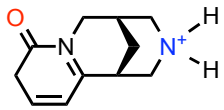
APPENDIX 1: Characterizing the Pharmacophore Binding Interactions of Cytisine in the $(\alpha4)_2(\beta2)_3$ Receptor*

A1.1 RESULTS AND DISCUSSION

Cytisine is a natural product found in various plant species, especially *Cytisus laburnum* (the golden rain tree), and is a potent agonist of nicotinic acetylcholine receptors (nAChRs). It has been used as a smoking cessation drug for decades in Eastern Europe (marketed as Tabex®)[1] and also served as the lead compound for Pfizer's smoking cessation drug varenicline (marketed as Chantix® in the U.S.).[2]



(–)-Cytisine

We sought to characterize the nicotinic pharmacophore binding interactions of cytsine at the α4β2 receptor. Chapter 2 describes studies that probe an important hydrogen bond between the CO of cytisine (the hydrogen bond acceptor of the nicotinic pharmacophore) and the backbone NH of β2L119 at both subunit stoichiometries of the pentameric α4β2 receptor, $(\alpha4)_2(\beta2)_3$ (A2B3) and $(\alpha4)_3(\beta2)_2$ (A3B2). In these studies, cytisine was affected more than any other agonist tested by perturbations to this interaction at the A2B3 stoichiometry (giving a remarkable 62-fold increase in EC_{50} where other agonists like ACh and nicotine give only 7-fold increases[3]). This is an intriguing finding given the remarkably low efficacy (~ 3% relative to ACh) of cytisine at this stoichiometry. Here we use unnatural amino acid mutagenesis to probe the remaining nicotinic pharmacophore interactions for cytisine at A2B3– a cation-π interaction to a

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conserved Trp in the α4 subunit (TrpB) and a hydrogen bond to the backbone CO of this same Trp.

We have well-established strategies for evaluating both interactions. The existence of a cation-π interaction can be established by successively fluorinating TrpB (**Figure A1.1A**).[4-6] Fluorines diminish the cation-π binding ability of the Trp side chain, and the effect is additive. A linear correlation between the observed potency of the agonist and the cation-π binding ability of the ring is the hallmark of a cation-π interaction.

To probe hydrogen bonding interactions to the protein backbone, we replace the appropriate amino acid with its α-hydroxy analog (**Figure A1.1B**).[5-7] This converts the backbone amide to an ester, with predictable consequences.[8-11] In the case of the hydrogen bonding interaction to the carbonyl of TrpB, we replace the *i+1* residue, Thr155, with its α-hydroxy analog, Tah (threonine, α-hydroxy). This attenuates the hydrogen bond accepting ability of the backbone carbonyl, as it is an ester carbonyl rather than an amide carbonyl. We and others have seen significant impacts for mutations of this sort. [5-11]

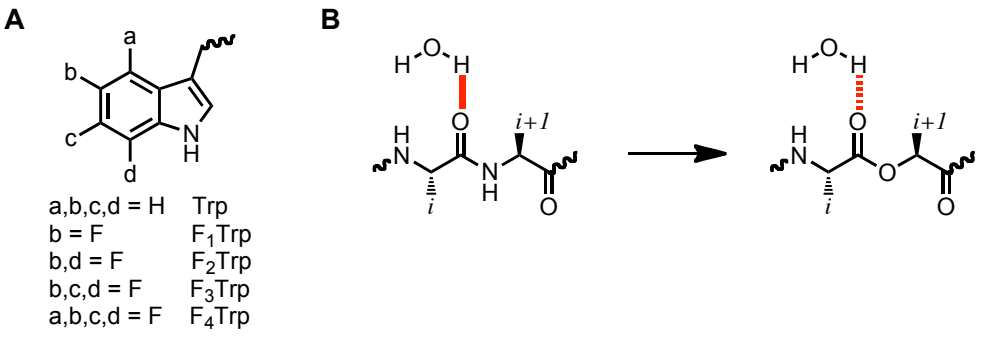

Figure A1.1. Unnatural amino acids used in this study. (A) Structures of the amino acids used to probe the cation-π interaction to TrpB and (B) the backbone ester mutation strategy for probing the hydrogen bond to the backbone CO of TrpB.

Studies of these interactions were challenging due to the very low efficacy of cytisine at the A2B3 stoichiometry. Several strategies were employed to overcome these challenges. We first injected large quantities of mRNA (~100-150 ng total per oocyte relative to the ~25 ng used in typical suppression experiments) and amino-acylated tRNA (up to 125 ng total per oocyte), and also used longer incubation times when necessary. In especially challenging cases, additional measures were taken including adding a second injection of mRNA and tRNA 24 hrs after the initial injection (with a total incubation time of 48-72 hrs), and allowing the injected oocytes to incubate at room temperature for 2-3 hrs prior to electrophysiological recordings. Even with these strategies, it was difficult to obtain adequate current sizes to construct a dose-response relation for F_4Trp (required from our studies of the cation-π interaction). In addition, the Hill coefficient obtained for this mutant is lower than what we typically see for this receptor (**Table A1.1**). Other alternative mutants were also tested, including 5-Br-Trp and 5-CN-Trp, but these mutants also gave low current sizes in electrophysiological recordings.

Despite these difficulties, a clear linear "fluorination plot" is seen for cytisine (shown in comparison to the corresponding plot for ACh[5] in **Figure A1.2**), providing strong evidence in support of a cation-π interaction between the N^+H of cytisine and the TrpB side chain. We have previously argued that the relative slope of a fluorination plot is an indication of the relative strength of a cation-π interaction.[7] We also routinely use the fold-shift in EC_{50} for the F_3Trp or F_4Trp mutants as an alternative measure of the strength of a cation-π interaction. The data in **Table A1.2** suggest mostly modest differences in the variations of fluorination plot slope values and F_3Trp/F_4Trp shifts in EC_{50} for all agonists that have been studied at A2B3.[5] The strongest interaction is seen

for (−)-Epi (discussed in Chapter 5 of this thesis), which gave a fluorination plot slope of 0.11 and a remarkable 180-fold increase in EC_{50} for the Trp-F_4 mutation.

Table A1.1. EC_{50} values and Hill coefficients for (−)-cytisine at the A2B3 stoichiometry. All studies gave current values at +70 mV (normalized to −110 mV) of ≤0.08, confirming the A2B3 stoichiometry. Errors are standard error of the mean. Mutations identified as "Leu" and "Trp" represent recovery of the wild-type receptor by nonsense suppression.

Mutation	EC_{50} (nM)	Hill (n_H)
WT	6.9 ± 0.29	1.4 ± 0.07
α4T155Thr	15 ± 0.7	1.2 ± 0.05
α4T155Tah	130 ± 9	1.2 ± 0.08
α4W154Trp	11 ± 0.4	1.1 ± 0.04
α4W154Trp-F_1	22 ± 1	1.1 ± 0.04
α4W154Trp-F_2	21 ± 1	1.1 ± 0.07
α4W154Trp-F_3	180 ± 7	1.3 ± 0.05
α4W154Trp-F_4	212 ± 60	0.62 ± 0.08
α4T155Thr	15 ± 0.7	1.2 ± 0.05
α4T155Tah	130 ± 9	1.2 ± 0.08

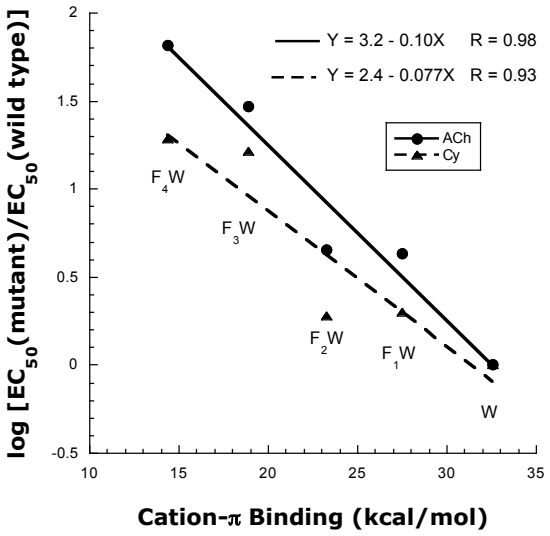

Figure A1.2. Fluorination plot for (−)-cytisine. Agonist potency is plotted against calculated gas-phase cation-π binding energies.[4] The corresponding plot for ACh is also shown based on data from studies by Xiu *et al*.[5]

Table A1.2. EC_{50} fold-shifts for Trp-F_3 and Trp-F_4 at TrpB, fluorination plot slopes and EC_{50} fold-shifts for Tah at Thr155 for all agonists that have been studied at A2B3. A fold-shift is the ratio of the EC_{50} of the mutant / EC_{50} of the natural amino acid (obtained by nonsense suppression). Data for ACh and nicotine (*S*-Nic) is from the studies of Xiu *et al*.[5] Varenicline (Var) data is courtesy of Nyssa L. Puskar and epibatidine (Epi) data is from Chapter 4 of this thesis.

Agonist	Trp-F_3 Fold-Shift	Trp-F_4 Fold-Shift	Fluorination Plot Slope	Tah Fold-Shift
ACh	30	65	0.10	1.1
S-Nic	13	47	0.089	19
Var	11	23	0.076	14
(+)-Epi	17	22	0.080	4.5
(−)-Epi	63	180	0.11	9.4
(+)-N-Methyl Epi	25	53	0.094	15
(−)-N-Methyl Epi	51	54	0.099	12
Cy	27	31	0.077	8.8

Cytisine was also impacted by the T155Tah mutation, providing compelling evidence for the hydrogen bond between cytisine's N^+H and the backbone CO of TrpB. The 8.8-fold increase in EC_{50} seen for cytisine is consistent with what is seen for other agonists at this stoichiometry (**Table A1.2**). Note that ACh has a quaternary N (not a protonatable amine) and therefore cannot participate in this interaction.

In summary, we have shown compelling evidence in support of a cation-π interaction and a hydrogen bond to the N^+H of cytisine. The strength of these interactions is on par with the strengths of the analogous interacts seen for ACh, nicotine, varenicline, and the epibatidine compounds.

A1.2 EXPERIMENTAL SECTION

The molecular biology and electrophysiology protocols used in these studies can be found in Chapters 2 and 4.

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