Synthesis and Biological Evaluation of Pyrroloindolines as Positive Allosteric Modulators of the $\alpha 1\beta 2\gamma 2$ GABA_A Receptor

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ABSTRACT: γ -Aminobutyric acid type A (GABA_A) receptors are key mediators of central inhibitory neurotransmission and have been implicated in several disorders of the central nervous system. Some positive allosteric modulators (PAMs) of this receptor provide great therapeutic benefits to patients. However, adverse effects remain a challenge. Selective targeting of GABA_A receptors could mitigate this problem. Here, we describe the synthesis and functional evaluation of a novel series of pyrroloindolines that display significant modulation of the GABA_A receptor, acting as PAMs. We found that halogen incorporation at the C5 position greatly increased the PAM potency relative to the parent ligand, while substitutions at other positions generally decreased potency. Mutagenesis studies suggest that the binding site lies at the top of the transmembrane domain.

KEYWORDS: GABA_A receptor, Cys-loop, positive allosteric modulator, ion channel, pyrroloindoline

Some key mediators of central inhibitory neurotransmission are γ -aminobutyric acid type A (GABA_A) receptors, and as such these receptors have been drug targets for numerous central nervous system (CNS) disorders.^{1–3} The GABA_A receptor is an anion-selective, pentameric, ligand-gated ion channel that is part of the larger Cys-loop receptor family. A functional receptor results from the assembly of five homologous subunits. A total of 19 homologous subunits exist, and they assemble into at least 30 different functional subtypes *in vivo*.⁴ Some types, including those comprised of $\alpha 1\beta 2\gamma 2$ subunits, are predominantly expressed at the postsynaptic termini and mediate phasic inhibition, while others are located at extrasynaptic sites and mediate tonic inhibition.^{4–6} The large diversity of subtypes and differential localization in the brain emphasize their importance, but also present a challenge, as current GABA_A receptor therapeutics modulate a broad range of subtypes, which can result in adverse effects.

Each GABA_A subunit consists of an N-terminal extracellular domain (ECD), a transmembrane domain (TMD) that comprises four transmembrane α -helices (M1–M4), an extracellular M2–M3 loop and C-terminus, and an intracellular domain composed predominantly of the M3–M4 loop.⁷ Receptor activation occurs upon binding of an agonist to the orthosteric site, which is located in the ECD at the $\beta + \alpha$ -subunit interfaces. This activation can be modulated by additional binding of other ligands to several allosteric sites on the pentameric complex.⁸ Positive allosteric modulators (PAMs) potentiate the evoked response by an agonist, while negative allosteric modulators (NAMs) inhibit that response.⁹ Over the years various modulators of GABA_A receptors have

been identified and several of the positive allosteric modulators are widely used to treat anxiety and panic disorders.^{9,10}

Although GABA_A receptor modulators have a proven therapeutic benefit, adverse effects remain a problem.^{11,12} Additionally, elucidating functions of individual subtypes is crucial for a better understanding of the GABA_A receptor's role in health and disease. Therefore, recent efforts have focused on finding subtype-selective modulators. Various novel modulators have been derived for the $\alpha + \beta$ - interface.^{13,14} For example, a series of pyrazolopyridinones developed by Blackaby et al. showed increased selectivity for $\alpha 3\beta 3\gamma 2$ over $\alpha 1\beta 3\gamma 2$.¹⁵ Two different series of pyrazoloquinolinones exhibited selectivity for $\alpha 6\beta 3\gamma 2$ and $\beta 1$ -containing receptors, respectively.^{16,17}

Physostigmine (**1**, Figure 1), also known as eserine or antilirium, is a reversible acetylcholinesterase inhibitor¹⁸ that has been used to treat glaucoma and delayed gastric emptying.^{19,20} In addition, it has been found to potentiate and inhibit nicotinic acetylcholine receptors, another member of the Cys-loop receptor family.^{21–23}

As a result of our interest in the synthesis of pyrroloindoline natural products, we have prepared a number of new, non-natural pyrroloindoline compounds.^{24–26} Given their structural similarity to other modulators of Cys loop receptors, we

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Figure 1. Chemical structures of selected pyrroloindolines.

screened a representative collection of these structures and found that compounds bearing aryl substitution at C8a can act as PAMs of GABA_A receptors.^{27,28} Here, we report the synthesis of pyrroloindoline (+)-2 (Figure 1) and modification of this scaffold by substitution at N1, C3a, C5, and C8a, yielding a novel series of GABA_A receptor ligands. All of the compounds were tested for agonism and allosteric modulation properties at the human $\alpha 1\beta 2\gamma 2$ GABA_A receptor, the most abundant GABA_A subtype in the adult brain, expressed in *Xenopus laevis* oocytes via two-electrode voltage clamp electrophysiology. Additionally, we performed mutagenesis experiments to identify the binding site of these ligands.

RESULTS AND DISCUSSION

The synthesis of the pyrroloindoline framework commenced with protection of tryptamine (3) to provide carbamate 4 (Scheme 1). Pd-catalyzed C2 arylation with iodobenzene

Scheme 1. Synthesis of the Pyrroloindoline Scaffold

under microwave conditions gave 2-phenyl tryptamine 5 in 77% yield.²⁹ Various approaches were investigated for effecting oxidative cyclization of 5.³⁰ Although there are many examples of related cyclizations of tryptamine and tryptophan derivatives,^{24,31–35} we found that many of these conditions were unsuitable for tryptamine 5, presumably due to the phenyl substituent at C2. After extensive experimentation, it was found that oxidative cyclization of 5 by treatment with *N*-chlorosuccinimide followed by water afforded C3a-hydroxy pyrroloindoline (±)-6 in 85% yield. Reduction of carbamate (±)-6 with Red-Al provided the N1-methyl pyrroloindoline (±)-2.³¹ Attempts to render the cyclization of 5 to 6

enantioselective have thus far been unsuccessful;³⁵ however, the enantiomers of both compounds (±)-6 and (±)-2 can be resolved using preparative SFC with a chiral stationary phase. X-ray crystallography confirmed the structure and absolute stereochemistry of (+)-2.

In a preliminary screen, five pyrroloindoline compounds, including (±)-2, were tested for modulation of eight pentameric ligand-gated ion channels (pLGICs): muscle type nAChR, $\alpha 4\beta 2$ nAChR, $\alpha 7$ nAChR, 5-HT_{3A} receptor, $\alpha 1\beta 2\gamma 2$ GABA_A receptor, $\alpha 1\beta 2$ GABA_A receptor, GluR2, and the glycine receptor. This assay identified pyrroloindoline (±)-2 as a potent PAM of the $\alpha 1\beta 2\gamma 2$ GABA_A receptor (Table S1 and Figure S1).^{27,28} Although no GABA_A receptor activity has been previously reported for physostigmine, compound (±)-2 appears to selectively potentiate $\alpha 1\beta 2\gamma 2$ GABA_A receptors over other Cys-loop receptors.

Based on the selective PAM profile of (±)-2, we decided to further characterize this ligand. We set out to determine if both enantiomers are active at the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. Enantiomer-specific effects would imply a specific drug–receptor interaction, rather than some more generic effect such as altering membrane properties. To assess functional effects, we used a similar two-electrode voltage clamp protocol to one previously described by Marotta et al.²⁸ Briefly, the current responses of three identical EC₅₀ doses of GABA were recorded, followed by a dose of the test-ligand at 40 μ M. After a 30 s incubation, a dose was applied containing both GABA at its EC₅₀ and the test-ligand at 40 μ M. Finally, two doses of GABA EC₅₀ were applied. The first three GABA doses establish a baseline of the GABA response at that concentration, and the purpose of the last two GABA doses is to verify proper functioning of the receptor post modulation and control for independent rise in current amplitude. Of the two (±)-2 enantiomers, only (+)-2 showed a meaningful potentiation of the EC₅₀ GABA dose, with a mean of 16 $\pm$ 4.1%, as shown in Figure 2B.

To determine activity at the $\alpha 1\beta 2$ subtype and consequent involvement of the $\gamma 2$ subunit in potentiation, we performed the same experiment for this subtype. For (±)-2 a mean potentiation of 28 $\pm$ 5.2% was observed (Figure 2). Similar to the observations for the $\alpha 1\beta 2\gamma 2$ subtype, (+)-2 showed increased potentiation over (−)-2 with mean values of 17 $\pm$ 2.6% and 9.2 $\pm$ 1.1%, respectively (Figure 2B and Table S2). These results demonstrate that the $\gamma 2$ subunit is not required for potentiation of the $\alpha 1\beta 2\gamma 2$ receptor by (±)-2.

The amplitude of potentiation is dependent on several factors, among which are both the PAM concentration and the GABA concentration at which we tested the modulation. Next, we determined the effect of 40 μ M (±)-2 on the GABA EC₅₀ (Δ EC₅₀((±)-2)) at the $\alpha 1\beta 2\gamma 2$ receptor. The observed (±)-2-induced shift in GABA EC₅₀ is 13 μ M as shown in Figure 3A and Table S3. This shift is comparable to the induced shift seen for this subtype by the benzodiazepine Triazolam, 16–50 μ M.³⁶ Moreover, we wanted to determine the potency of the pure enantiomer (+)-2. Well-studied modulators, such as flurazepam and zolpidem, have EC₅₀s in the nanomolar range when coapplied with GABA EC_{2–5}, being 270 nM and 340 nM, respectively.³⁷ The PAM tested here, (+)-2, appears to be less potent with an EC₅₀ of 110 μ M when coapplied with GABA EC₅, as shown in Figure 3B and Table S3.

Having established that pyrroloindoline (+)-2 acts as a PAM on the $\alpha 1\beta 2\gamma 2$ GABA_A receptor, further potentiation experiments used the GABA EC_{10–15} instead of EC₅₀. Using the

Figure 2. Functional effects of pyrroloindoline (±)-2 on the $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 2$ GABA_A receptor subtypes. (A) Wave forms of the $\alpha 1\beta 2\gamma 2$ current responses from a GABA EC₅₀ only dose, a 40 μ M (±)-2 only dose, and coapplication of GABA EC₅₀ and 40 μ M (±)-2. (B) Relative modulation of a GABA EC₅₀ response of the $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 2$ GABA_A receptor subtypes by (±)-2 and the individual enantiomers. *p < 0.05; ***p < 0.001 (one-way ANOVA).

EC_{10–15} allows for a larger potentiation window than EC₅₀, which enables the detection of more subtle functional differences between GABA_A mutants or pyrroloindoline analogues. Figures 2B and 4C illustrate this difference in modulation potency for (±)-2. For the $\alpha 1\beta 2\gamma 2$ subtype, (±)-2 causes a 62% potentiation of the GABA EC₁₀ response, while at the GABA EC₅₀ this is only 17%.

GABA activates the GABA_A receptor through binding in the ECD at the interface of the $\beta + \alpha$ subunits. Besides this orthosteric site, several allosteric binding sites have been established, of which the benzodiazepine site (BZ) in the ECD at the $\alpha + \gamma$ interface is the most well-known.³⁸ More recently, a distinct binding site in the ECD at the $\alpha + \beta$ interface has been identified for the ligand CGS9895.^{39,40} In addition to binding sites in the ECD, several anesthetics and neurosteroids affect channel activity through binding in the TMD. Recent X-ray crystal structures and cryo-EM structures have shed light on the TM residues involved in binding.^{41,42}

In order to determine the binding site for the PAM (±)-2, we performed mutagenesis on residues that have been implicated in binding of known modulators. For the first screen we selected $\alpha 1$ (H129R)³⁸ and $\alpha 1$ (Y209Q)^{40,43} to probe the BZ-site, $\beta 2$ (Q88C) to probe the $\alpha + \beta$ site,³⁹ and

Figure 3. Functional characterization of pyrroloindoline (±)-2 at the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. (A) (±)-2-induced shift in GABA EC₅₀. A 40 μ M concentration of (±)-2 was used here. (B) (+)-2 EC₅₀ coapplied with GABA EC₅ doses. The peak current at GABA EC₅ was subtracted from all responses.

triple mutant $\alpha 1$ (S297I) $\beta 2$ (N289I) $\gamma 2$ (S319I) to probe for anesthetic sites in the TMD.^{40,44} All three ECD mutants were potentiated to a similar extent as the WT receptor (mean $62 \pm 6.1\%$). However, the triple TMD mutant was not affected by (±)-2 (mean $1.0 \pm 3.0\%$) as shown in Figure 4C and Table S4. These results indicate that (±)-2 does not assert its potentiating effects through binding at the interfaces in the ECD but on one or more interfaces in the TMD.

To determine which specific interfaces are involved in binding in the TMD, we performed potentiation experiments for the single and double mutants of $\alpha 1\beta 2\gamma 2$, as well as the $\alpha 1\beta 2$ subtype (Figure 4B). The mean potentiation in both mutants with single mutations in the $\alpha 1$ and $\gamma 2$ subunits resembles that of the $\alpha 1\beta 2\gamma 2$ WT receptor. Only the single and double mutant receptors that contain a mutation in the $\beta 2$ subunit demonstrate greatly reduced potentiation, suggesting involvement of the $\beta 2$ subunit in binding. For the $\alpha 1\beta 2$ subtype, greater variability among the mutants has been observed, but the same general trend appears. The $\beta 2$ (N289I) mutation is located in the TMD, close to the top of TM2, at the $\beta + \alpha$ interface (Figure 4A). This residue has been implicated in the binding of several anesthetics, such as etomidate, propofol, and loreclezole.^{40,45}

Figure 4. Functional effects of pyrroloindoline (±)-2 on $\alpha 1\beta 2\gamma 2$ and $\alpha 1\beta 2$ GABA_A receptor mutants. (A) Side view of the human $\alpha 1\beta 2\gamma 2$ GABA_A receptor with the probed residues highlighted in pink (PDB ID: 6D6T). (B) Extracellular view into the pore with GABA and BZ sites indicated with arrows. (C) Relative modulation of GABA_{10–15} responses by (±)-2. ECD mutants and TMD mutants of $\alpha 1\beta 2\gamma 2$ are in blue and dark red, respectively. TMD mutants of $\alpha 1\beta 2$ are in light red. ** $p < 0.01$; **** $p < 0.0001$ (one-way ANOVA).

We performed potentiation experiments on these GABA_A mutants similarly as described in Figure 2; however, here we coapplied the 40 μ M PAM with an EC_{10–15} dose of GABA. To verify the EC_{10–15} values of the constructed mutant receptors, we determined the full dose–response relationships and found EC₅₀ values similar to those reported previously (Table S5).^{40,44}

Having characterized lead compound (+)-2, we aimed to optimize the potency of this ligand family by way of exploring substitutions at N1, C3a, C5, and C8a. Derivatization of the pyrroloindoline scaffold was undertaken with enantioenriched compounds 2 and 6 (Scheme 2). For each of the analogues synthesized, both enantiomers were prepared for evaluation of their ability to modulate GABA_A receptors; however, for simplicity, the chemistry is depicted on the (+) enantiomers of 2 and 6 in Scheme 2. From carbamate (+)-6, Pd-catalyzed deprotection of the Cbz group afforded the N1-H pyrroloindoline (+)-7. Derivatization of C3a was examined to interrogate the possibility of the C3a hydroxyl group acting as a hydrogen bond donor. Deoxyfluorination of (+)-6 using diethylamino-sulfur trifluoride followed by carbamate reduction with Red-Al furnished tertiary fluoride (+)-9. Methylation of the C3a hydroxy group of (+)-2 under standard conditions gave C3a-methoxy pyrroloindoline (+)-10.

Recognizing that physostigmine (1) possesses oxidation at C5, we also sought to derivatize this position of the pyrroloindoline framework with both electron-donating and electron-withdrawing substituents. Electrophilic aromatic substitution of (+)-2 with *N*-iodosuccinimide afforded aryl iodide (–)-11 (Scheme 2). Cu-catalyzed trifluoromethylation of the iodoarene gave (+)-12.⁴⁶ Bromination was also feasible using *N*-bromosuccinimide to furnish (+)-13. From the aryl bromide, Cu-catalyzed methoxylation provided methoxy analogue (+)-14.⁴⁷ Additionally, Buchwald–Hartwig coupling of aryl bromide (+)-13 gave morpholine (–)-15.⁴⁸

We also sought to introduce structural variations on the C8a aryl group. Using 1-bromo-4-iodobenzene, Pd-catalyzed C2 arylation of protected tryptamine 4 gave an aryl bromide analogue that was advanced to pyrroloindoline (±)-16 (see Scheme S1 for synthetic details).

Functional evaluation of the series of pyrroloindoline derivatives was conducted using two-electrode voltage clamp electrophysiology as described earlier for (±)-2. General trends will be discussed first. Most of the derivatives do not demonstrate agonist behaviors, except for compounds (–)-11 and (+)-13, which only activated the receptor with very low efficacy (Figure 6A). Generally, all derivatives demonstrate a similar activity pattern for the two enantiomers as we have observed for (±)-2, with only the *S,S*-enantiomer demonstrating activity. One exception to this is the aryl bromide 16, for which both enantiomers show substantial potentiation. It is also worth noting that morpholines (+)-15 and (–)-15, and aryl iodide (+)-11 and (–)-11, have reversed signs for their optical rotations as compared to all other derivatives.

Changes at N1 resulted in decreased potentiation relative to the enantiomerically pure parent ligand (+)-2 (125 ± 15%), with the N1-protio compound (+)-7 and the N1-Cbz compound (+)-6 giving potentiation values of 41 ± 3.0% and 9.6 ± 5.2%, respectively (Figure 5A and Table S6). Substitution of the C3a hydroxyl with fluorine ((+)-9) gives similar potentiation (100 ± 11%) to (+)-2, whereas methylation of the hydroxyl group ((+)-10) results in a substantial reduction in activity (21 ± 3.0%).

Next, we looked at the C5-substituted derivatives (Figure 5B and Table S6). The presence of a methoxy ((+)-14) or morpholino ((+)-15) substituent at C5 drastically reduced the potentiation efficacy to 17 ± 3.2% and –4.8 ± 1.8%, respectively. Potentiation by trifluoromethyl compound (+)-12 resembled that of (+)-2 at 108 ± 9.5%. Surprisingly, introduction of a halogen (Br or I) at C5 greatly increased potentiation with a modulation of 213 ± 21% for (+)-13 and 231 ± 24% for (–)-11, respectively (Figure 5B and 6A). This structure–activity relationship could indicate the presence of a halogen bonding binding interaction. These two ligands appear to have the largest potentiation effects on the $\alpha 1\beta 2\gamma 2$ GABA_A receptor at 40 μ M of all the derivatives evaluated here. Therefore, we attempted to determine a full dose–response relationship for these two PAMs; however, solubility problems at concentrations greater than 100 μ M prevented this (Figure S2). Additionally, we determined the GABA Δ EC₅₀ shift due to 40 μ M (–)-11 or (+)-13 at the $\alpha 1\beta 2\gamma 2$ receptor; we observed a 9 μ M and 16 μ M shift for (–)-11- and (+)-13, respectively (Figure 6B and Table S2). These values are similar to that observed for (±)-2.

Comparing the different effects of C5 substitution and structural variations at the C8a aryl group, recall that methoxy

Scheme 2. Derivatization of the Pyrroloindoline Framework

Figure 5. Functional effects of pyrroloindoline derivatives at the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. (A) N1, C2, C3-substituted derivatives. (B) C5-substituted derivatives. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ (one-way ANOVA).

analogue (+)-14 did not exhibit any PAM properties, nor did methoxyarene ($\pm$)-SI-5 (Table S5). However, bromide (+)-13 demonstrated increased PAM properties relative to (+)-2. Considering the spatial positioning of the bromine at C5, we asked whether a ligand with a 4-Br-Ph at C8a would also possess PAM properties. Indeed, both aryl bromides (+)-16 ($98 \pm 8.3\%$) and (−)-16 ($155 \pm 11\%$) showed comparable potency to (+)-2 (Figure S5 and Table S6). It is surprising that both enantiomers of 16 are active, and we hypothesize that the *R,R*-enantiomer (−)-16 might be able to bind in an “upside down” orientation, in which the bromine occupies the same position as the bromine of ligand (+)-13. Figure 7 depicts an overlay of the two chemical structures, (+)-13 and (−)-16, to illustrate this. (−)-16 is shown at a slightly rotated orientation (looking down C3a and C8a instead of C2) to mimic the orientation of (+)-13, which indeed resembles this structure. These results indicate that not only is the *para*-position of the C8a-phenyl substituent permissive to halide substitution, but it is possible that (−)-16, the *R,R*-enantiomer, is able to fit the binding pocket in the upside down orientation, unlike the other ligands tested in this study.

CONCLUSION

In this work, we described the synthesis of a series of pyrroloindoline compounds and functional evaluation for positive allosteric modulation at the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. First, we characterized the lead positive allosteric modulator ($\pm$)-2, which has an EC₅₀ of $110 \mu\text{M}$ and causes a $13 \mu\text{M}$ shift in GABA EC₅₀. Second, we performed mutagenesis studies to elucidate the binding site of this PAM. We found that the TMD triple mutant $\alpha 1(\text{S}297\text{I})\beta 2(\text{N}289\text{I})\gamma 2(\text{S}319\text{I})$ completely lost sensitivity to ($\pm$)-2, while ECD mutants displayed no meaningful change. This strongly suggests that the binding site is located in the TMD at the top of TM2.

Figure 6. Functional characterization of pyrroloindoline (–)-11 and (+)-13 at the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. (A) Wave forms of the $\alpha 1\beta 2\gamma 2$ current responses from a GABA EC₁₀ only dose, 40 μ M (–)-11 or (+)-13 only dose, and coapplication of GABA EC₁₀ and 40 μ M PAM. (B) PAM-induced shift in GABA EC₅₀. A 40 μ M concentration of (–)-11 and (+)-13 was used here.

Figure 7. Overlay of chemical structures of (+)-13 and (–)-16.

Next, we explored substitution of **2** at various positions to increase efficacy. Like the parent compound, most ligands demonstrated PAM properties only for the *S,S*-enantiomer.

The most potent PAMs tested here contain a bromine or iodine at C5, (+)-13, and (–)-11 respectively. Ligand (±)-16 demonstrated increased potentiation efficacy relative to the parent ligand (±)-2 for both enantiomers. We suspect that the (–) enantiomer, which is generally inactive for other compounds, may be able to fit into the binding pocket in an upside-down orientation due to its 4-Br-Ph substitution at C2.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsmchemlett.0c00340>.

Detailed experimental procedures, compound characterization data, and ¹H and ¹³C NMR spectra (PDF)

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Author Contributions

All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GABA_A, γ -aminobutyric acid type A; PAM, positive allosteric modulator; pLGIC, pentameric ligand-gated ion channel; BZ, benzodiazepine; SEM, standard error of the mean.

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