonist that demonstrates a mode of interaction with the M_1 receptor that differs from that of classic orthosteric ligands and may be mechanistically similar to AC-42 (63–64). As mentioned earlier, the unique clinical efficacy of clozapine is believed to be directly related to the concentration of the major circulating metabolite NDMC (60–65).

ANTIPSYCHOTIC POTENTIAL OF M_1 ALLOSTERIC MODULATION

Significant advances have been made in the last 2 years in the discovery and development of both M_1 PAMs and M_1 allosteric agonists that possess the profile required to perform in vivo proof-of-concept studies and dissect the contribution of selective M_1 activation in the preclinical and clinical efficacy of the M_1/M_4-preferring xanomeline 5. M_1 PAMs representing multiple chemotypes 19–22 have recently been identified in a high-throughput functional screen conducted at Vanderbilt (Fig. 5) (94). None of these ligands display agonist activity, but function as pure PAMs inducing parallel leftward shifts of the ACh concentration-response curves (CRCs). PAMs 19–23 possess varying degrees of M_1 selectivity (relative to the other mAChRs) with some showing complete selectivity. As anticipated, none of these M_1 PAMs bind at the orthosteric site, and were shown to increase ACh affinity at M_1. Interestingly, these PAMs displayed differential regulation of coupling of the M_1 receptor to different signaling pathways (94). While not in vivo tools, these ligands represented a marked improvement, and demonstrated that all allosteric M_1 activation is not equivalent.

A major breakthrough resulted with the discovery of the M_1 PAM coined BQCA (benzylquinolone carboxylic acid) 24 (Fig. 5) by researchers at Merck. BQCA is a potent (human M_1 EC_{50} = 845 nM, 129-fold leftward shift of the ACh CRC), highly M_1-selective PAM (no agonism, potentiation or antagonism of $M_{2–5}$ observed up to 100 μM) with exceptional pharmacokinetics and CNS exposure (95). BQCA does not bind at the orthosteric ACh site, and site-directed mutagenesis experiments identified an allosteric binding site for BQCA involving residues Y179 and W400. Similar to the pre-clinical profile of xanomeline, BQCA increased c-Fos expression in critical brain regions, dose-dependently reverses amphetamine-induced hyperlocomotion in mice and reverses

scopolamine-induced memory deficits in contextual fear conditioning. The procognitive phenotype of BQCA was further noted in rat sleep studies, where it modified sleep architecture (increased wakefulness with concomitant decrease in delta sleep). Quite unexpectedly, BQCA also increased blood flow to the cerebral cortex, a process formerly associated with M_5 from studies in knockout mice, and another example of the critical need for selective tools to elucidate receptor function (95). Further work with BQCA by the Vanderbilt group demonstrated that activation of the M_1 receptor by BQCA induces a robust inward current and an increase in spontaneous excitatory postsynaptic currents in medial prefrontal cortex (mPFC) pyramidal cells, effects which are absent in acute slices from M_1 receptor knockout mice (96). To evaluate the effect of BQCA on intact and functioning brain circuits, multiple single-unit recordings were obtained from the mPFC of rats that showed BQCA increases firing of mPFC pyramidal cells in vivo. BQCA also restored discrimination reversal learning in a transgenic mouse model of Alzheimer's disease and was found to regulate nonamyloidogenic amyloid precursor protein processing in vitro, suggesting that M_1 receptor PAMs have the potential to provide both symptomatic and disease-modifying effects in Alzheimer's disease patients. This latter study with BQCA provides compelling evidence that M_1 activation induces a dramatic excitation of PFC neurons and suggests that selectively activating the M_1 mAChR subtype may ameliorate impairments in cognitive function. A library of BQCA analogues 25 was also prepared, and a robust structure–activity relationship was observed, with multiple analogues displaying comparable potency, efficacy and fold-shift to BQCA (96).

In parallel, the second-generation M_1 allosteric agonists have generated equally exciting and impressive support for an antipsychotic and precognitive role of selective M_1 activation (Fig. 6). TBPB 26 is a potent (EC_{50} = 280 nM), highly selective (> 30 μM vs. $M_{2–5}$) and centrally penetrant M_1 allosteric agonist (27) (Table I). A second systemically active M_1 allosteric agonist, 77-LH-28-1 27, was identified from a series of AC-42 analogues (97). 77-LH-28-1 is also highly M_1 selective (though weak agonism of M_3 is noted), but may more accurately be referred to as a bitopic ligand. Of these, TBPB has activity in multiple electrophysiology, expression and animal models used to predict antipsychotic efficacy for the positive symptoms of schizophrenia. Activation of M_1 by TBPB was shown to potentiate NMDA receptor currents, induce c-Fos expression in key brain regions, increase dopamine turnover, reverse amphetamine-induced hyperlocomotion and reverse prepulse inhibition to a degree comparable to xanomeline and other atypical antipsychotics (27). In addition, TBPB showed efficacy in reversing scopolamine-induced memory deficits in contextual fear conditioning at low doses. Similar to BQCA, TBPB was also shown to regulate nonamyloidogenic APP processing in vitro, suggesting that M_1 allosteric agonists have the potential to provide both symptomatic and disease-modifying effects in Alzheimer's disease patients (27, 97). This report was followed by accounts of TBPB analogues 28 and 29, wherein both full and partial M_1 allosteric agonists were identified (98–100). Surprisingly, molecular switches (halogen substitutions) were discovered that "dialed in" dopamine D_2 inhibition; thus, like NDMC, ligands could be designed to possess both D_2 inhibition and M_1 allosteric agonism, an attractive profile for an antipsychotic agent (98, 99).

Recently, a third generation of exquisitely potent (EC_{50}s 150–200 nM), selective (> 50 μM vs. $M_{2–5}$ and clean ancillary pharmacology) and centrally penetrant (brain:plasma ratio of

4.0) M_1 allosteric agonists, VU-0186470 30 and VU-0357017 31, were reported by the Vanderbilt group (100). Unlike all the predecessors that bind at an allosteric site in the 7 transmembrane domain, VU-0186470 and VU-0357017 act at a novel allosteric site in the third extracellular loop. Importantly, these new M_1 allosteric agonists potentiate NMDA currents and were shown to provide a full reversal of scopolamine-induced memory deficits in contextual fear conditioning at a 10 mg/kg dose (100). Preclinical antipsychotic efficacy is eagerly awaited. Thus, selective activation of M_1 by either a PAM or an allosteric agonist does mimic some of the effects of xanomeline in animal models that are relevant to clinical efficacy of antipsychotic agents.

ANTIPSYCHOTIC POTENTIAL OF M_4 ALLOSTERIC MODULATION

A major breakthrough for selective activation of M_4 occurred with the discovery of VU-0010010 32 as a highly selective M_4 PAM (> 30 μM vs. $M_{1,2,3,5}$) (101). VU-0010010 is an allosteric potentiator and does not activate M_4 directly but dramatically potentiates the response of the receptor to ACh. Extensive in vitro pharmacological characterization reveals that VU-0010010 binds to an allosteric site to increase both the affinity of M_4 for ACh and efficiency of coupling of M_4 to G proteins (102). More recently, two related compounds were reported, VU-0152099 33 and VU-0152100 34, that are also highly selective for rat M_4 (rat M_4 EC_{50}s in the 350–400 nM range, > 30 μM vs. $M_{1,2,3,5}$, 30-to 70-fold leftward shift of the ACh CRC), readily cross the blood–brain barrier and have pharmacokinetic properties making them highly suitable for behavioral studies (103). Interestingly, both VU-0152100 and VU-0152099 almost fully reverse amphetamine-induced hyperlocomotion in rats relying on endogenous ACh, suggesting that M_4 PAMs have efficacy in at least one model used to predict antipsychotic efficacy similar to xanomeline (103).

Chan and coworkers reported the discovery of a similar, but structurally distinct M_4 PA M , termed LY-2033298 35 (101). This compound exhibits highly selective and potent human M_4 potentiator activity, whereas VU-0010010, VU-0152099 and VU-0152100 show equivalent potency at both human and rat M_4. Interestingly, LY-2033298 has also been shown to possess in vivo efficacy in reducing conditioned avoidance response and in reversal of apomorphine-induced disruption of prepulse inhibition, two additional rodent paradigms predictive of antipsychotic drug efficacy and in which xanomeline showed similar positive effects. Importantly, due to the weaker activity of LY-2033298 at the rat (vs. human) receptor, in vivo studies in rats required co-dosing with a low dose of oxotremorine for the PAM to potentiate receptor function; in contrast, the VU compounds were efficacious in vivo relying solely on endogenous ACh (101, 103).

A second generation of M_4 PAMs from Vanderbilt incorporated basic residues in the Western portion and noted a unique species difference, wherein VU-0152129 36 and VU-0359509 37 (Fig. 7) were an order of magnitude more potent on human versus rat M_4 (human EC_{50}s of 100 nM and 183 nM, respectively; rat EC_{50}s of 2.0 μM and 3.8 μM, respectively, but with > 50-fold shifts on both cell lines) (104). Despite the weaker potency on rat M_4, both compounds still provided modest reversal of amphetamine-induced hyperlocomotion activity in rats, relying on endogenous ACh for receptor potentiation.

Collectively, the studies with M_4 allosteric modulators clearly demonstrate that selective activation of M_4 is a viable target for the development of novel antipsychotic agents. Moreover, the observed efficacy in three pre-clinical antipsychotic models where xanomeline affords similar positive results implicates M_4 as a major contributor to the mechanism of action of xanomeline.

ANTIPSYCHOTIC POTENTIAL OF M_5 ALLOSTERIC MODULATION

Though implicated as a potential antipsychotic target from a combination of knockout and genetic studies mentioned earlier, M_5 is far less advanced than M_1 and M_4, due to a lack of ligands to study receptor function. In fact, prior to 2009, no selective M_5 ligands existed. Interestingly, the *p*-bromobenzyl-substituted isatin M_1 PAM screening hit VU-0119498 19 (Fig. 8) was found to exhibit allosteric potentiator activity at not only M_1, but also M_3 and M_5 receptors with comparable potency and efficacy in Ca^{2+} mobilization assays, but lacked potentiator effects at M_2 and M_4 (94). Chemical optimization of this $M_1/M_3/M_5$ PAM led to the discovery of VU-0238429 38, the first M_5-preferring ligand, an M_5 PAM (M_5 EC_{50} = 1.1 μM, > 30 μM vs. $M_{1–4}$, 14-fold shift) (105). Similar to other mAChR PAMs, VU-0238429 did not compete for binding at the orthosteric ACh binding site and increased affinity of M_5 for ACh (105). Further optimization of VU-0238429 led to the discovery of the phenoxybenzyl analogue VU-0400265 39, which displayed similar potency (M_5 EC_{50} = 1.9 μM) but was completely selective for M_5 in functional assays with recombinant cells expressing each of the other mAChRs (106). This initial disclosure is very exciting, and in vivo data with this class of M_5 PAMs, and hopefully M_5 NAMs, will be essential to determine the antipsychotic potential of selective M_5 modulation.

CONCLUSIONS

Historical clinical data from over 50 years ago suggested a cholinergic hypothesis for the pathophysiology of schizophrenia. Preclinical and clinical data with xanomeline, an M_1/M_4-preferring orthosteric agonist, suggest mAChR activation has high antipsychotic potential. Only recently has science evolved to a point where highly selective small molecules can be developed to activate individual mAChRs, by virtue of allosteric modulation. Collectively, the studies with M_1 and M_4 allosteric modulators, both allosteric agonists and PAMs, clearly demonstrate the potential of targeting allosteric sites for developing highly selective activators of individual mAChR subtypes and suggest that both M_1 and M_4 may provide viable targets for the development of novel antipsychotic agents. Moreover, these preclinical animal model studies suggest that the antipsychotic efficacy of xanomeline is due to a synergy of M_1 and M_4 activation, although M_4 may play a dominant role. The antipsychotic potential of M_3 is unclear, but with the emergence of new selective M_5 PAMs, we may soon know the contribution, if any, of selective M_5 activation, and potentially M_5 NAMs on the etiology of schizophrenia. Based on all of these data, muscarinic allosteric modulation will clearly be a theme driving antipsychotic drug discovery efforts for decades to come.

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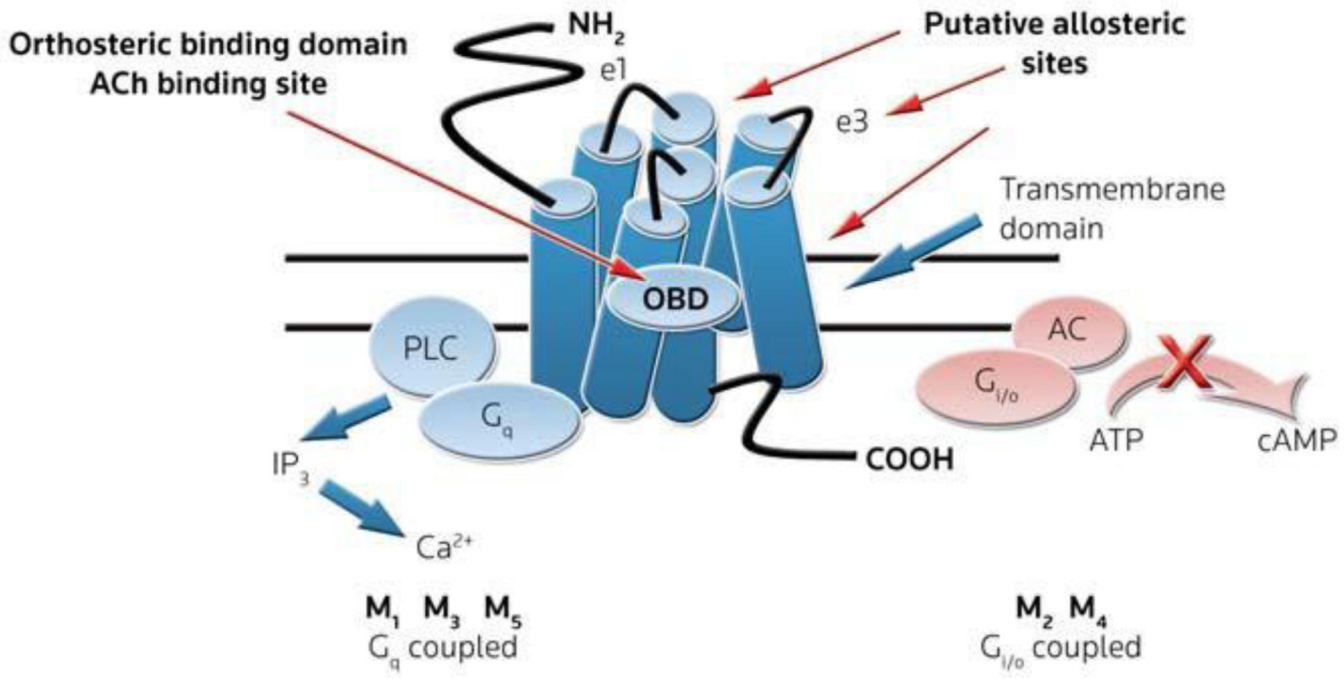


Figure 1.

Schematic Illustration of the structures and effector systems of the muscarinic acetylcholine (ACh) receptor subtypes M_1–M_5. The orthosteric binding domain (OBD) is highlighted within the transmembrane domain and putative allosteric binding sites are denoted. PLC, phospholipase C; AC, adenylyl cyclase.

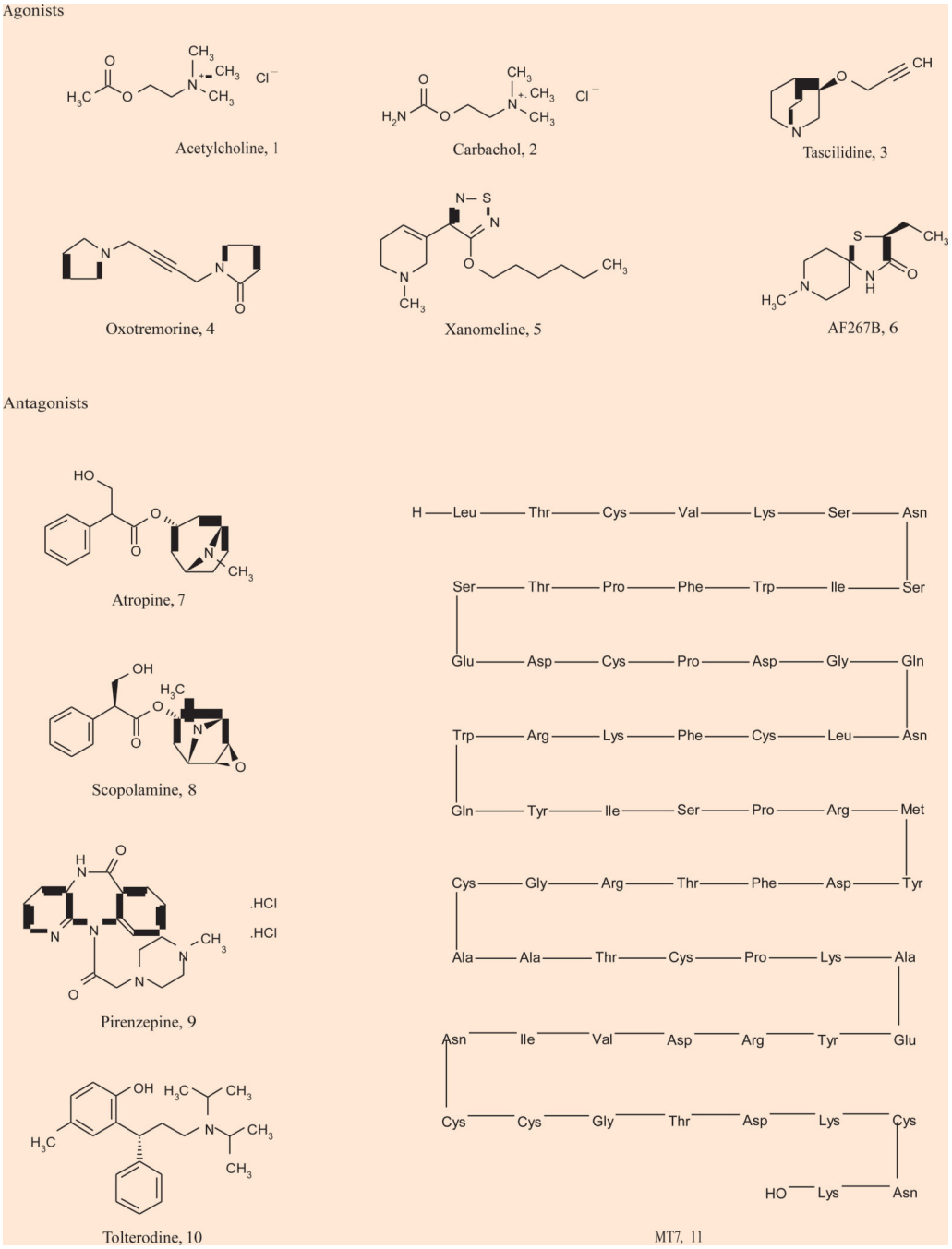


Figure 2.
Representative orthosteric agonists and antagonists of muscarinic acetylcholine receptors.

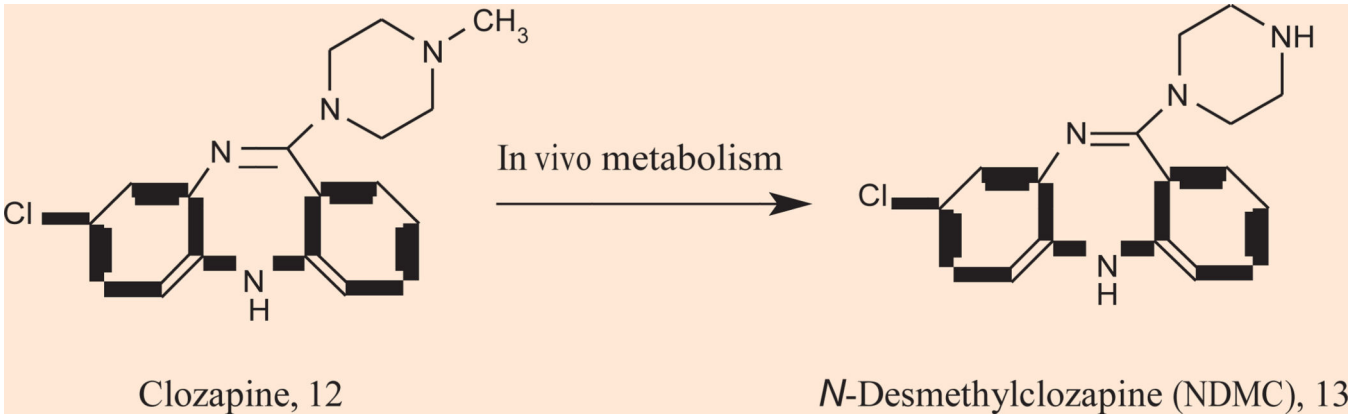


Figure 3.
Chemical structure of clozapine 12 and its major metabolite *N*-desmethylclozapine (NDMC) 13.

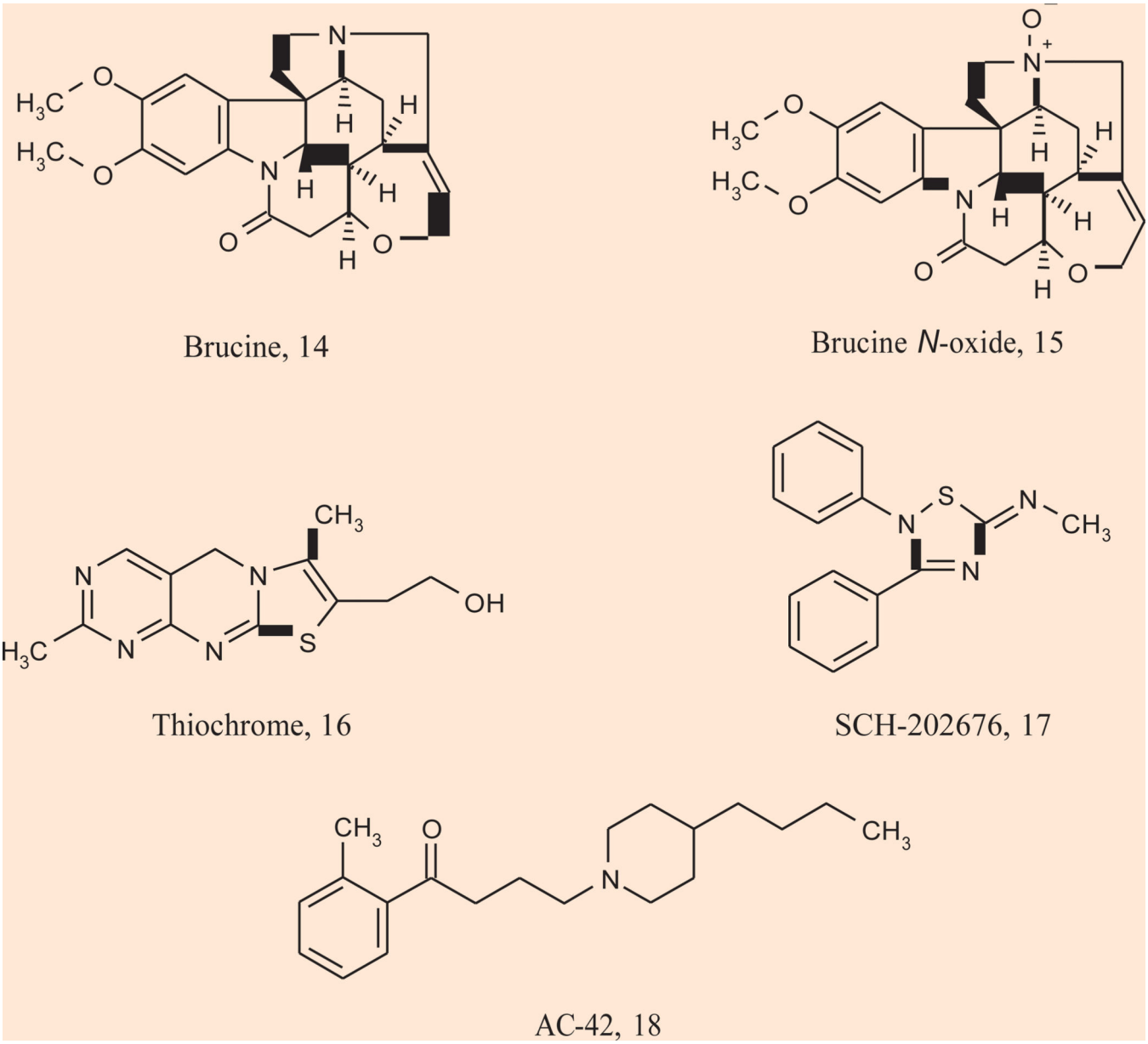


Figure 4.

Structures of the first-generation allosteric ligands (early positive allosteric modulators and allosteric agonists) of muscarinic receptor subtypes M_1 and M_4.

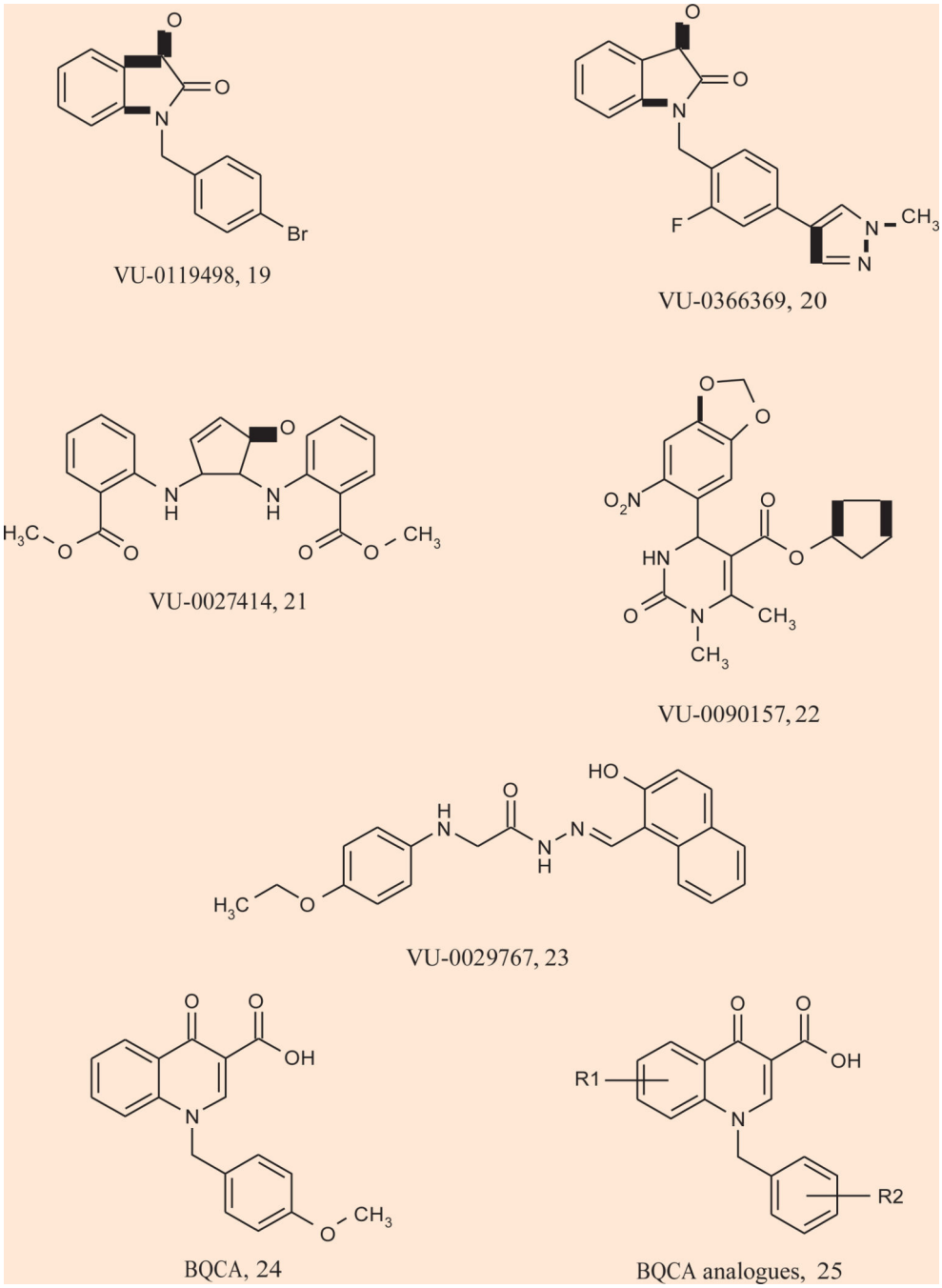


Figure 5.
Structures of M_1 positive allosteric modulators.

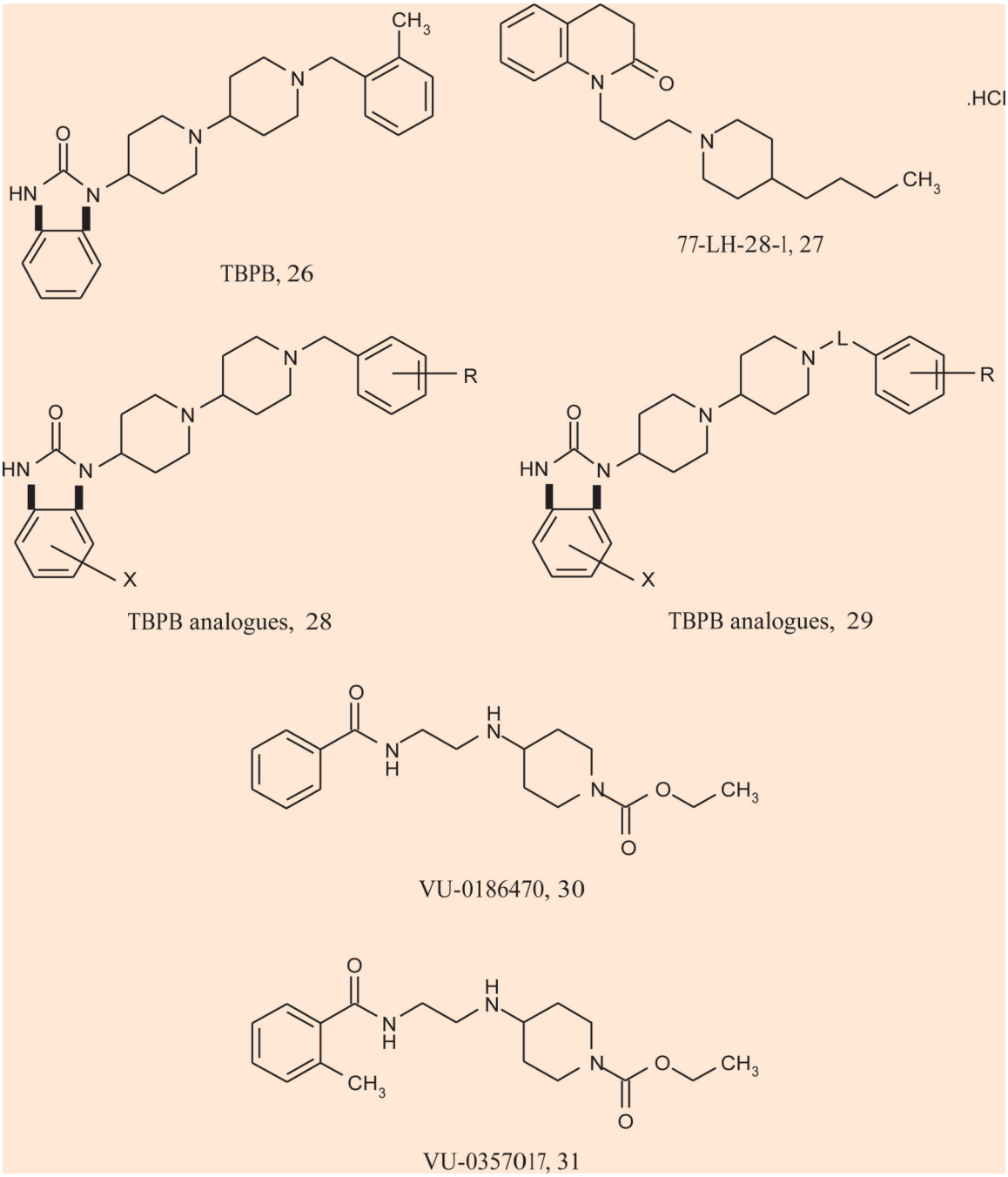


Figure 6.
Structures of M_1 allosteric agonists.

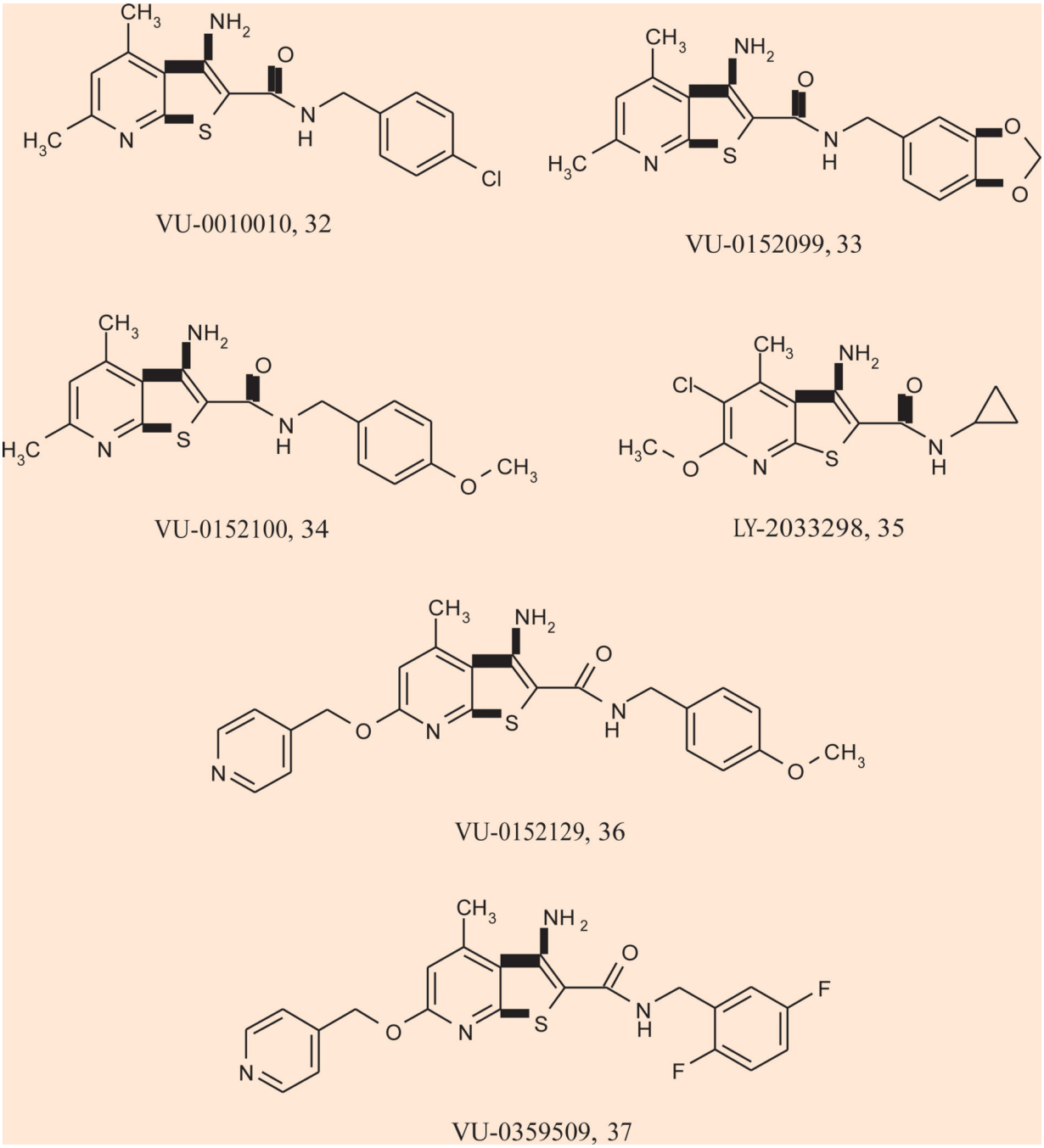
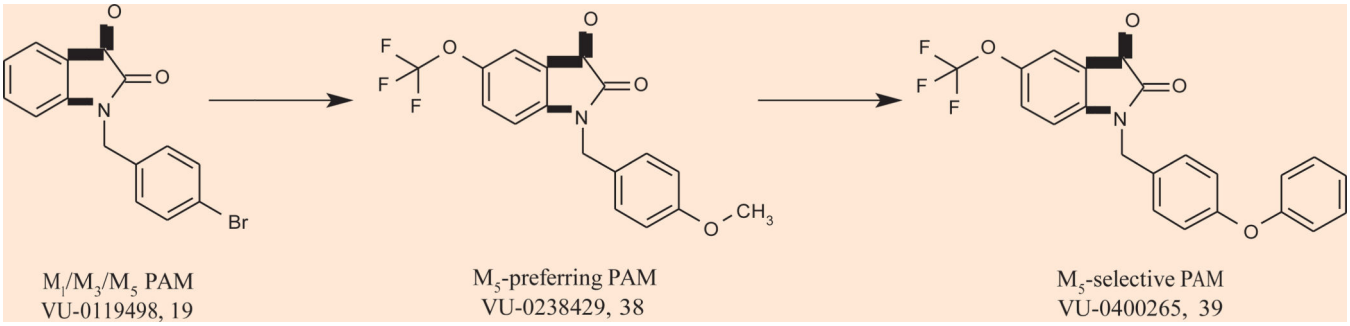


Figure 7.
Structures of M_4 positive allosteric modulators.



$M_1/M_3/M_5$ PAM
VU-0119498, 19

M_5-preferring PAM
VU-0238429, 38

M_5-selective PAM
VU-0400265, 39

Figure 8.

Structure of the first M_5-preferring ligand VU-0238429 38, an M_5 positive allosteric modulator (PAM).

Table I

Abbreviated summary of muscarinic acetylcholine receptor (mAChR) ligands discovered by Vanderbilt University.

Compound	Type/mode	Potency in cell-based Ca^{2+} assay	Efficacy in rodent behavioral models	Electrophysiology/ neurochemistry
TBPB (26)	M_1 agonist	158 nM at rat M_1, > 30 μM vs. all other mAChRs	Amphetamine-induced hyperlocomotion, apomorphine-induced PPI	Potentiates NMDAR current in CA1, induces fos expression in forebrain
VU-0357017 (31)	M_1 agonist	198 nM at rat M_1, > 30 μM vs. all other mAChRs	Scopolamine-induced disruption of contextual fear conditioning (acquisition)	Potentiates NMDAR current in CA1
VU-0366369 (20)	M_1 PAM	830 nM at rat M_1, > 30 μM vs. all other mAChRs	Not determined/unpublished	Not determined/unpublished
VU-0152100 (34)	M_4 PAM	380 nM at rat M_4, > 30 μM vs. all other mAChRs	Amphetamine-induced hyperlocomotion	Not determined/unpublished
VU-0238429 (38)	M_5 PAM	1.1 μM at human M_5, > 30 μM vs. all other mAChRs	Not determined/unpublished	Not determined/unpublished