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Synthesis and Structure-Activity Characterization of Selective Agonists for Alpha-7 nAChRs

A thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Xiaoxuan (Alice) Wu

Committee in charge:

Professor Palmer Taylor, Chair
Professor Kimberly Cooper, Co-chair
Professor Stanley Lo

2021

The thesis of Xiaoxuan (Alice) Wu is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2021

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ABSTRACT OF THE THESIS

Synthesis and Structure-Activity Characterization of Selective Agonists for Alpha-7 nAChRs

by

Xiaoxuan (Alice) Wu

Master of Science in Biology

University of California San Diego, 2021

Professor Palmer Taylor, Chair
Professor Kimberly Cooper, Co-chair

Previous research on the possible target for treatment of neurodegenerative diseases such as Alzheimer's has indicated the value of studying selective agonist to nicotinic acetylcholine receptors (nAChRs). Following this route, we made a strong effort in studying the ligand binding to nAChRs, especially the α7 subtype. We have characterized the central pyrimidine ring and the 2-(Di-picolyl) substituted at the 4 position of the pyrimidine as important motifs for the selective agonistic activity. To further explore the characteristics of crucial motif, we synthesized 7 intermediate and 8 final compounds accordingly, each rationally designed to confer a unique chemical property. The analogue library was tested in HEK cells transfected with cDNAs encoded for α7- nAChR, α4β2-nAChR, and a serotonin receptor (5-HT_{3A}) along with a florescent reporter. We observed the symmetry in the 2-(Di-picolyl) motif, the hydrophilicity of the second position, as well as the presence of the amino group in the second position are crucial for agonistic activity. Final compounds were rationally designed following these observations and one leading compound was shown to be able to cross the blood-brain barrier. In conclusion, the structure of the

compound has determining effect on the agonistic activity, efficacy as well as its ability to traverse the physiological blood-brain barrier. This structure-activity information is extremely valuable and should be considered in future ligand design.

Introduction

Dementias

The mammalian brain is a highly complex organ. Being human relies on the normal brain function for simple daily tasks. Malfunction can lead to severe neurological disorders. Approximately 600 neurological disorders have been characterized globally. Among them, many patients acquire disorders or death of multifunctional neuronal cells (termed neurons) in the brain, leading to a group of diseases also known as Dementias (Alzheimer's disease facts and figures, 2013). Given the unique non-dividable features of neurons, cell death and damage can eventually impair the patients' cognitive functions and catastrophically affect patients' daily lives, and lead to death in severe cases. As a globally prevalent disease, dementia is reported to affect 35.6 million people worldwide in 2010. Currently, around 50 million globally are affected, and the number is expected to more than double in the year 2050 (Prince 2013).

Among various types of dementia, Alzheimer's Diseases (AD) is the most common kind, characterized by accumulation of amyloid-beta peptide(A) and misfolded tau protein, as well as cholinergic dysfunction (2013 Alzheimer's disease facts and figure 2013). According to statistics presented by the World Health Organization (WHO), in late 2020, as many as 60-70% of dementia cases involve AD, with most patients above the age of 60 (Dementia 2020). Patients with AD experience impaired memory, trouble understanding, communicating and thinking, trouble breathing and eating, and significant change of personality. As the disease sporadically progresses along with ageing, it often causes patients to withdraw from society, become bed-bound, and, in severe cases, become infected by pneumonia, leading to death (2013 Alzheimer's disease facts and figure 2013).

Given AD's enormous societal impact, a considerable effort had emerged aiming to discover effective treatments in the past 30 years. One practical approach is the target of the neurotransmission process. To rescue the decrease of cholinergic transmission due to cholinergic neuron loss in the basal forebrain, acetylcholinesterase inhibitors were first developed. However, many of these inhibitors produced clinically insignificant outcomes. Although effective in clinical trials, other drugs developed as an agonist to the muscarinic receptor were shown to have undesirable side effects due to their lack of selectivity (Mangialasche 2010). Nicotinic acetylcholine receptors are also a drug target. The drug developed as the agonists to its subtypes is shown to improve symptoms and have neuroprotective effect to some degree (Mangialasche 2010).

Signaling targets

The nicotinic acetylcholine receptors (nAChRs) are pentameric, ligand-gated ion channels that are stimulated by acetylcholine. In mammalian brains, a total of 11 types of subunits have been characterized. These subunits assemble to form pentamers with different compositions, giving rise to unique functional and pharmacological properties for each receptor subtype. nAChRs are highly abundant in the autonomic and central nervous systems in neuronal and nonneuronal cell types. Their expression is primarily observed in the presynaptic terminals, indicating its unique role in modulating the release of both acetylcholine and other neurotransmitters (MacDermot 1999). The modulatory roles make this receptor a great research candidate for many neuronal degenerative diseases, AD and schizophrenia and Parkinson's disease. Most recently, it has been found to be relevant in inflammation by regulating the cholinergic anti-inflammatory pathway in immune cells (Bagdas 2018).

Among the nAChR superfamily, the α7 subtype is one of the most abundant. Its uniqueness comes from the five identical α7 subunits. It has five potential orthosteric sites localized at the subunit interfaces, between the C loop containing the principal face and the neighboring complementary face (Kaczanowska et al. 2017). The α7-nAChR can be activated by choline; it has a high permeability to calcium ion and can be rapidly desensitized within a millisecond timeframe when exposed to a high concentration of agonist. Agonists are exogenous compounds that bind to the orthosteric binding site and trigger the activation of the receptor. Enhanced expression of the α7-nAChR subtype is shown to correlate with increased attention and improved post-stroke cognitive functional deficits in animal models (Yuan 2020).

Various α7-nAChR agonists have been developed as a treatment for symptomatic AD in the past two decades, yet this process faces extreme complications due to a wide range of the non-CNS modulatory functions of nAChR receptor subtypes, the unique protective mechanism and complexity of the brain itself. Encenicline, a partial agonist selective against the α7-nAChR, has been shown to enhance cognition in clinical trials effectively. However, it has been suspended due to its gastrointestinal severe side effects in the elderly patient during clinical trials (Phase 3) in 2014 (Hoskin 2018). Another agonist, ABT-418, developed to have activity toward complications including nausea. Another agonist targeting the α7-nAChR, such as ABT-126 (Nelonicline), showed admirable efficacy in early clinical trials, but failed in phase 2 trials because of insufficient efficacy. Although evidence supporting the cholinergic pathway as a potential treatment option for AD continue to increase, developed agonists still face

complications such as undesirable non-CNS effects (Florian 2016). Therefore, pharmaceuticals of unique structure with higher specificity toward the α7-nAChR is needed to minimize CNS and non-CNS complications.

The primary goal in the Taylor Lab has been searching for compounds that selectively activate the α7 subtypes, thereby improving the side effect profile of this pharmaceutical category. Based on previous crystallography studies, 2,4,6-substituted pyrimidines ligands are shown to have a relatively more selective agonistic effect on α7 AChR, compared to α4β2 AChR and 5HT3A serotonin receptors, which are also abundant at similar sites as α7 (Camacho 2019). Following this method, more candidate compounds were synthesized. An attempt to modify the central ring was also conducted to enrich the existing agonist structure. The agonistic/antagonistic activities of the compounds were evaluated *in vitro* with cell-based fluorescent assays, which faithfully reflects the activation of the receptor by detecting cellular calcium influx in intact cells. Selected compounds were also injected in mice for evaluation of their ability to cross the blood-brain barrier.

Results

Previous research with structure-activity analysis coupled with the Acetylcholine Binding Protein (AChBP) X-ray crystal structures from mollusks had shown the positional importance of the three 2-aminopyrimidine nitrogens at the center ring as well as the necessity of the di-picolyl motif (Kaczanowska, 2017). To further explore the effect of the presence and number of the nitrogens, the position of the functional group, the symmetry and ability to form hydrogen bond, seven compounds A1-A7 (Chart 1) with variations in the second and fourth position were synthesized using Scheme 1. Commercially available 4,6-dichloropyrimidines with variation at the second position was reacted with selected secondary amine. All amines are analogues of di(2-picolyl) amine, which was demonstrated to be structurally advantageous for activation of α7-nAChR in previous research (Kaczanowska, 2017). Each analogue was designed to contain a discrete alteration. A-1 was the original lead compound; A-2 lacked both of the nitrogen on the di(2- picolyl) amine motif; A-3 and A-6 had increase of hydrophobicity on the second position; A-4 lacked one of the hydrogen on the di(2-picolyl) amine motif, thus creating an asymmetry on the fourth position, A-5 lacked the amino group on the second position; and A-7's amino group and the di(2-picolyl) amine system was swapped.

Agonist and antagonist activities of all seven compounds were evaluated through a Quick Screen using the neurotransmitter fluorescent engineered reporter (CNiFER) with co-transfection of α7-nAChRs. To slowdown rapid

desensitization of the receptor, all agonistic activity screening was conducted with the addition of an α7-nAChR-positive allosteric modulator (PAM). In this case, PNU120596 was added to enhance signal while measurement was taken. Agonist and antagonist activities of all seven compounds were displayed in Figure 2a. Three compounds (A-1, A-3, and A-6) reached the threshold level for agonistic activity. A-6 displayed moderate level of antagonistic activity right below the antagonistic activity threshold.

To test the selectivity of all seven compounds toward α7-nAChRs, their activities were tested using CNiFER with overexpression of α4β2-nAChRs or 5HT3Rs, which were also known to be highly abundant in the CNS. Epibatidine and 5-hydroxytryptamine were measured against α4β2-nAChRs or 5HT3Rs respectively to provide positive control in the agonistic test. Dihydro- β-erythroidine (DHBE) and tropisetron were used against the two receptors respectively for a positive control in the antagonist test. Agonist and antagonist activities of all seven compounds were displayed in Figure 2b and 2c. No compound had any agonist measurements passing the agonist threshold for both receptors. A-3, A-4, and A-7 displayed moderate antagonistic activity toward α4β2-nAChRs, and A6 demonstrated moderate antagonistic activity toward 5HT3Rs.

To further explore the pharmacokinetics and animal-drug interaction, A-4 and A-5 were used as intermediates to synthesize 8 final compounds (Chart 2) following Scheme 2. AL-4C and AC-171C were chosen for animal injection to test their ability to traverse the blood brain barrier. Both compounds were injected intraperitoneally with 1mg per mouse. Brain and blood samples were collected at 3 minutes, 30 minutes, and 1 hour post injection for tissue concentration analysis using Mass Spectrometry. AL-4C demonstrated the ability to cross the blood brain barrier. At 3 minutes post injection, the concentration of AL-4C was detected at the level of 10000ng/ml. The concentration decreases as time advance and reaches a level of 1230ng/ml at the 1-hour timeline. Concentration of AL-4C in the blood shows inverse relationship compared to brain tissue concentration, going from 956 to 10400ng/ml.

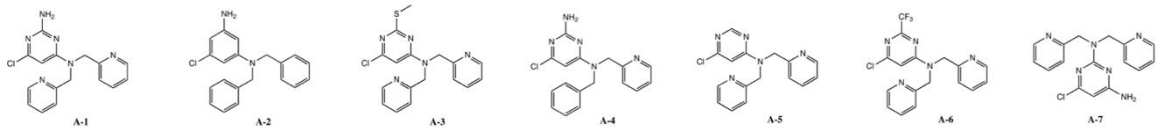
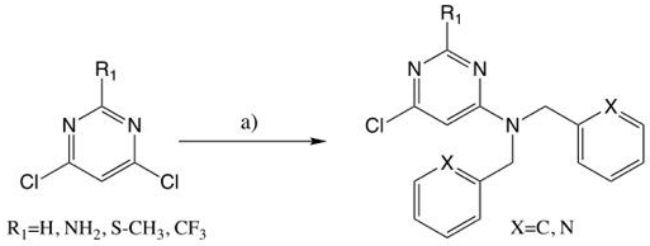
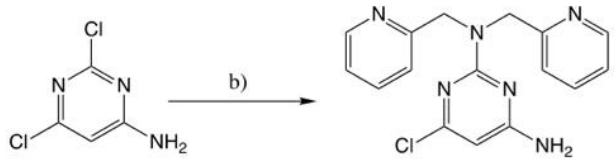


Figure 1. Analogues designed with appropriate amines and alteration on second position. A-2 and A-4 lacked nitrogen(s) on the di(2- picolyl) amine motif; A-3 and A-6 had increase of hydrophobicity; A-5 lacked the amino group on the second position; and A-7's amino group and the di(2-picolyl) amine system was swapped.



a) Amine, DIPEA, DMF



b) Di-(2-picolyl) amine, DIPEA, isopropanol

Scheme 1. synthesis of analogues. Reagents and conditions: a) di(2-picolyl)amine, DIPEA, DMF, 80 ℃; b)di(2-picolyl)Amine, DIPEA, isopropanol.

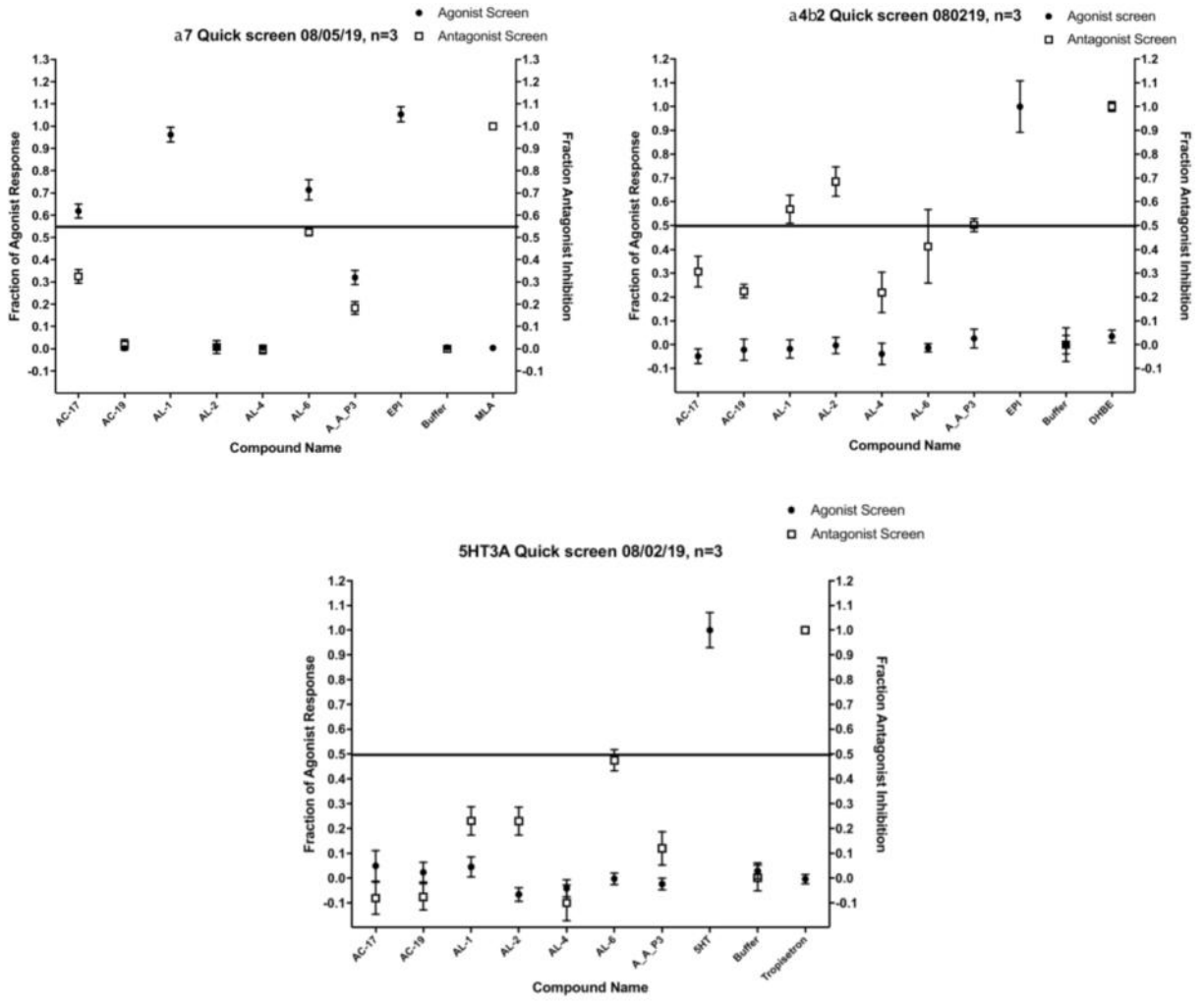


Figure 2. Agonist and antagonist activities of pyrimidine analogue series . This rapid screen assay was performed with cells co-transfected with α7-nAChRs, α4β2-nAChRs or $5HT_3$Rs and a fluorescent reporter that faithfully reflect calcium ion influx, thus the activation of the receptor(s). The level of fluorescence was measured by FLUXStation instrument. Fraction of agonist response was measured with 13 μM candidate agonist co-administrated with 10 μM MLA. Fraction of antagonist response was determined by a 100mM epibatidine response after incubation with 10 μM candidate antagonist for 30 minutes. Epibatidine or 5HT was used as positive controls (1.0 fraction of agonist response) in the agonistic assay and MLA, DHBE, or Tropisetron was used for positive control (1.0 fraction of antagonist response) in the antagonistic assay. Compounds with fractional response above the 0.5 fraction line are considered as partial of full agonists.

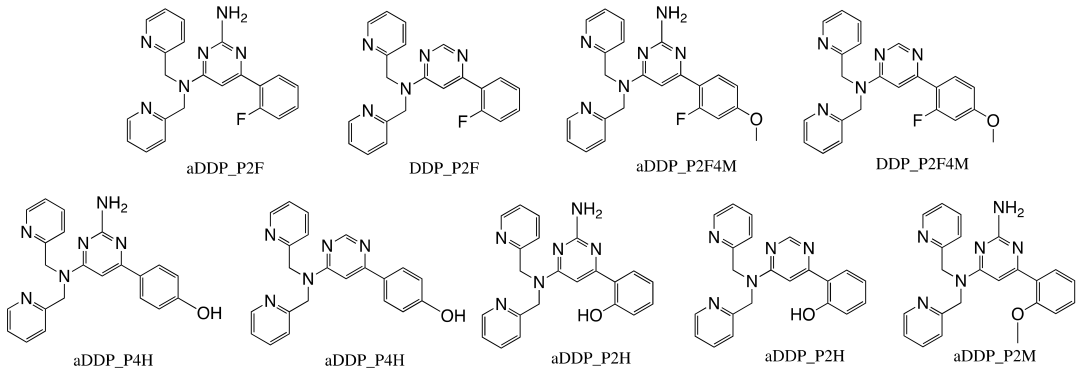
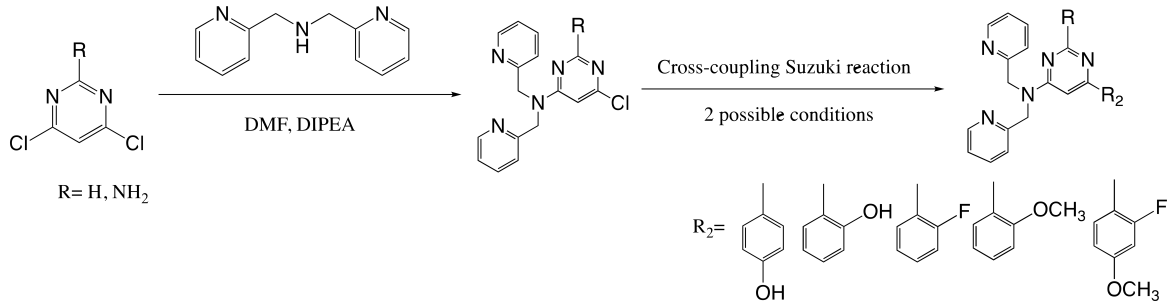


Figure 3. Analogues designed with di(2-picolyl) and alteration on 2 and 6 positions.



Scheme 2. Synthesis of final analogues. a) di(2-picolyl)amine, DIPEA, DMF, 80℃; b) boronic acid, Pd(dppf)Cl_2, K_2CO_3, DMA, 149℃; c) boronic acid, PD(PPH3)4, NA2CO3, THF, reflux.

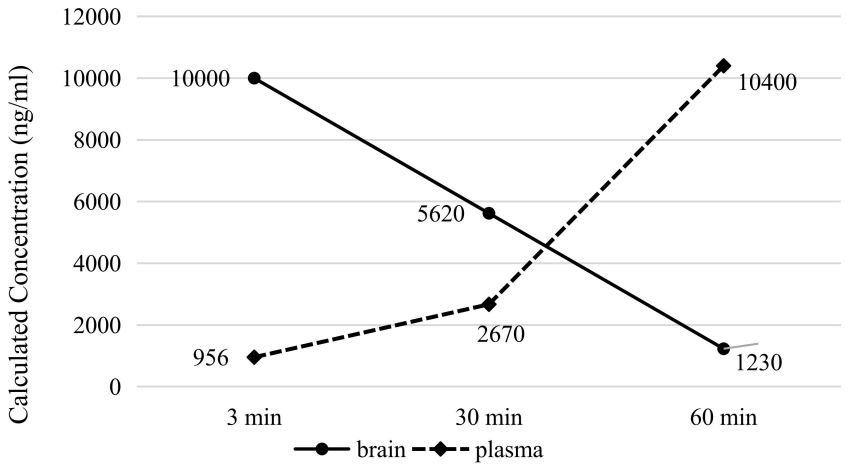


Figure 4. Calculated Concentration Post AL-4C IP Injection. Plasma and brain concentration calculated from samples collected in mouse 3 minutes, 30 minutes, and one hour post injection of AL-4C with 1mg per mouse concentration.

Discussion

In this study, we present a total of seven structural templates with a pyrimidine center ring and functional substitution at the 2 and 4 position as potential activators of α7-nAChR. All were synthesized and tested in the Quick Screen using LGIC Cell Lines. Three of the synthesized analogues demonstrated ability to selectively activate α7-nAChR being AC-17, AL-1, and AL-6, pointing to the structural importance of the amino substitution at the 2- position. The di(2-picolyl) motif also needed to obtain its symmetry in order to maximize its critical activation activity toward the α7-nAChR. Swapping the position of the amino group and the di(2-picolyl) amine system also slightly affect activation on α7-nAChR. An increase of hydrophobicity in the second position can enhances the fraction of agonistic response toward α7-nAChR. On the contrary, removing the amino group in the second position cause a decrease in the fraction of agonistic response on α7-nAChR.

While some compounds display full agonistic quality such as AL-1, other compound like AL-6 demonstrated a unique effect of both an agonist and an antagonist. AL-6 elicits over 0.7 of fraction of agonistic response in the agonistic screen and yet displayed a close to 0.5 fraction of antagonistic response in α7-nAChR.

We hypothesized that this could be explained by the fact that AL-6 functions as a partial agonist. In a study done by Antonio-Tolentino in 2020 also demonstrated compounds with similar structures behaves like a partial agonist against α7-nAChR while still demonstrated favorable preclinical data against schizophrenia (Antonio-Tolentino, 2020). Given the close structural similarity in AL-6, although being a partial agonist, the potential of AL-6 is still worth further exploration.

Our compounds are unique from other typical agonists since they do not contain a strongly basic center. Several studies involving redesigned agonists toward the nAChR have shown that compounds with the ability to cross the blood brain barrier and to reach the target site typically contains amines or imines (Pattaporn, 2016 & Horenstein, 2016). These compounds, like nicotine, epibatidine, and anabaseines, mostly contains a strong basic center that interact with the principal interface on the receptor and another weakly basic or dipole motif that binds to the complementary interface (Kaczanowska et al. 2017). Our compounds contrast with these compounds with all of the nitrogen present in a neutral and non-ionized form under physiological pH.

From A-4 and A-5, 8 more final compounds with 4^{th} and 6^{th} position substitution were synthesized to expand the analogue library. Out of them, AL-4 and AC-171C were injected intraperitoneally to test their ability to cross the blood brain barrier. While no signals for AC-171C was detected in neither the brain nor the blood sample, the MS data for AL-4C showed success blood brain barrier transversions of the compound. Though AL-4C was detected at 3 minutes time point in the brain. It was transported back to the blood system quickly, leaving around 1230 ng/ml AL-4C in the brain at the one-hour time point. Both compounds demonstrated difficulties to dissolve with <10% DMSO while maintaining approximately pH7. This might attribute to the failure of detection of any AC-171C data. It's possible to say that some AL-4C compound was able to enter the brain, but more animal repetition would be needed to confirm this hypothesis. The solubility issue for both compounds should also be take into consideration for the improving accuracy in future animal experiments.

Much about the structure-activity of these compounds remains unknown. To fully understand the structural-activity relationships for establishing a larger library of analogues, concentration-response curve can be generated for the three compounds that passed the quick screen on α7-nAChR. pK_A values can also be assessed. Whether the physiochemical properties of these compounds pass the membrane permeation and blood-brain barrier thresholds also remain to be determined with larger analogue library before further advancements as lead compounds can be made.

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