A Review of the Updated Pharmacophore for the Alpha 5 GABA(A) Benzodiazepine Receptor Model

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1. Introduction

The gamma-aminobutyric acid A (GABA_A) receptor is a heteropentameric chloride ion channel. This channel is generally made up of two α-subunits, two β-subunits, and a single γ-subunit arranged in an αβαβγ fashion. The GABA_A receptors (GABA_A R) are responsible for a myriad of brain functions. Positive allosteric modulators (PAMs) and negative allosteric modulators (NAMs) act on the benzodiazepine (BZ) site of the GABA_A R which can change the conformation of the receptor to inhibit or excite the neurons associated with the ion channel. To date, researchers have been unable to get an X-ray crystal structure of a functional Bz/GABA_A R ion channel. Recently, Miller and Aricescu [1] have reported the crystal structure of a homopentameric GABA_A R containing the β3-subunit at 3 Å resolution. Although this work provides great promise that other heteropentameric GABA_A Rs will be crystallized in the near future, molecular modeling and structure-activity-relationships (SARs) still remain key tools to find better subtype-selective binding agents.

2. Subtype Selective Ligands for α5 GABA(A)/Bz Receptors

Interest in BzR/GABA(A) α5 subtypes began years ago when it was realized that α5β3γ2 Bz/GABA(A) subtypes are located
primarily in the hippocampus. More recently this interest has been confirmed by the report of Möhler et al. [2–5] on α5 “knock-in mice.” This group has provided strong evidence that hippocampal extrasynaptic α5 GABA(A) receptors play a critical role in associative learning as mentioned above [6–11].

Earlier we synthesized a series of α5 subtype selective ligands (RY-023, RY-024, RY-079, and RY-080) based on the structure of Ro 15-4513 and reported their binding affinity [6], as well as several ligands by Atack et al. [12]. These ligands are benzodiazepine receptor (BzR) negative modulators in vivo and a number of these compounds have been shown to enhance memory and learning [13]. One of these ligands was shown by Bailey et al. [6] to be important in the acquisition of fear conditioning and has provided further evidence for the involvement of hippocampal GABA(A)/BzR in learning and anxiety [13]. This is in agreement with the work of DeLorey et al. [7] in a memory model with a ligand closely related to α5 subtype selective inverse agonists RY-024 and RY-079 including PWZ-029 (1).

In order to enhance the α5 subtype selectivity, the bivalent form of RY-80 (3) was prepared to provide XLi-093 (4) [13]. The binding affinity of XLi-093 in vitro was determined on α1, α2, α3, α4, α5, α6 LTK cells and is illustrated in Figure 1. This bivalent ligand exhibited little or no affinity at α1–4, α3, α2 BzR/GABA(A) subtypes, but this α5 ligand had a Kᵣ of 15 nM at the α5β3γ2 subtype [14]. Since this receptor binding study indicated bivalent ligand XLi-093 bound almost exclusively to the α5 subtype, the efficacy of this ligand on GABA(A) receptor subtypes expressed in Xenopus oocytes was investigated by Sieghart, Furtmueller, Li, and Cook [14, 15]. Analysis of the data indicated that XLi-093 up to a concentration of 1 µM did not trigger chloride flux in any one of the GABA(A) subtypes tested. At 1 µM XLi-093 did not modulate GABA induced chloride flux in α1β3γ2, α2β3γ2, or α3β3γ2 receptors, but very slightly inhibited chloride flux in α5β3γ2 subtypes. At 1 µM, XLi-093 barely influenced benzodiazepine (Valium) stimulation of GABA-induced current in α1β3γ2, α2β3γ2, and α3β3γ2 BzR but shifted the diazepam dose response curve to the right in α5β3γ2 receptors in a very significant manner [16]. Importantly, bivalent ligand XLi-093 was able to dose dependently and completely inhibit diazepam-stimulated currents in α5β3γ2 receptors. This was the first subtype selective benzodiazepine receptor site antagonist at α5 receptors. This bivalent ligand XLi-093 provided a lead compound for all of the bivalent ligands in this research [16].

Illustrated in Figure 2 is XLi-093 (4) aligned excellently within the pharmacophore-receptor model of the α5β3γ2 subtype [14, 16–19]. The fit to the pharmacophore-receptor and the binding data indicate that bivalent ligands will bind to BzR subtypes [14, 19]. It is believed that the dimer enters the binding pocket with one monomeric unit docking while the other monomer tethered by a linker extends out of the protein into the extracellular domain. If this is in fact true that the second imidazole unit is protruding into the extracellular domain of the BzR/GABA(A) α5 binding site,
Table 1: Full PDSP panel receptor binding reported (Roth [138]) for XLI-093 and XLI-356.

| Cook code | 5ht1a | 5ht1b | 5htld | 5htle | 5ht2a | 5ht2b | 5ht2c | 5ht3 | 5ht5a | 5ht6 | 5ht7 | α1A | β1B | α2A | α2B |
|-----------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|------|------|------|------|
| XLI093    | *     |       | *     |       | *     |       | *     |       | *     |       |       | *    | *    |     |     |
| XLI356    |       | *     |       | *     |       |       |       | *     |       |       | *     | *    | *    |     |     |

| Cook code | α2C | Beta1 | Beta2 | CB1 | CB2 | D1 | D2 | D3 | D4 | D5 | DAT | DOR | H1 | H2 | H3 |
|-----------|-----|-------|-------|-----|-----|----|----|----|----|----|-----|-----|----|----|----|
| XLI093    | *   |       |       | *   |     | *  | *  | *  | *  | *  | *   | *   |    |    |    |
| XLI356    |     |       |       | *   |     | *  | *  | *  | *  | *  | *   | *   |    |    |    |

| Cook code | H4 | Imidazoline | KOR | M1 | M2 | M3 | M4 | M5 | MDR | MOR | NET | NMDA | SERT | σ1 | σ2 |
|-----------|----|-------------|-----|----|----|----|----|----|-----|-----|-----|------|------|----|----|
| XLI093    | *  | *           | 2,024.00 | *  | *  | *  | *  | *  | *  | *  | *   | *    | *    | *  |
| XLI356    | *  | *           | 6,118.00  | *  | *  | *  | *  | *  | *  | *  | *   | *    | *    |    |

Data (“secondary binding”) are \( K_i \) values. \( K_i \) values are reported in nanomolar concentration, Case Western Reserve University. "*" indicates "primary missed" (<50% inhibition at 10 \( \mu \)M). See full data of the PDSP screen in the report of Clayton [22].

It could have a profound effect on the ligand design. This means other homodimers or even heterodimers may bind to BzR/GABA(A)ergic sites.

In this vein, Wenger, Li, and Cook et al. [13, 20, 21] earlier described preliminary data that XLI-093, an \( \alpha_5 \) subtype selective antagonist, enhances performance of C57BL/6J mice under a titrating delayed matching to position schedule of cognition, as illustrated in Figure 3 [14, 16–19]. This indicates, however, that this agent does cross the blood brain barrier.

Bivalent ligands have a preferred linker of 3–5 methylene units, between the two pharmacophores (see XLI-093). This was established by NMR experiments run at low temperatures, X-ray crystallography, and molecular modeling of the ligands in question and will be discussed [14, 17, 18].

Based on this data, additional \( \alpha_5 \)-subtype selective ligands have been prepared (see Figure 4). The basic imidazoazobenzodiazepine structure has been maintained [7]; however substituents were varied in regions around the scaffold based on molecular modeling [6]. These are now the most \( \alpha_5 \) subtype selective ligands ever reported [22]. Moreover, the ability to increase the subtype selectivity can be done by selecting specific substituents on these ligands to new agents with 400–1000-fold affinity has occurred principally at \( \alpha_5 \) subtypes. This is an important step forward to understanding the true, unequivocal physiological responses mediated by \( \alpha_5 \) subtypes in regard to cognition (amnesia), schizophrenia, anxiety, and convulsions, all of which in some degree are influenced by \( \alpha_5 \) subtypes. Based on the ligands in Figures 4 and 5, affinity has occurred principally at \( \alpha_5 \) subtypes. In addition, since XLI-093 bound very tightly only to \( \alpha_5 \) BzR subtypes, the bivalent nature and functionality presented here can be incorporated into other dimeric ligands.

As shown previously in Figure 3, \( \alpha_5 \)-antagonist XLI-093 (4) was shown to enhance cognition. In another study, a reduction of the two acetylenic groups of XLI-093 resulted in ethyl groups [14], providing a new bivalent ligand (XLI-356, 10) which shows \( \alpha_5 \)-selective binding with very low affinity for \( \alpha_1 \) subtypes (Figure 5). Efficacy (oocyte) data shows XLI-356 is an \( \alpha_5 \) negative allosteric modulator [7, 13]. DeLorey et al. have recently shown in mice that XLI-356 does potent reverse scopolamine induced memory deficits [7]. This bivalent \( \alpha_5 \) inverse agonist enhanced cognition in agreement with work reported from our laboratory on monovalent inverse agonists RY-10 [6] and RY-23 [7].

The dimers XLI-093 (4) and XLI-356 (10) were sent to Case Western Reserve (NIMH supported PDSP program, Roth et al.) for full panel receptor binding and they do not bind to other receptors at levels of concern (Table 1).

Although XLI-093 (4) was found to be an antagonist at the \( \alpha_5 \) subtype, XLI-356 (10) was found to be a weak agonist-antagonist. XLI-356 was found to reverse scopolamine induced memory deficits in mice. When XLI-356 was looked at in audio cued fear conditioning, the results show no activity. This suggests that the effect of XLI-356 is selective through \( \alpha_5 \) receptors which are abundant in the hippocampus which is highly associated with contextual memory. Audio cued memory instead is amygdala-based and should not be affected by an \( \alpha_5 \) subtype selective compound [39–42].

As illustrated in Figure 6, scopolamine (1 mg/kg) reduced freezing (i.e., impairs memory) generally due to coupling the
**Figure 4:** Binding data of selected imidazobenzodiazepines [22].

| Ligand | R<sub>1</sub> | α1 | α2 | α3 | K<sub>i</sub> (nM)<sup>a</sup> | α4 | α5 | α6 |
|--------|-------------|----|----|----|----------------|----|----|----|
| 1a<sup>b</sup> | CH<sub>2</sub>OCH<sub>3</sub> | >300 | >300 | >300 | ND | 38.8 | >300 |
| 1b<sup>b</sup> | CH<sub>2</sub>OCH<sub>3</sub> | 920 | ND | ND | ND | 30 | ND |
| 1c<sup>b</sup> | CH<sub>2</sub>Cl | 362.4 | 180.3 | 328.2 | ND | 6.185 | ND |
| 5 | CH<sub>2</sub>Cl | >300 | >300 | >300 | ND | 28.5 | >300 |
| 6 | CH<sub>2</sub>OEt | >300 | >300 | >300 | ND | 82.7 | >300 |
| 7 | CH<sub>3</sub> | >89 | >70 | >91 | ND | 3.7 | >301 |
| 8 | CO<sub>2</sub>Et | >1000 | >1000 | >1000 | >1000 | 64 | >1000 |
| 9 | CO<sub>2</sub>Et | >2000 | >2000 | >2000 | >2000 | 176 | >2000 |

<sup>a</sup>Data shown here are the means of two determinations which differed by less than 10%. ND: not determined (presumably similar to α6).

<sup>b</sup>Ligands 1a–c are binding datasets of PWZ-029b from three separate laboratories. This figure is modified from that illustrated in reference [22] to indicate the α5 subtype selectivity.

**Figure 5:** Binding data of selected imidazobenzodiazepines substituted with an E-ring as compared to XLi-356 (10).

| Ligand | α1 | α2 | α3 | K<sub>i</sub> (nM)<sup>a</sup> | α4 | α5 | α6 |
|--------|----|----|----|----------------|----|----|----|
| XLi-356 (10) | 1852 | 4203 | 8545 | ND | 101 | 5000 |
| RY-068 (11) | >500 | 877 | 496 | ND | 37 | >1000 |
| RY-062 (12) | >1000 | >1000 | >500 | ND | 172 | >2000 |
| RY-069 (13) | 692 | 622 | 506 | ND | 19 | >1000 |
| RY-I-29 (14) | >1000 | >1000 | >1000 | ND | 157 | >1000 |

<sup>a</sup>Data shown here are the means of two determinations which differed by less than 10%. ND: not determined (presumably greater than 1000 nM; similar to α6).
context (the cage) with a mild shock. XLI-356 (10 mg/kg) attenuated the impairment of memory returning the freezing to the levels on par with subjects dosed with vehicle. In audio cued memory the response was activated by sound, not the context. XLI-356 was not able to reverse this type of memory effect which is amygdala driven. A similar effect was observed for XLI-093 by Harris et al. [43]. XLI-093 is the most selective antagonist for α5 subtypes reported to date [13, 43] and is a very useful α5 antagonist used by many in vivo [22, 44, 45].

Molecular modeling combined with this knowledge was used to generate new lead compounds aimed at the development of α5-subtype selective positive and negative allosteric modulators to study cognition as well as amnesia mediated by the hippocampus. All of these compounds have been prepared based on the structure of current α5-subtype selective ligands synthesized in Milwaukee [46] (see Figures 4 and 5), as well as the binding affinity (15 nM)/selectivity of bivalent α5 antagonist XLI-093 (4) [13].

In efforts to enhance α5-selectivity in regards to cognition, Cook, Bailey, and Helmstetter et al. have employed RY-024 to study the hippocampal involvement in the benzodiazepine receptor in learning and anxiety [14, 19]. Supporting this Harris, DeLorey et al. show in mice that α5 NAMs (I) and RY-10 potently reversed scopolamine-induced memory impairment. These α5 NAMs provide insight as to how GABA_A, Rs influence contextual memory, an aspect of memory affected in age associated memory impairment and especially in Alzheimer’s disease [13, 62–64]. In addition, Savić et al. have used the α1 preferring antagonist, BCCt, in passive avoidance studies, in which midazolam’s amnesic effects are shown to be due to interaction of agonist ligands at α5 in addition to α1β3γ2 BzR subtypes [24, 65].

### 3. PWZ-029: A Negative Allosteric Modulator

PWZ-029 (I) has been studied extensively as an α5-GABA_A inverse agonist and in certain experimental models has been shown to enhance cognition. The binding data from three separate laboratories (Table 2) have all shown that it exhibits remarkable selectivity for the α5 subunit-containing receptors, all greater than 60-fold compared to the next subunit.

Electrophysiological efficacy testing done by Sieghart et al. in oocytes demonstrated that PWZ-029 (I) acts as a negative allosteric modulator at the α5-subunit, with a very weak agonist activity at the α1, α2, and α3 subunits (Figure 7).

At a pharmacologically relevant concentration of 0.1 μM, PWZ-029 exhibits moderate negative modulation at the α5-subunit, while showing little or no effect at the α1, α2, or α3-subunits.

Milić et al. reported on the effects of PWZ-029 in the widely used novel object recognition test, which differentiates between the exploration time of novel and familiar objects. As shown by significant differences between the exploration times of the novel and familiar object (Figure 8(a)), as well as the respective discrimination indices (Figure 8(b)), all the three tested doses of PWZ-029 (2, 5 and 10 mg/kg) improved object recognition in rats after the 24 h delay period. Additionally, in the procedure with the 1 h delay between training and testing, the lowest of the tested doses of PWZ-029 (2 mg/kg) successfully reversed the deficit in recognition memory induced by 0.3 mg/kg scopolamine (Figure 9) [25].

The results of the described study showed for the first time that inverse agonism at α5-GABA_A receptors may be efficacious in both improving cognitive performance in

**Table 2: Affinity of PWZ-029 (I); Kᵢ (nM)*.**

| Code     | MW | α1   | α2   | α3   | α4   | α5   | α6   |
|----------|----|------|------|------|------|------|------|
| PWZ-029 (I) | 291.73  | >300  | >300  | >300  | ND   | 38.8 | >300 |
| PWZ-029 (I) | 291.73  | 920   | ND    | ND    | ND   | 30   | ND   |
| PWZ-029 (I) | 291.73  | 362   | 180   | 328   | ND   | 6    | ND   |

*Data from three separate laboratories.

**Figure 6:** Visual and audio cued data for XLI-356 (10). This figure was modified from that in [22].

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**Contextual memory**

| Freezing (%) | VEH | Scopolamine | XLI356 + scopolamine |
|--------------|-----|-------------|----------------------|
| 0            | 75  | 50          | 25                   |
| **p > 0.05** |     |             |                      |

**Audio cued memory**

| Freezing (%) | VEH | Scopolamine | XLI356 + scopolamine |
|--------------|-----|-------------|----------------------|
| 0            | 75  | 50          | 25                   |
| **p > 0.05** |     |             |                      |
**Figure 7:** Oocyte electrophysiological data of PWZ-029 (I) [24].

**Figure 8:** The effects of PWZ-029 (I) (2, 5 and 10 mg/kg) on (a) time exploring familiar and novel objects and (b) discrimination indices in the novel object recognition test using a 24 h delay (mean ± SEM). Significant differences are indicated with asterisks (paired-samples $t$-test, novel versus familiar, **$P < 0.05$, ***$P < 0.01$, ****$P < 0.001$). A significant difference from zero is indicated with hashes (one sample $t$-test, ##$P < 0.01$). The number of animals per each treatment group was 10. SOL = solvent [25].
unimpaired subjects and ameliorating cognitive deficits in pharmacologically impaired subjects, as assessed in two protocols of the same animal model [25].

In a recent by Rowlett et al. [26], negative allosteric modulator PWZ-029 was evaluated in female rhesus monkeys (n = 4) in an Object Retrieval test with Detours (ORD; Figure 10 for details). It was administered via i.v. catheters in ORD trained monkeys and evaluated for cognition enhancement. A successful trial was determined by the ability of the subject to obtain a food reward within a transparent box with a single open side, with varying degrees of difficulty (“easy” or “difficult” or “mixed” as a combination of both) based on food placement within the box. In “mixed” trials using PWZ-029, no significant results were observed when compared to vehicle (Figure II(a)). “Difficult” trials, however, exhibited an increasing dose-dependent curve for successful trials (Figure II(b)). These results were attenuated by a coadministration α5-antagonist XLi-093 (Figure II(c)). PWZ-029 was also shown to dose-dependently reverse the cholinergic deficits that were induced by scopolamine (Figure II(d)) [26].

These findings suggest that PWZ-029 can enhance performance on the ORD task, only under conditions in which baseline performance is attenuated. The effects of PWZ-029 were antagonized in a surmountable fashion by the selective α5-GABA_A ligand, XLi-093, consistent with PWZ-029’s effects being mediating via the α5-GABA_A receptor. The results are consistent with the view that α5 GABA_A receptors may represent a viable target for discovery of cognitive enhancing agents.

In addition, we have new data showing that modulation of α5-GABA_A Rs by PWZ-029 rescues Hip-dependent memory in an AD rat model [PMID: 23634826] as evidenced by a significant decrease in the latency to reach the hidden platform (memory probe trials) on spatial water maze task (Figure 12). Roche has employed a similar strategy at α5 subtypes and recently has a drug in the clinic to treat symptoms of dementia in Down syndrome patients. It is well known many Down syndrome patients develop Alzheimer’s disease or a dementia with a very similar etiology. This is aimed at treating early onset Alzheimer’s patients.

4. PWZ-029 Docking within α5γ2 GABA_A Receptor Subunit Homology Model

These studies with PWZ-029 led to the molecular model rendering of the compound docked within the α5γ2 BzR subtype (Figures 13–16). The model figures have the following features:

The docking of PWZ-029 within the GABA_A/BzR shows the molecule bound and interacting with specific amino acids. The A and B rings of the benzodiazepine framework undergo a π-stacking interaction with HIS 105, indicated by the magenta coloring. At the other end of the molecule the methoxy lone pair and imidazole nitrogen lone pair act as a hydrogen bond acceptors with THR 210 and TYR 213, respectively. These interactions are shown by the aqua-blue descriptors.
### Methods

**Subjects:**

Rhesus monkeys (*Macaca mulatta*)

\[ N = 5 \]

Implanted with chronic i.v. catheters

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**Procedure:**

Object retrieval with detours (ORD)

15 trials per session

Easy, difficult, and mixed trials (see diagram)

Tests:

(i) PWZ-029 · mixed trials

(ii) PWZ-029 · difficult trials

(iii) PWZ-029 + XL-093 · difficult trials

(iv) Scopolamine · mixed trials

(v) PWZ-029 + scopolamine · mixed trials

**Measurements:**

% correct trials—trial in which food is obtained with the first reach

Errors: barrier reaches (hand in contact with closed side of box) and incomplete trials (food grasped but dropped)

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**Figure 10:** ORD methods and procedure [26].

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### 5. Subtype Selective Agonists for \( \alpha_5 \) GABA\(_4\)/Bz Receptors

Mohler has proposed that \( \alpha_5 \) selective inverse agonists or \( \alpha_5 \) selective agonists might enhance cognition [5, 13, 16–18, 86]. This is because of the extrasynaptic pyramidal nature of \( \alpha_5\beta_3\gamma_2 \) subtypes, located almost exclusively in the hippocampus. Because of this, a new “potential agonist” which binds solely to \( \alpha_5\beta_3\gamma_2 \) subtypes was designed by computer modeling (see Figure 17). This ligand (DM-I-81, 9) has an agonist framework and binds only to \( \alpha_5\beta_3\gamma_2 \) subtypes [13, 17, 18, 86]. The binding potency at \( \alpha_5 \) subtypes is 176 nM. Although the \( 8 \)-pendant phenyl of DM-I-81 was lipophilic and bound to the \( L_2 \) pocket, additional work on the \( 8 \)-position of this scaffold has been abandoned and generally left as an acetylene or halide function, with a few exceptions. The steric bulk of the \( 8 \)-phenyl moiety was felt detrimental to activity and potency which may have led to the weak binding affinity.

### 6. \( \alpha_5 \) Positive Allosteric Modulators in Schizophrenia

In addition to inverse agonists, a number of other \( \alpha_5 \)-GABA\(_4\)R positive allosteric modulators (PAMs) have been synthesized. These compounds, such as SH-053-2\( ^{\text{F}}\)-R-CH\(_3\) (2), have been shown to decrease the firing rate of synapses controlling cognition and can be used to treat schizophrenia.

The following is reported by Gill, Cook, and Grace et al. [27–38]. There are a number of novel benzodiazepine-positive allosteric modulators (PAMs), selective for the \( \alpha_5 \) subunit of the GABA\(_4\) receptor, including SH-053-2\( ^{\text{F}}\)-R-CH\(_3\) (2), which has been tested for its ability to effect the output of the HPC (hippocampal) in methylazoxymethanol (MAM)–treated animals, which can lead to hyperactivity in the dopamine system [27–38]. In addition, the effect of this compounds (2) response to amphetamine in MAM-animals on the hyperactive locomotor activity was examined. Schizophrenic-like symptoms can be induced into rats when treated prenatally with DNA-methylating agent, methylazoxymethanol, on gestational day (GD) 17. These neurochemical outcomes and changes in behavior mimic those found in schizophrenic patients. Systemic treatment with (2) resulted in a reduced number of spontaneously active DA (dopamine) neurons in the VTA (ventral tegmental area) of MAM animals (Figure 18) to levels seen in animals treated with vehicle (i.e., saline). To confirm the location of action, 2 was also directly infused into the ventral HPC (Figure 19).
and was shown to have the same effect. Moreover, HPC neurons in both SAL and MAM animals showed diminished cortical-evoked responses following α5-GABA_A R PAM treatment. This study is important for it supports a treatment of schizophrenia that targets abnormal HPC output, which in turn normalized dopaminergic neuronal activity [27–38]. This is a novel approach to treat schizophrenia.

The pathophysiology of schizophrenia has identified hippocampal (HPC) dysfunction as a major mediator as reported by many including Anthony Grace [27–38]. This included morphological changes, reduced HPC volume, and GAD67 expression [27, 28] that have been reported after death in the brains of patients with schizophrenia. Both HPC activation and morphology changes have been identified that can precede psychotic symptoms or correlate with severity of cognitive deficits [29–33]. This has been shown in a cognitive test during baseline and activation.

Many animal models of schizophrenia were essential to behavioral pathology and have delivered new knowledge about the network disturbances that contribute to CNS disorder. This study shows that the offspring of MAM-treated animals showed both structural and behavioral abnormalities. These were consistent with those observed in patients with schizophrenia. The animals had reduced limbic cortical and HPC volumes with increased cell packing density and showed increased sensitivity to psychostimulants [34–36]. In addition, the startle response in prepulse inhibition was reduced in MAM-treated animals and deficits in latent inhibition were observed [35]. Furthermore, a pathological rise in spontaneous dopamine (DA) activity by the ventral tegmental area (VTA) was observed that could be attributed to aberrant activation within the ventral HPC [36]. It was suggested that reductions in parvalbumin- (PV-) stained interneurons might be the reason for the hyperactivation of the HPC and disruption of normal oscillatory activity in the HPC and cortex of MAM animals [38, 61]. At least this is the prevailing hypothesis at the moment put forth by many investigators (see references cited in [27–38]).

Selective α5-GABA_A R positive allosteric modulator (2) was successful in reversing the pathological increase in tonic DA transmission in methylazoxymethanol rats by targeting abnormal hippocampal activity. In addition, the α5-PAM was
Figure 12: PWZ-029 rescues spatial memory deficits in AD model as evidenced by a decrease in the latency to reach the hidden platform (probe test) in the water maze relative to vehicle (VEH, *p < 0.05).

Figure 13: PWZ-029 docked within α5γ2 BzR binding site (BS).

Figure 14: PWZ-029 docked with amino acid residues.

Figure 15: PWZ-029 docked with A.A. residue interactions.

Figure 16: PWZ-029 docked with interactions. (1) HIS 105 π-stacking interaction with centroid of PWZ-029. (2) TYR 213 phenol OH hydrogen bonding to imidazole nitrogen lone pair. (3) THR 210 OH and lone pair on methoxy of PWZ029. (4) α5 ribbon being green. (5) γ2 ribbon being yellow. (6) Hydrogen bonding being aqua blue. (7) π-stacking being magenta.

able to reduce the behavioral sensitivity to psychostimulants observed in MAM rats (Figures 20 and 21). This suggests that novel α5-partial allosteric modulators should be effective in alleviating dopamine-mediated psychosis. However, if this drug can also restore rhythmicity within HPC-efferent structure, it may also affect other aspects of this disease state such as cognitive disabilities and negative symptoms. This study, using the MAM-model to induce symptoms of schizophrenia, shows that the use of α5-GABA_A R targeting compounds could be an effective treatment in schizophrenic patients. The selective targeting solely of α5β3γ2 subunits, as opposed to unselective BZDs such as diazepam, could provide relief from the psychotic symptoms without producing adverse effects such as sedation [27–38].

As reported by Gill, Grace et al. [36, 38, 47–61]. Often initial antipsychotic drug treatments (APD) for schizophrenia are ineffective, requiring a brief washout period prior to secondary treatment. The impact of withdrawal from initial APD on the dopamine (DA) system is unknown. Furthermore, an identical response to APD therapy between normal and pathological systems should not be assumed. In another study by Gill, Grace et al., α5 positive allosteric modulator SH-053-2F-R-CH_3 (2) was used in the MAM neurodevelopmental model of schizophrenia which was used to study impact of withdrawal from repeated haloperidol (HAL) on the dopamine system [36, 38, 47–61].

The following studies were designed to provide insight as to why a new drug to treat schizophrenia may be effective in Phase II clinical trials but fail in Phase III because of the large number of patients required for the study. Many of these patients in Phase III studies have altered neuronal pathways in the CNS because of long-term treatment with antipsychotics (sometimes 10–20 years) [36, 38, 47–61].

Importantly, spontaneous dopamine activity reduction was observed in saline rats withdrawn from haloperidol with an enhanced locomotor response to amphetamine, indicating the development of dopamine supersensitivity. In addition, PAM treatment, as well as ventral HPC inactivation, removed the depolarization block of DA neurons in withdrawn HAL treated SAL rats. In contrast, methylazoxymethanol rats withdrawn from HAL displayed a reduction in spontaneous dopamine activity and enhanced locomotor response that
was unresponsive to PAM treatment with SH-053-2′F-R-CH₃ or ventral HPC inactivation [36, 38, 47–61].

Prior HAL treatment withdrawal can restrict the efficacy of subsequent pharmacotherapy in the MAM model of schizophrenia. This is an extremely important result indicating that testing a new drug for schizophrenia in humans treated for years with both typical and atypical antipsychotics may result in a false negative with regard to treatment. Studies that support this hypothesis follow here [36, 38, 47–61].

Novel therapeutics for the treatment of schizophrenia that exhibit initial promise in preclinical trials often fail to demonstrate sufficient efficacy in subsequent clinical trials. In addition, relapse or noncompliance from initial treatments is common, necessitating secondary antipsychotic intervention [47, 48]. Studies have shown that between 49 and 74% of schizophrenia patients discontinue the use of antipsychotic drug (APD) treatments within 18 months due to adverse side-effects [48, 49]. Current pharmacotherapies for schizophrenia target the pathological increase in dopamine system activity, as mentioned above. Common clinical practice for secondary antipsychotic application involves a brief withdrawal period from the initial APD. Unfortunately, the success of even secondary treatments is far from being optimal with the rehospitalization of patients being a common occurrence. The impact of repeated antipsychotic treatment and subsequent withdrawal on the dopamine system has not been adequately assessed [36, 38, 47–61].

As indicated above, schizophrenia is a complex chronic psychiatric illness characterized by frequent relapses despite ongoing treatment. The search for more effective pharmacotherapies for the treatment of schizophrenia continues unabated. It is not uncommon for novel pharmaceuticals to demonstrate promise in preclinical trials but fail to show an adequate response in subsequent clinical trials. Indeed, evaluating the benefits of one APD versus another is complicated by clinical trials beset with high attrition rates and poor
efficacy in satisfactorily reducing rehospitalization [47, 49–52].

Previous work from the Gill, Grace et al.’s laboratory [36, 38, 47–61] with the MAM model of schizophrenia has identified a potential novel therapeutic, a α5GABAAR PAM. The dopamine system pathology in the MAM model is likely the result of excessive output from the ventral HPC [36]. The α5GABAAR PAM was identified as a potential therapeutic due to the relatively selective expression of α5GABAAR in the ventral HPC and its potential for reducing HPC activity [53–60]. When either administered systemically or directly infused into the ventral HPC, the α5GABAAR PAM (SH-053-2′F-R-CH₃) was effective in reducing the dopamine system activation in MAM rats [38]. Anthony Grace, Gill et al. showed α5GABAAR PAM treatment was also effective in reducing the enhanced behavioral response to amphetamine in MAM rats, as stated above. Data from the present study sought to delineate whether the α5GABAAR PAM (SH-053-2′F-R-CH₃) would remain effective in MAM rats withdrawn from prior neuroleptic treatment, a common occurrence in the patient population. In both SAL and MAM rats, there was a reduction in the spontaneous activity of dopamine neurons in the VTA after 7 days withdrawal from repeated HAL treatment. However, MAM rats continued to exhibit a greater activation of the dopamine system in comparison to SAL rats. Treatment with the α5GABAAR PAM was no longer effective in reducing the activity of dopamine neurons in the VTA in withdrawn HAL treated MAM rats. In contrast, α5GABAAR PAM treatment in the withdrawn HAL treated SAL rats instead increased the spontaneous activity of dopamine in the VTA (Figures 22–25) [36, 38, 47–61].

Similar to the effects seen following α5GABAAR PAM treatment, ventral HPC inactivation in withdrawn HAL treated SAL rats restored normal dopamine system activity by increasing the number of spontaneously active dopamine neurons. The disparate effect of withdrawal from HAL on

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**Figure 17:** The α5 selective agonist DM-I-81 (9), bound within the α1 and α5 subtypes. Binding data shown as $K_i$ (nM).

| Binding data | α1 | α2 | α3 | α4 | α5 | α6 |
|--------------|----|----|----|----|----|----|
| DM-I-81 (9)  | >2000 | >2000 | >2000 | >2000 | 176 | >2000 |

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DM-I-81 aligned in the included volume of the pharmacophore/receptor model for the α1β3γ2 (blue) and α5γ3β2 (red) subtypes
the dopamine system between SAL and MAM rats provides a vital clue for the inconsistencies between preclinical trials for novel therapeutics that utilize normal subjects and subsequent clinical trials in a patient population [36, 38, 47–61].

The data suggests underlying dopamine system pathology alters the impact of withdrawal from prior repeated HAL in the MAM model of schizophrenia. In addition, subsequent novel APD treatment loses efficacy following withdrawal from repeated HAL in MAM animals. This certainly has relevance to Phase III clinical trials of new drugs to treat schizophrenia [36, 38, 47–61].

7. GABA<sub>α</sub>5 Positive Allosteric Modulators Relax Airway Smooth Muscle

Emala, Gallos, et al. [66–75] have found that novel α5-subtype selective GABA<sub>α</sub> positive allosteric modulators relax airway smooth muscle from rodents and humans. The clinical need for new classes of bronchodilators for the treatment of bronchoconstrictive diseases such as asthma remains a major medical issue. Few novel therapeutics have been approved for targeting airway smooth muscle (ASM) relaxation or lung inflammation in the last 40 years [66]. In fact, several asthma-related deaths are attributed, in part, to long-acting β-agonists (LABA) [67]. Adherence to inhaled corticosteroids, the first line of treatment for airway inflammation in asthma, is very poor [68, 69]. Therapies that break our dependence on β-agonism for ASM relaxation would be a novel and substantial advancement.

These ASM studies were undertaken due to a pressing clinical need for novel bronchodilators in the treatment of asthma and other bronchoconstrictive diseases such as COPA. There are only three drug classes currently in clinical
use as acute bronchodilators in the United States (methylxanthines, anticholinergics, and β-adrenoceptor agonists) [70]. Thus, a novel therapeutic approach that would employ cellular signaling pathways distinct from those used by these existing therapies involves modulating airway smooth muscle (ASM) chloride conductance via GABA_A receptors to achieve relaxation of precontracted ASM [71, 72]. However, widespread activation of all GABA_A receptors may lead to undesirable side effects (sedation, hypnosis, mucus formation, etc.). Thus, a strategy that selectively targets a subset of GABA_A channels, those containing α subunits found to be expressed in airway smooth muscle, may be a first step in limiting side effects. Since human airway smooth muscle contains only α4 or α5 subunits [72], ligands with selectivity for these subunits are an attractive therapeutic option. Concern regarding nonselective GABA_A receptor activation is not limited to the airway or other peripheral tissues. GABA_A receptor ligands are classically known for their central nervous system effects of anxiolysis, sedation, hypnosis, amnesia, anticonvulsion, and muscle relaxant effects. Such indiscriminate activation of GABA_A receptors in the CNS is exemplified by the side effects of classical benzodiazepines (such as diazepam) which were the underpinning for the motivation of a search for benzodiazepine (BZD) ligands that discriminate among the α subunits of GABA_A receptors [73–75].

A novel approach to identify novel benzodiazepine derivatives to selectively target GABA_A channels containing specific α subunits was developed by Cook et al. in the 1980s that employed a pharmacophore receptor model based on the binding affinity of rigid ligands to BDZ/GABA_A receptor sites (as reviewed in 2007 [23]). From this series

**Figure 19:** Hippocampal (HPC) infusion of SH-053-2’F-R-CH_3 (1 μM/side; patterned bars) normalizes the aberrant increase in the number of spontaneously firing dopamine neurons (expressed as cells/track) in methylazoxymethanol acetate (MAM-) treated animals (a). There was no effect of SH-053-2’F-R-CH_3 treatment in control animals (open bars, (a)–(c)) or on firing rate in MAM animals (dark bars; (b)). Hippocampal (HPC) infusion of SH-053-2’F-R-CH_3 significantly reduced the percentage of spikes occurring in bursts of dopamine (DA) neurons in MAM and control animals (c) (* p < 0.05, two-way ANOVA, Holm-Sidak post hoc; N = 7 rats/group) [27–38].
of receptor models for $\alpha_{1-6}\beta3\gamma2$ subtypes a robust model for $\alpha5$ subtype selective ligands emerged, the result of which included the synthesis of a novel $\alpha5\beta3\gamma2$ partial agonist modulator, SH-053-2 F-R-CH$_3$ (2). The discovery of this and related ligands selective for $\alpha5$ BDZ/GABA$_A$-ergic receptors and the realization that only $\alpha4$ and $\alpha5$ subunits are expressed in GABA$_A$ channels on human airway smooth muscle yielded an ideal opportunity for targeting these $\alpha5$-subunit containing GABA$_A$ channels for bronchorelaxation [66–75].

The GABA$_A$$\alpha5$ subunit protein was first localized to the ASM layer of human trachea while costaining for the smooth muscle specific protein $\alpha$ actin (Figure 26). The first panel of Figure 26 shows GABA$_A$$\alpha5$ protein stained with fluorescent green and blue fluorescent nuclear staining (DAPI). The second panel is the same human tracheal smooth muscle section simultaneously stained with a protein specific for smooth muscle, $\alpha$ actin, and the third panel is a merge of the first two panels showing costaining of smooth muscle with GABA$_A$$\alpha5$ and $\alpha$ actin proteins. The fourth panel is a control omitting primary antibodies but including nuclear DAPI staining [66–75].

After demonstrating the protein expression of GABA$_A$ receptors containing the $\alpha5$ subunit, functional studies of isolated airway smooth muscle were performed in tracheal airway smooth muscle from two species. Human airway smooth muscle suspended in an organ bath was preconstricted with a concentration of acetylcholine that was the EC$_{50}$ concentration of acetylcholine for each individual airway smooth muscle preparation. The induced contraction was then relaxed with a $\beta$-agonist (isoproterenol) in the absence or presence of the GABA$_A$$\alpha5$ ligand SH-053-2 F-R-CH$_3$ (2). Figure 27(a) shows that the amount of relaxation

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**Figure 20:** Extracellular recording traces illustrate the reduction in evoked responses in the ventral hippocampal (HPC) to entorhinal cortex stimulation in both MAM- and saline-treated animals (a, b). Treatment with SH-053-2 F-R-CH$_3$ (0.1 mg/kg, i.v.) decreases the evoked excitatory response (dashed lines) of ventral HPC neurons to entorhinal cortex stimulation in both MAM- and saline-treated animals (c) ($^{*} p < 0.05$ for saline and $^+ p < 0.05$, two-way repeated measures ANOVA, Holm-Sidak post hoc) [27–38].

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induced by 10 nM isoproterenol was significantly increased if 50 μM SH-053-2-F-R-CH₃ (2) was also present in the buffer superfusing the airway smooth muscle strip. Studies were also performed in airway smooth muscle from another species, guinea pig, that measured direct relaxation of a different contractile agonist, substance P. As shown in Figure 27(b), the amount of remaining contractile force 30 minutes after a substance P-induced contraction was significantly reduced in airway smooth muscle tracheal rings treated with SH-053-2-F-R-CH₃ (2) [66–75].

Following these studies in intact airway smooth muscle, cell based studies were initiated in cultured human airway smooth muscle cells to directly measure plasma membrane chloride currents and the effects of these currents on intracellular calcium concentrations. SH-053-2-F-R-CH₃ (2) induced a CT current in vitro using conventional whole cell patch clamp techniques [66–75]. These electrophysiology studies were then followed by studies to determine the effect of these plasma membrane chloride currents on intracellular calcium concentrations following treatment of human airway smooth muscle cells with a ligand whose receptor couples through a Gq protein pathway, a classic signaling pathway that mediates airway smooth muscle contraction.

SH-053-2-F-R-CH₃ (2) attenuated an increase in intracellular calcium concentrations induced by a classic Gq-coupled ligand, bradykinin (Figure 28(a)) [66–75]. The attenuation by SH-053-2-F-R-CH₃ (2) was significantly blocked by the GABAₐ antagonist gabazine (Figure 28(b)) indicating that SH-053-2-F-R-CH₃ (2) was modulating GABAₐ receptors for these effects on cellular calcium [66–75].

The major findings of these studies are that human airway smooth muscle expresses α5 subunit containing GABAₐ receptors that can be pharmacologically targeted by a selective agonist. The GABAₐ α5 subunit selective ligand SH-053-2-F-R-CH₃ (2) relaxed intact guinea pig airway smooth muscle contracted with substance P and augmented β-agonist-mediated relaxation of intact human airway smooth muscle. The mechanism for these effects was likely mediated by plasma membrane chloride currents that contributed to an attenuation of contractile-mediated increases in intracellular calcium, a critical event in the initiation and maintenance of airway smooth muscle contraction [66–75].

8. Recent Discovery of Alpha 5 Included Volume Differences: L₄ Pocket as Compared to Other Bz/GABAergic Subtypes

The findings in both the MAM-model of schizophrenia and the relaxation of airway smooth muscle have led to the study of SH-053-2-F-R-CH₃ and related compounds bound within the α5-GABAₐ/BzR (Figure 29). The SH-053-R-CH₃ (15) and SH-053-S-CH₃ (16) isomers have been previously described [23]. These compounds along with SH-053-2-F-R-CH₃ and SH-053-2-F-S-CH₃ have been tested for binding affinity and show selectivity for the α5-subunit (Table 3).

From examination of Figure 30 and Tables 3 and 4, it is clear the (R)-isomers bound to the α5 subtype while the (S)-isomers were selective for α2/α3/α5 subtypes.

From this data, these compounds were then used in examining the α5-binding pocket, most specifically the fluoroseries. In regard to molecular modeling, depicted in Figure 30 is the included volume and ligand occupation of the SH-053-2-F-S-CH₃ (17) and SH-053-2-F-R-CH₃ (2) enantiomers in the α5 subtype as well as the α2 subtype. It
is clear a new pocket ($L_4$) has been located in the $\alpha_5$ subtype permitting 2 as well as 17 to bind to the $\alpha_5$ subtype. Examination of both ligands in the $\alpha_2$ subtype clearly illustrates the analogous region in the $\alpha_2$ subtype is not present and thus does not accommodate 2 for the pendant phenyl which lies outside the included volume in the space allocated for the receptor protein itself [23].

9. BzR GABA(A) Subtypes

In terms of potency, examination of the values in Table 4 [87], it is clear the R-isomer (2) shows more selectivity towards the $\alpha_5$-subunit, while the S-isomer (17) is potent at the $\alpha_2/3/5$ subunits. It is important, as postulated earlier [23], that the major difference in GABA(A)/Bz receptors subtypes stems
from differences in asymmetry in the lipophilic pockets $L_1$, $L_2$, $L_3$, $L_4$, and $L_{Di}$ in the pharmacophore/receptor model and indicates even better functional selectivity is possible with asymmetric BzR ligands.

The synthetic switching of chirality at the C-4 position of imidazobenzodiazepines to induce subtype selectivity was successful. Moreover, increase of the potency of imidazobenzodiazepines can be achieved by substitution of the 2'-position hydrogen atom with an electron rich atom (fluorine) on the pendant phenyl ring in agreement with Haefely et al. [88], Fryer [89, 90], and our own work [22, 91]. The biological data on the two enantiomeric pairs of benzodiazepine ligands...
confirm the ataxic activity of BZ site agonists is mediated by α1β2γ2/3γ2 subtypes, as reported in [23, 91–93]. The antianxiety activity in primates of the S isomers was preserved with no sedation. In only one study in rodents was any sedation observed; the confounding sedation was observed in both the S isomer (functionally selective for α2, α3, and α5 receptor subtypes) and R isomer (essentially selective for α5 subtype) and may involve at least, in part, agonist activity at α5 BzR subtypes. There are some α5 BzR located in the spinal cord which might be the source of the decrease in locomotion with SH-053-2F-R-CH3 and SH-053-2F-S-CH3; however, this is possibly some type of stereotypical behavior. Hence in agreement with many laboratories including our own [23, 92, 93] the best potential nonselective, nonnanesic, antianxiety agents stem from ligands with agonist efficacy at α2 subtypes essentially silent at α1 and α5 subtypes (to avoid sedation) [91]. It must be pointed out again; however, in primates Fischer et al. [87] observed a potent antianxiety effect with no sedation with the 2'F-S-CH3 (17) isomer, while the 2'F-R-CH3 (2) isomer exhibited only a very weak anxiolytic effect.

Numerous groups have done modeling and SAR studies on different classes of compounds which have resulted in a few different pharmacophore models based on the benzodiazepine binding site (BS) of the GABA_A receptor [94]. These models are employed to gain insight in the interactions between the BS and the ligand. These have been put forth by Loew [7, 95, 96], Crippen [97, 98], Codding [76, 77, 99–101], Fryer [89, 90, 94], Gilli and Borea [102–105], Tebib et al. [106], and Gardner [107], as well as from Professors Sieghart, Cromer, and our own laboratory [21, 39, 40, 76, 78–82, 108–118].

The Milwaukee-based pharmacophore/receptor model is a comprehensive building of the BzR using radioligand binding data and receptor mapping techniques based on 12 classes of compounds [20, 23, 39, 40, 42, 111, 119–122]. This model (Figure 31) [79] has brought together previous models which have used data from the activity of antagonists, positive allosteric modulators, and negative allosteric modulators and included the new models for the “diazepam-insensitive” (DI) sites [123]. Four basic anchor points, H1, H2, A2, and L1, were assigned, and 4 additional lipophilic regions were defined as L2, L3, L4, and the new L5 (see captions in Figure 31 for details); regions S1, S2, and S3 represent negative areas of steric repulsion. As previously reported, the synthesis of both partial agonists and partial inverse agonists has been achieved by using parts of this model [99, 100, 104, 105, 119, 124–127].

The cloning, expression, and anatomical localization of multiple GABA(A) subunits have facilitated both the identification and design of subtype selective ligands. With the availability of binding data from different recombinant receptor subtypes, affinities of ligands from many different structural classes of compounds have been evaluated.

Illustrated in Figure 31 is the [3,4-c]quinolin-3-one CGS-9896 (18) (dotted line), a diazadindole (19) (thin line), and diazepam (20) (thick line) fitted initially to the inclusive pharmacophore model for the BzR. Sites H1 (Y210) and H2 (H102) represent hydrogen bond donor sites on the receptor protein complex while A2 (T142) represents a hydrogen bond acceptor site necessary for potent inverse activity in vivo. L1, L2, L3, L4, and L5(D1) are four lipophilic regions in the binding pharmacophore. Descriptors S1, S2, and S3 are regions of negative steric repulsion.

Based on SAR data obtained for these ligands at 6 recombinant BzR subtypes [128–132], an effort has been undertaken to establish different pharmacophore/receptor models for BzR subtypes. The alignment of the twelve different structural classes of benzodiazepine receptor ligands was earlier based on the least squares fitting of at least three points. The coordinates of the four anchor points (A2, H1, H2, and L1) employed in the alignment are outlined in Figure 32.

### Table 3: Binding affinity at αβγ2 GABA_A receptor subtypes (values are reported in nM).

| Compound | α1 | α2 | α3 | α4 | α5 | α6 |
|----------|----|----|----|----|----|----|
| SH-053-R-CH3 (15) | 2026 | 2377 | 1183 >5000 | 949.1 >5000 |
| SH-053-S-CH3 (16) | 1666 | 1263 | 1249 >5000 | 206.4 >5000 |
| SH-053-2F-R-CH3 (2) | 759.1 | 948.2 | 768.8 >5000 | 95.17 >5000 |
| SH-053-2F-S-CH3 (17) | 350 | 141 | 1237 >5000 | 19.2 >5000 |

*Data shown here are the means of two determinations which differed by less than 10%.

### Table 4: Oocyte electrophysiological data of benzodiazepines* [87].

| Compound | α1 | α2 | α3 | α4 | α5 | α6 |
|----------|----|----|----|----|----|----|
| SH-053-2F-R-CH3 (2) | 111/154 | 124/185 | 125/220 | 183/387 |
| SH-053-2F-S-CH3 (17) | 116/164 | 170/348 | 138/301 | 218/389 |

*Efficacy at αβγ2 GABA_A receptor subtypes as % of control current at 100 nM and 1μM concentrations. Data presented as percent over baseline (100) at concentrations of 100 nM/1 μM.
Herein are described the results from ligand-mapping experiments at recombinant BzR subtypes of 1,4-benzodiazepines, imidazobenzodiazepines, β-carbolines, diindoles, pyrazoloquinolinones, and others [126]. Some of the differences and similarities among these subtypes can be gleaned from this study and serve as a guide for future drug design.

10. **α1 Updates**

10.1. Beta-Carbolines. A series of 3,6-disubstituted β-carbolines was prepared and evaluated for their in vitro affinity at ααβγy2 GABA(A)/BzR subtype by radioligand binding assays in search of ααβγy2 subtype selective compounds (Figure 33). A potential therapeutic application of such antagonist analogs is to treat alcohol abuse [133, 134]. Analogues of βCCt (21) were synthesized via a carbonyldiimidazole-mediated method by Yin et al. [85] and the related 6-substituted β-carboline-3-carboxylates including WYS8 (27) were synthesized from 6-iodo βCCt (29). Bivalent ligands (42 and 43) were also synthesized to increase the scope of the structure-activity relationships (SAR) to larger ligands. An...
Figure 26: Protein expression of the GABA$_A$ $\alpha_5$ subunit in intact human trachea-bronchial airway smooth muscle. Representative images of human tracheal airway smooth muscle sections using confocal microscopy are depicted following single, double, and triple immunofluorescence labeling. The antibodies employed were directed against the GABA$_A$ $\alpha_5$ subunit (green), $\alpha$-smooth muscle actin (SMA; red), and/or the nucleus via DAPI counterstain (blue). Panels illustrate the following staining parameters from left to right: (1st) costaining of DAPI and GABA$_A$ $\alpha_5$ subunit; (2nd) $\alpha$-SMA staining alone; (3rd) triple-staining of GABA$_A$ $\alpha_5$, $\alpha$-SMA, and DAPI; (4th) DAPI nucleus counterstain, with primary antibodies omitted as negative control. Modified from [66–75].

Figure 27: SH-053-2’F-R-CH$_3$ (2) mediated activation of $\alpha_5$ subunit containing GABA$_A$ channels induces relaxation of precontracted airway smooth muscle. (a) SH-053-2’F-R-CH$_3$ (2) (SH-053) potentiates $\beta$-agonist-mediated relaxation of human airway smooth muscle. Cotreatment of human airway smooth muscle strips with SH-053-2’F-R-CH$_3$ (2) (50 $\mu$M) significantly enhances isoproterenol (10 nM) mediated relaxation of an acetylcholine EC$_{50}$ contraction compared to isoproterenol alone ($N = 8$ / group, $$ = p < 0.01$). Modified from [66–75]. (b) SH-053-2’F-R-CH$_3$ (2) activation of $\alpha_5$ containing GABA$_A$ receptors induces direct relaxation of substance P-induced airway smooth muscle contraction. Compiled results demonstrating enhanced spontaneous relaxation (expressed as % remaining force at 30 minutes following a 1 $\mu$M substance P mediated contraction) following treatment with SH-053-2’F-R-CH$_3$ (2) compared to treatment with vehicle control ($n = 4$ - 5 / group, $$ = p < 0.01$) [66–75].

Initial SAR on the first analogs demonstrated that compounds with larger side-groups at C6 were well tolerated as they projected into the $L_D$ domain (see 42 and 43) [85]. Moreover, substituents located at C3 exhibited a conserved stereo interaction in lipophilic pocket $L_1$, while N2 likely participated in hydrogen bonding with $H_1$. Three novel $\beta$-carboline ligands (21, 23, and 27) permitted a comparison of the pharmacological properties with a range of classical benzodiazepine receptor antagonists (flumazenil, ZK93426) from several structural groups and indicated these $\beta$-carbolines were “near GABA neutral antagonists.” Based on the SAR, the most potent (in vitro) $\alpha_1$ selective ligand was the 6-substituted acetylenyl $\beta$CCl (WYS8, 27). In a previous study both 21 and 23 were able to reduce the rate at which rats self-administrated alcohol in alcohol preferring and HAD rats but had little or no effect on sucrose self-administration [85].
3-PBC (23) was also active in baboons [134]. This data has been used in updating the pharmacophore model in the α1-subtype.

11. The Updated Included Volume Models

Illustrated in Figure 34 is the included volume of the updated pharmacophore receptor model of the α1β3γ2 subtype of Clayton [22]. The current model for the α1β3γ2 subtype has several new features. The cyclopropyl group of CD-214 extended 2 Å past the A2 descriptor slightly increasing its volume. The trimethylsilyl group of QH-II-82 and WYS7 illustrates how well bulky groups are tolerated near the entrance of the binding pocket. Despite not being as potent, dimers of beta carbolines, WYS2 and WYS6, bound to α1 subtypes at 30 nM and 120 nM, respectively. Their ability to bind, albeit weakly, supports the location of the binding site entrance from the extracellular domain. The included volume of the α1β3γ2 subtype was previously 1085.7 cubic angstroms. The volume has now been measured as 1219.2 cubic angstroms. Volume measurements should be used carefully as the binding site is not enclosed and the theoretical opening near LDI is not clearly demarcated. Dimers were excluded from the included volume exercise because although they bound to the receptor, they represented compounds which were felt to extend outside the receptor binding pocket when docked to the protein. Where appropriate,
The left image of figure rotated 90°. It can be clearly seen that 17 fits within the included volume.

The left image of figure rotated 90°. It can be clearly seen that the conformation of 2 is such that the pendant 6-phenyl sticks outside the included volume.

The left image of figure rotated 90°. It can be clearly seen that 17 fits within the included volume.

The left image of figure rotated 90°. It can be clearly seen that 2 fits within the included volume.

**Figure 30:** Included volume and ligand occupation of the SH-053-2'F-S-CH$_3$ 17 and SH-053-2'F-R-CH$_3$ 2 enantiomers in the α5 and γ2 pharmacophore/receptor models. This figure was modified and reproduced from that reported by Clayton et al. in [22, 23].
their monomers were included in the included volume analysis. Ligands considered for the included volume in Table 5 exhibited potent binding at α1 subtypes (K_i ≤ 20 nM) but were not necessarily subtype selective. The binding data for ligands at α2-6-subtypes follow (Tables 6–10; structures located in Clayton [22] and Supporting Information, Appendix III in Supplementary Material available online at http://dx.doi.org/10.1155/2015/430248).

12. The α1β3γ2 Receptor Subtype
The focus of this research was aimed at diazepam sensitive receptors; additional features to the α4β3γ2 and α6β3γ2 receptors were not identified (see Table 5, Figures 34 and 35). The major new feature identified for the α5β3γ2 receptor was a new L_4 pocket. This new lipophilic pocket was identified with SH-053-R-CH_3 (15) and SH-053-S-CH_3 (16) chiral enantiomers as well as the Z F analogs [74, 135, 136].
| Ligands | R₆ | R₃     | α₁  | α₂  | α₃  | α₄  | α₅  | α₆  |
|---------|----|--------|-----|-----|-----|-----|-----|-----|
| 21(βCCt) | H  | CO₂tBu | 0.72 | 15  | 18.9 | 1000  | 111  | >5,000 |
| 22(βCCE) | H  | CO₂Et  | 1.2  | 4.9 | 5.7  | ND   | 26.8 | 2,700 |
| 23(3-PBC) | H  | OnPr   | 5.3  | 52.3 | 68.8 | 1000  | 591  | >1,000 |
| 24(WYB14) | TMS | CO₂tBu | 6.8  | 30  | 36   | 2000  | 108  | 1000  |
| 25(WY-B-25) | TMS | CO₂CH₂CF₃ | 17  | 59  | 88   | 200  | 1444 | >3000 |
| 26(WY-B-99-1) | TMS | CO₂Et  | 4.4  | 4.5 | 5.58 | 2000  | 47   | 2000  |
| 27(WYS8) | H  | CO₂tBu | 0.972 | 111 | 102  | 2000  | 1473 | 1980  |
| 28(WY-B-26-2) | H  | CO₂CH₂CF₃ | 4.5  | 44.6 | 42.7 | 2000  | 124  | 2000  |
| 29(iodo-βCCt) | I  | CO₂tBu | 14.4 | 44.9 | 123  | >4000 | 65.3 | >4000 |
| 30(WY-B-20) | I  | CO₂CH₂CF₃ | 12  | 39  | 47   | 2000  | 122  | 3000  |
| 31(iodo-βCCE) | I  | CO₂Et  | 4.8  | 31  | 34   | 1000  | 286  | 1000  |
| 32(WY-B-08) | I  | CO₂CH₂(CF₃)₂ | 78  | 301 | 131  | 3000  | 681  | 3000  |
| 33(WYS13) | G  | CO₂tBu | 2.4  | 13  | 27.5 | NA   | 163  | 5000  |
| 34(WYB27-1) | S  | CO₂CH₂CF₃ | 26  | 143 | 117  | 3000  | 127  | 2000  |
| 35(WYS12) | S  | CO₂tBu | 37  | 166 | 314  | NA   | 2861 | 5000  |
| 36(WYB27-2) | S  | CO₂CH₂CF₃ | 9.2 | 13  | 72   | 2000  | 449  | 2000  |
| 37(WYS15) | S  | CO₂tBu | 3.63 | 2.02 | 44.3 | NA   | 76.5 | 5000  |
| 38(WYB29-2) | S  | CO₂CH₂CF₃ | 25  | 137 | 125  | 2000  | 299  | 2000  |
| 39(CMA57) | F  | CO₃H₇  | 3.7  | 27  | 40   | NA   | 254  | >2500 |
| 40(CM-A-82a) | C(CH₃)₃ | CO₂tBu | 2.78 | 8.93 | 24.5 | 1000  | 7.49 | 1000  |
| 41(CM-A-87) | F  | CO₂tBu | 1.62 | 4.54 | 14.7 | 1000  | 4.61 | 1000  |
| 42(WY-S-2) | Bcct | MOM   | 30  | 124 | 100  | >300  | >300 | >4000 |
| 43(WY-S-6) | Bcct | | 120 | 1059 | 3942 | 5000  | 5000 | 5000  |

*Affinity of compounds at GABA(A)/BzR recombinant subtypes was measured by competition for [³H]flunitrazepam or [³H] Ro-15-4513 binding to HEK cell membranes expressing human receptors of composition α₁β₃γ₂, α₂β₃γ₂, α₃β₃γ₂, α₄β₃γ₂, α₅β₃γ₂, and α₆β₃γ₂ [85]. Data represent the average of at least three determinations with a SEM of ±5%.*

**Figure 33:** *Affinities (Kᵢ = nM) of 3,6-disubstituted β-carbolines at αₓβ₃γ₂ (x = 1–3, 5, 6) receptor subtypes [85]. The structures versus code numbers of all ligands in the tables of this review can be found in the Ph.D. thesis of Terry Clayton (Ph.D. thesis, University of Wisconsin-Milwaukee, Milwaukee, WI, December, 2011) [22] and in the Supporting Information.*
Table 5: These ligands bound with potent affinity for $\alpha_1$; ligands bound with $K_i$ values < 20 nM at this subtype.

| Cook code* | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\alpha_4$ | $\alpha_5$ | $\alpha_6$ |
|------------|------------|------------|------------|------------|------------|------------|
| WY-TSC-4 (WYS8) | 0.007 | 0.99 | 1.63 | 51.04 | | |
| SH-TSC-2 (BCCT) | 0.03 | 0.0419 | 0.035 | 69.32 | | |
| QH-II-090 (CGS-8216) | 0.05 | 0.08 | 0.12 | 0.25 | 17 | |
| XLI-286 | 0.051 | 0.064 | 0.118 | 0.684 | | |
| QH-II-077 | 0.06 | 0.08 | 0.05 | 0.12 | 4 | |
| QH-II-092 | 0.07 | 0.03 | 0.04 | ND | 0.17 | ND |
| JYI-57 | 0.076 | 0.076 | 0.131 | ND | 0.036 | ND |
| QH-II-085 | 0.08 | 0.06 | 0.02 | ND | 0.08 | ND |
| XHE-II-024 | 0.09 | 0.18 | 0.32 | 14 | 0.24 | 11 |
| PWZ-007A | 0.11 | 0.1 | 0.09 | ND | 0.2 | 10 |
| CGS8216 | 0.13 | ND | ND | ND | 46 | |
| SPH-121 | 0.14 | 1.19 | 1.72 | ND | 4 | 479 |
| QH-II-075 | 0.18 | 0.21 | 0.25 | ND | 1.3 | 40 |
| PZII-028 | 0.2 | ND | 0.2 | ND | 0.32 | 1.9 |
| CGS9895 | 0.21 | ND | ND | ND | 9.3 | |
| PWZ-0071 | 0.23 | 0.17 | 0.12 | ND | 0.44 | 17.31 |
| XHE-III-24 | 0.25 | ND | 8 | 222 | 10 | 328 |
| JYI-42 | 0.257 | 0.146 | 0.278 | ND | 0.256 | ND |
| CGS9896 | 0.28 | ND | ND | ND | ND | 181 |
| JYI-64 (C17H12N4FBr) | 0.305 | 1.11 | 0.62 | ND | 0.87 | 5000 |
| PZII-029 | 0.34 | ND | 0.79 | ND | 0.52 | 10 |
| BRETAZENIL | 0.35 | 0.64 | 0.2 | ND | 0.5 | 12.7 |
| FG8205 | 0.4 | 2.08 | 1.16 | ND | 1.54 | 227 |
| YT-5 | 0.421 | 0.6034 | 36.06 | ND | 1.695 | ND |
| 6-PBC | 0.49 | 1.21 | 2.2 | ND | 2.39 | 1343 |
| QH-146 | 0.49 | ND | 0.76 | ND | 7.7 | 10000 |
| DM-II-90 (C17H12N4BrCl) | 0.505 | 1 | 0.63 | ND | 0.37 | 5000 |
| SPH-165 | 0.63 | 2.79 | 4.85 | ND | 10.4 | 1150 |
| BCCt | 0.72 | 15 | 18.9 | ND | 110.8 | 5000 |
| SH-I-048A | 0.774 | 0.1723 | 0.383 | ND | 0.11 | ND |
| alprazolam | 0.8 | 0.59 | 1.43 | ND | 1.54 | 10000 |
| Ro15-1788 | 0.8 | 0.9 | 1.05 | ND | 0.6 | 148 |
| WYS10 C14H9F3N2O2 | 0.88 | 36 | 25.6 | ND | 548.7 | 15.3 |
| WY-B-15 | 0.92 | 0.83 | 0.58 | 2080 | 4.42 | 646 |
| WY-A-99-2 (WYS8) | 0.972 | 11 | 102 | 2000 | 208 | 1980 |
| XHE-III-06a | 1 | 2 | 1 | 5 | 1.8 | 37 |
| Xli366 C22H21N3O2 | 1 | ND | ND | ND | ND | ND |
| JYI-59 (C22H13N3O2F4) | 1.08 | 2.6 | 11.82 | ND | 11.5 | 5000 |
| WYSCI C16H16N2O2 | 1.094 | 5.44 | 12.3 | ND | 69.8 | 21.2 |
| MLI-I-70 | 1.1 | 1.2 | 1.1 | ND | 40.3 | 1000 |
| SVO-8-30 | 1.1 | 5.3 | 5.3 | 2.8 | 0.6 | 15 |
| BCCE | 1.2 | 4.9 | 5.7 | ND | 26.8 | 2700 |
| XHE-III-04 | 1.2 | 2 | 1 | 219 | 0.4 | 500 |
| XLI350 C17H11C1N2O | 1.224 | 1.188 | ND | ND | 2.9 | ND |
| XHE-III-49 | 1.3 | 5.5 | 4.2 | 38.7 | 11.3 | 85.1 |
| PWZ-009A1 | 1.34 | 1.31 | 1.26 | ND | 0.84 | 2.03 |
| DM-239 | 1.5 | ND | 0.53 | ND | 0.14 | 6.89 |
| XLI351 C21H21C1N2OSi | 1.507 | 0.967 | ND | ND | 1.985 | ND |
| XLI352 C18H13C1N2O | 1.56 | 0.991 | ND | ND | 1.957 | ND |
| Cook code       | α1  | α2  | α3  | α4  | α5  | α6  |
|----------------|-----|-----|-----|-----|-----|-----|
| TG-4-39        | 1.6 | 34  | 24  | 5.6 | 1.4 | 23  |
| TG-II-82       | 1.6 | 2.9 | 2.8 | ND  | 1   | 1000|
| CM-A87         | 1.62| 4.54| 14.73| 1000| 4.61| 1000|
| QH-II-082      | 1.7 | 1.8 | 1.6 | ND  | 6.1 | 100 |
| JYI-49 (C20H12N3O2F4Br) | 1.87| 2.38| ND  | ND  | 6.7 | 3390|
| LJD-III-15E    | 1.93| 14  | 19  | ND  | 70.8| 1000|
| SPH-38         | 2   | 5.4 | 10.8| ND  | 18.5| 3000|
| XHE-I-093      | 2   | 7.1 | 8.9 | 1107| 20  | 1162|
| MSA-IV-35      | 2.1 | 16  | 21  | ND  | 995 | 3000|
| JYI-39 (C23H23N3O3S) | 2.176| 205 | ND  | ND  | 34  | 12.7|
| FLUNITRAZEPAM  | 2.2 | 2.5 | 4.5 | ND  | 2.1 | 2000|
| YCT-5          | 2.2 | 11.46| 16.3| ND  | 200 | 10000|
| TJH-IV-51      | 2.39| 17.4| 14.5| ND  | 316 | 10000|
| WYS13 C20H18N2O3 | 2.442| 10 | 21  | ND  | 163 | 5000|
| YT-III-25      | 2.531| 5.786| 5.691| ND  | 0.095 | ND |
| XHE-III-14     | 2.6 | 10  | 13  | 2   | 7   |     |
| WYS9 C16H15N2O2 | 2.72 | 22.2| 23.1| ND  | 562 | 122 |
| JYI-47         | 2.759| 2.282| 0.511| ND  | 0.427 | ND |
| CM-A82a        | 2.78| 8.93| 24.51| 1000| 7.49| 1000|
| TG-4-29        | 2.8 | 3.9 | 2.7 | 2.1 | 0.18| 3.9 |
| XLI268 C27H18BrN4 | 2.8145| 0.6862| ND  | ND  | 0.6243 | ND |
| JYI-54 (C24H15N3O3F4) | 2.89 | 172 | 6.7 | ND  | 57  | 1890 |
| MMB-II-74      | 3   | 24.5| 41.7| 500 | 125.7| 1000|
| MMB-III-016    | 3   | 1.97| 2   | 1074| 0.26| 211 |
| MMB-III-16     | 3   | 1.97| 2   | 1074| 0.26| 211 |
| QH-II-080b     | 3   | 3.7 | 4.7 | ND  | 24  | 1000|
| YCT-7A         | 3   | 23.8| 30.5| ND  | 240 | 10000|
| JYI-32 (C20H15N3O2BrF) | 3.07| 4.96| ND  | ND  | 2.92 | 52.24|
| RoI5-4513      | 3.3 | 2.6 | 2.5 | ND  | 0.26| 3.8 |
| XHE-II-017     | 3.3 | 10  | 7   | 258 | 17  | 294 |
| XLI-JY-DMH ANX3| 3.3 | 0.58| 1.9 | ND  | 4.4 | 5000|
| MLT-II-18      | 3.4 | 11.7| 11  | ND  | 225 | 10000|
| TJH-V-88       | 3.41| 30  | ND  | 140.9| 10000|
| XLI-2TC        | 3.442| 1.673| 44.08| ND  | 1.121|     |
| WYS15 C22H20N2O2 | 3.63| 2.02| 44.3| ND  | 76.5| 5000|
| CM-A57         | 3.7 | 27  | 40  | ND  | 254 | 1000|
| XHE-II-006b    | 3.7 | 15  | 12  | 1897| 144 | 1000|
| JYI-60         | 3.73| 1.635| 4.3 | ND  | 1.7 | 5000|
| RY-008         | 3.75| 7.2 | 4.14| ND  | 1.11| 44.3|
| MLT-II-18      | 3.9 | 12.2| 24.4| ND  | 210 | 10000|
| OMB-18         | 3.9 | 1.2 | 3.4 | 1733| 0.8 | 5   |
| WY-B-09-1      | 3.99| 8   | 32  | 1000| 461 | 2000|
| SHU-1-19       | 4   | 12  | 7   | 48  | 14  | 84  |
| ZK 93423       | 4.1 | 4.2 | 6   | ND  | 4.5 | 1000|
| WY-B-23-2 (WYS11) | 4.2 | 37.7| 39  | 2000| 176 | 69.4|
| WY-B-23-2 (WYS11) | 4.2 | 37.7| 73  | ND  | 176 | 69.4|
| WY-B-99-1      | 4.4 | 4.5 | 5.58| 2000| 47  | 2000|
| WY-B-26-2      | 4.45| 44.57| 42.66| 2000| 124 | 2000|
| XHE-II-006a    | 4.7 | 4.4 | 20  | 1876| 89  | 3531|
| Cook code                      | α1  | α2  | α3  | α4   | α5   | α6   |
|-------------------------------|-----|-----|-----|------|------|------|
| CM-B01                        | 4.8 | 31  | 34  | 1000 | 286  | 1000 |
| PWZ-085                       | 4.86| 13  | 8.5 | ND   | 0.55 | 40   |
| MTL-II-16                     | 5.05| 10.41| 18.4| ND   | 260  | 10000|
| 3 PBC                         | 5.3 | 52.3| 68.8| ND   | 591  | 1000 |
| MA-3-PROPOXYL                 | 5.3 | 52.3| 68.8| ND   | 591  | 1000 |
| TJH-IV-43                     | 5.42| 30.19| 48.9| ND   | 475  | 10000|
| DMCM                          | 5.69| 8.29| 4   | ND   | 1.04 | 134  |
| DM-139                        | 5.8 | ND  | 169 | ND   | 9.25 | 325  |
| XHE-II-073A (R ENRICHED)      | 5.9 | 11  | 10  | 15   | 1.18 | 140  |
| MSR-I-032                     | 6.2 | 18.7| 4   | ND   | 3.3  | 74.9 |
| JYI-70 (C19H13N4F)            | 6.3 | 2.1 | ND  | ND   | 0.56 | 5000 |
| XLI343 C20H19ClN2OSi           | 6.375| 17.71| ND  | ND   | 150.5| ND   |
| 3 EBC                         | 6.43| 25.1| 28.2| ND   | 826  | 1000 |
| DM-146                        | 6.44| ND  | 148 | ND   | 4.23 | 247  |
| DM-215                        | 6.74| ND  | 7.42| ND   | 0.293| 8.28 |
| ZG69A                         | 6.8 | 16.3| 9.2 | ND   | 0.85 | 54.6 |
| ZG-69a (Ro15-1310)            | 6.8 | 16.3| 9.2 | ND   | 0.85 | 54.6 |
| WY-B-14 (WYS7)                | 6.84| 30  | 36  | 2000 | 108  | 1000 |
| YT-II                         | 6.932| 0.8712| 3.518| ND   | 5.119| ND   |
| SVO-8-67                      | 7   | 41  | 26  | 15   | 2.3  | 191  |
| MTL-II-34                     | 7.04| 15.95| 22.3| ND   | 158  | 1000 |
| SPH-195                       | 7.2 | 168.5| 283.5| ND   | 271  | 10000|
| XHE-I-065                     | 7.2 | 17  | 18  | 500  | 57   | 500  |
| ZG-234                        | 7.25| 22.14| 9.84| ND   | 0.3  | 5.25 |
| SH-I-04                       | 7.3 | 6.136| 5.1 | ND   | 7.664| ND   |
| XHE-I-038                     | 7.3 | 5   | 34  | ND   | 132  | 1000 |
| XHE-III-13                    | 7.3 | ND  | 71  | 880  | 1.6  | 311  |
| WY-B-25                       | 7.6 | 40  | 66  | 2000 | 263  | 2000 |
| CM-A49 (R)                    | 7.7 | 32.5| 43  | ND   | 69   | 1000 |
| SVO-8-14                      | 8   | 25  | 8   | 6.9  | 0.9  | 14   |
| TG-4-29                       | 8.3 | 10.2| 6.9 | ND   | 0.4  | 7.61 |
| XHE-II-002                    | 8.3 | 18  | 13  | 3.9  | 1.5  | 11   |
| WY-B-14 (WYS7)                | 8.5 | 165 | 245 | ND   | 1786 | 5000 |
| XHE-II-011                    | 9   | 60  | 39  | 3233 | 90   | 1000 |
| WY-B-27-2                     | 9.19| 111 | 72  | 2000 | 449  | 2000 |
| QH-II-063                     | 9.4 | 9.3 | 31  | ND   | 7.7  | 3000 |
| JC184 C13H9BrN2OS              | 9.606| 10.5| ND  | ND   | 6.709| ND   |
| ZG-208                        | 9.7 | 11.2| 10.9| ND   | 0.38 | 4.6  |
| PY-1-31                       | 10  | 45  | 19  | ND   | 6    | 1000 |
| WY-B-23-1                     | 10  | 33  | 43  | 1000 | 189  | 2000 |
| RY-098                        | 10.1| 22.2| 16.5| ND   | 1.68 | 100  |
| Hz148 C18H15N3                 | 10.98| 5000| ND  | ND   | 256  | 5000 |
| SVO-8-20                      | 11  | 40  | 28  | 19   | 8.6  | 138  |
| XHE-II-073B (S ENRICHED)      | 11  | 17  | 12  | 33   | 2.1  | 269  |
| SH-I-085                      | 11.08| 4.866| 13.75| ND   | 0.24 | ND   |
| PWZ-096                       | 11.1| 36  | 16.9| ND   | 1.07 | 51.5 |
| ZG-168                        | 11.2| 10.7| 9.2 | ND   | 0.47 | 9.4  |
| CM-A77                        | 11.51| 51.9| 105.16| 1000 | 42.62| 1000 |
| WY-B-20                       | 12  | 39  | 47  | 2000 | 122  | 3000 |
| ABECARNIL                     | 12.4| 15.3| 7.5 | ND   | 6    | 1000 |
Table 5: Continued.

| Cook code\(^a\) | \(\alpha_1\) | \(\alpha_2\) | \(\alpha_3\) | \(\alpha_4\) | \(\alpha_5\) | \(\alpha_6\) |
|-----------------|----------|----------|----------|----------|----------|----------|
| SH-I-89S        | 12.78    | 8.562    | 8.145    | ND       | 3.23     | ND       |
| ZG-213          | 12.8     | 49.8     | 30.2     | ND       | 3.5      | 22.5     |
| EDC-I-071       | 12.9     | 83.1     | ND       | ND       | 314      | 5000     |
| MMB-III-14      | 13       | 13       | 6.9      | 333      | 1.1      | 333      |
| DM-173          | 13.1     | ND       | 38.1     | ND       | 0.78     | 118      |
| XLI-348         | 13.56    | 11.17    | 1.578    | ND       | 82.05    | ND       |
| EDC-I-093       | 13.6     | 423      | ND       | ND       | 2912     | 5000     |
| diazepam        | 14       | 20       | 15       | ND       | 11       | ND       |
| XLI223 C22H20BrN3O2 | 14     | 8.7      | 18       | 1000     | 10       | 2000     |
| WYSC2 C15H11F3N2O2 | 14.14  | 113      | 170      | ND       | 518      | 61.2     |
| SH-I-030        | 14.42    | 11.04    | 19.09    | ND       | 1.89     | ND       |
| CM-A100         | 14.49    | 44.91    | 123.8    | 1000     | 65.31    | 1000     |
| RY-033          | 14.8     | 56       | 25.3     | ND       | 1.72     | 22.9     |
| HJ-I-037        | 15.07    | 8.127    | 28.29    | ND       | 0.818    | ND       |
| YT-6            | 15.31    | 87.8     | 60.49    | ND       | 1.039    | ND       |
| EDC-II-044      | 15.4     | ND       | 293      | ND       | 323      | 1000     |
| CM-A58          | 16       | 120      | 184      | ND       | 1000     | 1012     |
| QH-II-067a      | 16       | 31       | 32       | ND       | 199      | 3000     |
| CD-214          | 16.4     | 48.2     | 42.5     | ND       | 9.8      | 168      |
| JYI-06 (C23H23N3O4) | 16.5    | 5.48     | 5000     | ND       | 12.6     | 5000     |
| CM-A50 (S)      | 17       | 59       | 88       | ND       | 144      | 1000     |
| RY-061          | 17       | 13       | 6.7      | ND       | 0.3      | 31       |
| ZG-224          | 17.1     | 33.7     | 50       | ND       | 2.5      | 30.7     |
| ZG-63A          | 17.3     | 21.6     | 29.1     | ND       | 0.65     | 4        |
| DM-II-30 (C20H13N3O2BrF3) | 17.6   | 13.4     | 28.51    | ND       | 7.8      | 5000     |
| CM-A64          | 18       | 60       | 116      | ND       | 216      | 1000     |
| RY-071          | 19       | 56       | 91       | ND       | 7.2      | 266      |
| WZ-113          | 19.2     | 13.2     | 13.4     | ND       | 11.5     | 300      |
| YT-III-23       | 19.83    | 23.65    | 19.87    | ND       | 1.105    | ND       |
| CM-E09b         | 20       | 22       | 19       | 55       | 0.45     | 69       |
| MMB-II-90       | 20       | 24       | 5.7      | 9        | 0.25     | 36       |

\(^a\) Affinity of compounds at GABA\(_A\)/BzR recombinant subtypes was measured by competition for \(^3\)H\]flunitrazepam or \(^3\)H\]Ro15-4513 binding to HEK cell membranes expressing human receptors of compositions \(\alpha_1\)\(\beta_3\)\(\gamma_2\), \(\alpha_2\)\(\beta_3\)\(\gamma_2\), \(\alpha_3\)\(\beta_3\)\(\gamma_2\), \(\alpha_4\)\(\beta_3\)\(\gamma_2\), \(\alpha_5\)\(\beta_3\)\(\gamma_2\), and \(\alpha_6\)\(\beta_3\)\(\gamma_2\) [139]. Data represent the average of at least three determinations with a SEM of \(\pm\)5%. The structures of these ligands are in the Ph.D. thesis of Clayton (2011) [22] and Supporting Information.

13. The \(\alpha_2\)\(\beta_3\)\(\gamma_2\) Receptor Subtype
See Table 6 and Figures 36 and 37.

14. The \(\alpha_3\)\(\beta_3\)\(\gamma_2\) Receptor Subtype
See Table 7 and Figures 38 and 39.

15. The \(\alpha_4\)\(\beta_3\)\(\gamma_2\) Receptor Subtype
See Table 8 and Figures 40 and 41.

16. The \(\alpha_5\)\(\beta_3\)\(\gamma_2\) Receptor Subtype
The multiple volume contours displayed in Figures 34–47 were created using the mvolume function (multiple volume contour function) in Sybyl and compounds with binding affinity at the receptor less than or equal to 20 nM. To create the overlays, first, the display (dsp) and contour (cnt) files were created for the \(\alpha_5\)\(\beta_3\)\(\gamma_2\) receptor subtype and the \(\alpha_1\)\(\beta_3\)\(\gamma_2\) receptor subtype by overlaying the compounds for each of these receptors (see Table 9 and Figures 42–45). Using the mvolume function, a logical expression was entered to create the surfaces making up the union as well as the included volume for each receptor subtype itself. It is clear from the
Table 6: Ligands with potent affinity for \( \alpha_2 \); ligands bound with \( K_i \) values <20 nM at this subtype. The structures of these ligands are in the Ph.D. thesis of Clayton (2011) [22].

| Cook code | \( \alpha_1 \) | \( \alpha_2 \) | \( \alpha_3 \) | \( \alpha_4 \) | \( \alpha_5 \) | \( \alpha_6 \) |
|-----------|----------------|----------------|----------------|---------------|--------------|---------------|
| QH-II-092 | 0.07           | 0.03           | 0.04           | ND            | 0.17         | ND            |
| SH-TSC-2 (BCCT) | 0.03   | 0.0419         | 0.035          | ND            | 69.32        | ND            |
| QH-II-085 | 0.08           | 0.06           | 0.02           | ND            | 0.08         | ND            |
| XLI-286  | 0.051          | 0.064          | 0.118          | ND            | 0.684        | ND            |
| JYI-57   | 0.076          | 0.076          | 0.131          | ND            | 0.036        | ND            |
| QH-II-090 (CGS-8216) | 0.05  | 0.08           | 0.12           | ND            | 0.25         | 17            |
| QH-II-077 | 0.06           | 0.08           | 0.05           | ND            | 0.12         | 4             |
| PWZ-007A | 0.11           | 0.1            | 0.09           | ND            | 0.2          | 10            |
| JYI-42   | 0.257          | 0.146          | 0.278          | ND            | 0.256        | ND            |
| PWZ-0071 | 0.23           | 0.17           | 0.12           | ND            | 0.44         | 17.31         |
| SH-I-048A | 0.774          | 0.1723         | 0.383          | ND            | 0.11         | ND            |
| XHE-II-024 | 0.09           | 0.18           | 0.32           | 14            | 0.24         | 11            |
| QH-II-075 | 0.18           | 0.21           | 0.25           | ND            | 1.3          | 40            |
| XLI-41DMHANX3 | 3.3            | 0.58           | 1.9            | ND            | 4.4          | 5000          |
| alprazolam | 0.8          | 0.59           | 1.43           | ND            | 1.54         | 10000         |
| YT-5     | 0.421          | 0.6034         | 36.06          | ND            | 1.695        | ND            |
| BRETRENZIL | 0.35           | 0.64           | 0.2            | ND            | 0.5          | 12.7          |
| XLI268 C17H13BrN4 | 2.8145     | 0.6862         | ND             | ND            | 0.6243       | ND            |
| WY-B-15  | 0.92           | 0.83           | 0.58           | 2080          | 4.42         | 646           |
| YT-II    | 6.932          | 0.8712         | 3.518          | ND            | 5.119        |               |
| RoI5-1788 | 0.8           | 0.9            | 1.05           | ND            | 0.6          | 148           |
| XLI351 C21H21CIN2OSi | 1.507   | 0.967          | ND             | ND            | 1.985        | ND            |
| WY-TSC-4 (WYS8) | 0.007   | 0.99           | 1.63           | ND            | 51.04        | ND            |
| XLI352 C18H13CIN2O | 1.56          | 0.991          | ND             | ND            | 1.957        | ND            |
| DM-II-90 (C17H12N4BrCl) | 0.505 | 1             | 0.63           | ND            | 0.37         | 5000          |
| JYI-64 (C17H12N4FBr) | 0.305       | 1.111          | 0.62           | ND            | 0.87         | 5000          |
| XLI350 C17H11CIN2O | 1.224         | 1.188          | ND             | ND            | 2.9          | ND            |
| SPH-121  | 0.14           | 0.19           | 1.72           | ND            | 4            | 479           |
| MLT-I-70 | 1.1            | 1.2            | 1.1            | ND            | 40.3         | 10000         |
| OMB-18   | 3.9            | 1.2            | 3.4            | 1733          | 0.8          | 5             |
| 6-PBC    | 0.49           | 1.21           | 2.2            | ND            | 2.39         | 1343          |
| YT-III-271 | 32.54          | 1.26           | 2.35           | ND            | 103          | ND            |
| PWZ-009A1 | 1.34          | 1.31           | 1.26           | ND            | 0.84         | 2.03          |
| DM-II-72 (C15H10N20BrCl) | 5000     | 1.37           | ND             | ND            | 2.02         | 5000          |
| JYI-60 (C17H11N2O2F) | 3.73         | 1.635          | 4.3            | ND            | 1.7          | 5000          |
| XLI-26C | 3.442          | 1.673          | 44.08          | ND            | 1.121        | ND            |
| QH-II-082 | 1.7           | 1.8            | 1.6            | ND            | 6.1          | 100           |
| TC-YT-II-76 | 101.1        | 1.897          | 5.816          | ND            | 11.99        | ND            |
| MMB-III-016 | 3          | 1.97           | 2              | 1074          | 0.26         | 211           |
| MMB-III-16 | 3            | 1.97           | 2              | 1074          | 0.26         | 211           |
| XHE-III-06a | 1           | 2              | 1              | 5             | 1.8          | 37            |
| XHE-III-04 | 1.2          | 2              | 1.1            | 219           | 0.4          | 500           |
| WYS15 C22H20N2O2 | 3.63         | 2.02           | 44.3           | ND            | 76.5         | 5000          |
| FG8205   | 0.4            | 2.08           | 1.16           | ND            | 1.54         | 227           |
| JYI-70 (C19H13N4F) | 6.3           | 2.1            | ND             | ND            | 0.56         | 5000          |
| JYI-47   | 2.759          | 2.282          | 0.511          | ND            | 0.427        | ND            |
| JYI-49 (C20H12N3O2F4Br) | 1.87       | 2.38           | ND             | ND            | 6.7          | 3390          |
| FLUNITRAZEPAM | 2.2          | 2.5            | 4.5            | ND            | 2.1          | 2000          |
| JYI-59 (C22H13N3O2F4) | 1.08         | 2.6            | 11.82          | ND            | 11.5         | 5000          |
Table 6: Continued.

| Cook code | \(\alpha_1\) | \(\alpha_2\) | \(\alpha_3\) | \(\alpha_4\) | \(\alpha_5\) | \(\alpha_6\) |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Ro15-4513 | 3.3       | 2.6       | 2.5       | ND        | 0.26      | 3.8       |
| SPH-165   | 0.63      | 2.79      | 4.85      | ND        | 10.4      | 1150      |
| YT-II-76  | 95.34     | 2.797     | 0.056     | ND        | 0.04      | ND        |
| TG-II-82  | 1.6       | 2.9       | 2.8       | ND        | 1         | 1000      |
| QH-II-080b| 3         | 3.7       | 4.7       | ND        | 24        | 1000      |
| TG-4-29   | 2.8       | 3.9       | 2.7       | 2.1       | 0.18      | 3.9       |
| PS-1-3-B  | ND        | 4.198     | 3.928     | ND        | ND        | ND        |
| ZK 93423  | 4.1       | 4.2       | 6         | ND        | 4.5       | 1000      |
| XHE-II-006a | 4.7     | 4.4       | 20        | 1876      | 89        | 3531      |
| WY-B-99-1 | 4.4       | 4.5       | 5.58      | 2000      | 47        | 2000      |
| CM-I-A87  | 1.62      | 4.54      | 14.73     | 1000      | 4.61      | 1000      |
| OMB-19    | 22        | 4.6       | 20        | 3333      | 3.5       | 40        |
| SH-I-085  | 11.08     | 4.866     | 13.75     | ND        | 0.24      | ND        |
| BCCE      | 1.2       | 4.9       | 5.7       | ND        | 26.8      | 2700      |
| JYI-32(C20H15N3O2BrF) | 3.07 | 4.96      | ND        | ND        | 2.92      | 52.24     |
| XHE-I-038 | 7.3       | 5         | 34        | ND        | 132       | 1000      |
| SVO-8-30  | 1.1       | 5.3       | 5.3       | 2.8       | 0.6       | 15        |
| SPH-38    | 2         | 5.4       | 10.8      | ND        | 18.5      | 3000      |
| WYSC1C16H16N2O2 | 1.094 | 5.44      | 12.3      | ND        | 69.8      | 21.2      |
| JYI-06(C23H23N3O4) | 16.5 | 5.48      | 5000      | ND        | 12.6      | 5000      |
| XHE-III-49| 1.3       | 5.5       | 4.2       | 38.7      | 11.3      | 85.1      |
| YT-III-25 | 2.531     | 5.786     | 5.691     | ND        | 0.095     | ND        |
| SH-I-04   | 7.3       | 6.136     | 5.1       | ND        | 7.664     | ND        |
| XHE-I-093 | 2         | 7.1       | 8.9       | 1107      | 20        | 1162      |
| RR-008    | 3.75      | 7.2       | 4.14      | ND        | 1.11      | 44.3      |
| DMH-D-053 (C43H30N6O4) | 236 | 7.4       | 272       | 5000      | 194.2     | 5000      |
| WY-B-09-1 | 3.99      | 8         | 32        | 1000      | 461       | 2000      |
| HJ-I-037  | 15.07     | 8.127     | 28.29     | ND        | 0.818     | ND        |
| DMCM      | 5.69      | 8.29      | 4         | ND        | 1.04      | 134       |
| SH-I-89S  | 12.78     | 8.562     | 8.145     | ND        | 3.23      | ND        |
| XL1223C22H20BrN3O2 | 14     | 8.7       | 18        | 1000      | 10        | 2000      |
| CM-A82a   | 2.78      | 8.93      | 24.51     | 1000      | 7.49      | 1000      |
| QH-II-063 | 9.4       | 9.3       | 31        | ND        | 7.7       | 3000      |
| XHE-II-017| 3.3       | 10        | 7         | 258       | 17        | 294       |
| TG-4-29   | 8.3       | 10.2      | 6.9       | ND        | 0.4       | 7.61      |
| MLT-II-16 | 5.05      | 10.41     | 18.4      | ND        | 260       | 10000     |
| JCI84C13H9BrN2OS | 9.606 | 10.5      | ND        | ND        | 6.709     | ND        |
| ZG-168    | 11.2      | 10.7      | 9.2       | ND        | 0.47      | 9.4       |
| XHE-II-073A (R ENRICHED) | 5.9 | 11        | 10        | 15        | 1.18      | 140       |
| XLI-8TC   | 21.52     | 11.01     | 2.155     | ND        | 4.059     | ND        |
| SH-I-030  | 14.42     | 11.04     | 19.09     | ND        | 1.89      | ND        |
| XLI-348   | 13.56     | 11.17     | 1.578     | ND        | 82.05     | ND        |
| ZG-208    | 9.7       | 11.2      | 10.9      | ND        | 0.38      | 4.6       |
| YT-TC-3   | 141.4     | 11.43     | 118.1     | ND        | 29.22     | ND        |
| YC-T-5    | 2.2       | 11.46     | 16.3      | ND        | 200       | 10000     |
| MLT-II-18 | 3.4       | 11.7      | 11        | ND        | 225       | 10000     |
| XHE-II-053-ACID | 50.35 | 11.8      | 44        | ND        | 5.9       | 5000      |
| SHU-1-19  | 4         | 12        | 7         | 48        | 14        | 84        |
| RY-067    | 21        | 12        | 10        | ND        | 0.37      | 42        |
Table 6: Continued.

| Cook code  | a1    | a2    | a3    | a4    | a5    | a6    |
|-----------|-------|-------|-------|-------|-------|-------|
| DM-III-01(C18H12N3O2Br) | 5000  | 12    | ND    | ND    | 4.73  | 5000  |
| MLT-II-18 | 3.9   | 12.2  | 24.4  | ND    | 210   | 10000 |
| SH-053-2’F | 21.99 | 12.34 | 34.9  | ND    | 0.671 | ND    |
| WYS13 C20H18N3O2 | 2.442 | 13    | 27.5  | ND    | 163   | 5000  |
| PWZ-085   | 4.86  | 13    | 8.5   | ND    | 0.55  | 40    |
| MMB-III-14 | 13    | 13    | 6.9   | 333   | 1.1   | 333   |
| RY-061    | 17    | 13    | 6.7   | ND    | 0.3   | 31    |
| WZ-I13    | 19.2  | 13.2  | 13.4  | ND    | 11.5  | 300   |
| YT-II-83  | 32.74 | 13.22 | 24.1  | ND    | 3.548 | ND    |
| DM-II-30(C20H13N3O2BrF3) | 17.6  | 13.4  | 28.51 | ND    | 7.8   | 5000  |
| LJD-III-15E | 1.93  | 14    | 19    | ND    | 70.8  | 1000  |
| YT-III-272 | 295.9 | 14.98 | 10.77 | ND    | 103.3 | ND    |
| BccT      | 0.72  | 15    | 18.9  | ND    | 110.8 | 5000  |
| XHE-II-006b | 3.7   | 15    | 12    | 1897  | 144   | 1000  |
| ABECARNIL | 12.4  | 15.3  | 7.5   | ND    | 6     | 1000  |
| MLT-II-34 | 7.04  | 15.95 | 22.3  | ND    | 158   | 1000  |
| MSA-IV-35 | 2.1   | 16    | 21    | ND    | 995   | 3000  |
| JYI-04(C21H23N3O3) | 28.3  | 16    | ND    | 0.51  | 1.57  |       |
| PS-I-35 C23H22N5OBr | ND    | 16.03 | 24.41 | ND    | ND    | ND    |
| ZG69A     | 6.8   | 16.3  | 9.2   | ND    | 0.85  | 54.6  |
| ZG-69A (Ro15-1310) | 6.8   | 16.3  | 9.2   | ND    | 0.85  | 54.6  |
| YT-III-42 | 382.9 | 16.83 | 44.04 | ND    | 9.77  | ND    |
| XHE-I-065 | 7.2   | 17    | 18    | 500   | 57    | 500   |
| XHE-II-073B (S-ENRICHED) | 11    | 17    | 12    | 33    | 2.1   | 269   |
| Tjh-IV-51 | 2.39  | 17.4  | 14.5  | ND    | 316   | 10000 |
| SH-1-047  | 1710  | 17.52 | 1222  | ND    | 1599  | ND    |
| XlI34 C20H19ClN2OSi | 6.375 | 17.71 | ND    | 150.5 | ND    |       |
| XHE-II-002 | 8.3   | 18    | 13    | 3.9   | 1.5   | 11    |
| YT-III-38 | 1461  | 18.21 | 14.63 | ND    | 3999  |       |
| JYI-72(C22H21N4SiF) | 48.5  | 18.5  | ND    | 11.5  | 5000  |       |
| MSR-I-032 | 6.2   | 18.7  | 4     | ND    | 3.3   | 74.9  |
| JC208 C15H10N2O2Si | 22.42 | 18.89 | ND    | 5.039 | ND    |       |
| Diazepam  | 14    | 20    | 15    | ND    | 11    | ND    |

*a* Affinity of compounds at GABA<sub>A</sub>/BzR recombinant subtypes was measured by competition for [³H]flunitrazepam or [³H] Ro15-4513 binding to HEK cell membranes expressing human receptors of compositions a₁β₂γ₂, a₁β₂γ₂, a₁β₂γ₂, a₁β₂γ₂, a₁β₂γ₂, and a₁β₂γ₂ [22, 139]. Data represent the average of at least three determinations with a SEM of ±5%. ND: not determined.

17. The a₆β₃γ₂ Receptor Subtype

See Table 10 and Figures 46 and 47.

18. Updates to the Previous Model

In addition to the newly discovered L₄ pocket, the updated library of binding affinity led to two specific updates in the previous model (Figure 48).

19. QSAR

A nontraditional quantitative structure activity relationship (QSAR) approach was executed to observe steric and electrostatic preferences for each receptor subtype. A subset of the compounds used in each subtype pharmacophore/receptor model were chosen with a good cross section of scaffold variety. The compounds used in the COMFA maps are the imidazobenzodiazepines published previously [110, 137] and additionally alternative scaffolds which bound with <20 nM at the respective subtype [22].

The interest here was in creation of steric and electrostatic maps of the comparative molecular field analyses (COMFA) created from molecular spreadsheets. A variety of compounds selective for each subtype were selected and placed into a dataset used to build the CoMFA models. Activities (Kᵢ...
Table 7: Ligands with potent affinity for $\alpha_3$; ligands bound with $K_i$ values $< 20$ nM at this subtype. The structures of these ligands are in the Ph.D. thesis of Clayton (2011) [22].

| Cook code | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\alpha_4$ | $\alpha_5$ | $\alpha_6$ |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| QH-II-085 | 0.08      | 0.06      | 0.02      | ND        | 0.08      | ND        |
| SH-TSC-2 (BCCT) | 0.03 | 0.0419 | 0.035 | ND | 69.32 | ND |
| QH-II-092 | 0.07      | 0.03      | 0.04      | ND        | 0.17      | ND        |
| QH-II-077 | 0.06      | 0.08      | 0.05      | ND        | 0.12      | 4         |
| YT-II-76  | 95.34     | 2.797     | 0.056     | ND        | 0.04      | ND        |
| PWZ-007A  | 0.11      | 0.1       | 0.09      | ND        | 0.2       | 10        |
| XLI-286   | 0.051     | 0.064     | 0.118     | ND        | 0.684     | ND        |
| QH-II-090 (CGS-8216) | 0.05 | 0.08 | 0.12 | ND | 0.25 | 17 |
| PWZ-0071  | 0.23      | 0.17      | 0.12      | ND        | 0.44      | 17.31     |
| JYI-57    | 0.076     | 0.076     | 0.131     | ND        | 0.036     | ND        |
| BRETazenil | 0.35 | 0.64 | 0.2 | ND | 0.5 | 12.7 |
| PZII-028  | 0.2       | ND        | 0.2       | ND        | 0.32      | 1.9       |
| QH-II-075 | 0.18      | 0.21      | 0.25      | ND        | 1.3       | 40        |
| JYI-42    | 0.257     | 0.146     | 0.278     | ND        | 0.256     | ND        |
| XHE-II-024 | 0.09 | 0.18 | 0.32 | 14 | 0.24 | 11 |
| SH-I-048A | 0.774     | 0.1723    | 0.383     | ND        | 0.11      | ND        |
| JYI-55    | 41.39     | ND        | 0.504     | ND        | 24.75     | ND        |
| JYI-47    | 2.759     | 2.282     | 0.511     | ND        | 0.427     | ND        |
| DM-239    | 1.5       | ND        | 0.53      | ND        | 0.14      | 6.89      |
| WY-B-15   | 0.92      | 0.83      | 0.58      | 2080      | 4.42      | 646       |
| JYI-64 (CI7H12N4FBr) | 0.305 | 1.111 | 0.62 | ND | 0.87 | 5000 |
| DM-II-90 (CI7H12N4BrCl) | 0.505 | 1 | 0.63 | ND | 0.37 | 5000 |
| QH-146    | 0.49      | ND        | 0.76      | ND        | 7.7       | 10000     |
| PZII-029  | 0.34      | ND        | 0.79      | ND        | 0.52      | 10        |
| WYS19 C26H32N2O4Si | ND | ND | 0.89 | ND | ND | ND |
| XHE-III-06a | 1 | 2 | 1 | 5 | 1.8 | 37 |
| Ro15-1788 | 0.8       | 0.9       | 1.05      | ND        | 0.6       | 148       |
| MLT-I-70  | 1.1       | 1.2       | 1.1       | ND        | 40.3      | 1000      |
| XHE-III-04 | 1.2 | 2 | 1.1 | 219 | 0.4 | 500 |
| FG8205    | 0.4       | 2.08      | 1.16      | ND        | 1.54      | 227       |
| PWZ-009A1 | 1.34      | 1.31      | 1.26      | ND        | 0.84      | 2.03      |
| alprazolam | 0.8 | 0.59 | 1.43 | ND | 1.54 | 10000 |
| XLI-348   | 13.56     | 11.17     | 1.578     | ND        | 82.05     | ND        |
| QH-II-082 | 1.7       | 1.8       | 1.6       | ND        | 6.1       | 100       |
| WY-TSC-4 (WYS8) | 0.007 | 0.99 | 1.63 | ND | 51.04 | ND |
| SPH-121   | 0.14      | 1.19      | 1.72      | ND        | 4         | 479       |
| XLI-JY-DMHANX3 | 3.3 | 0.58 | 1.9 | ND | 4.4 | 5000 |
| MMB-III-016 | 3 | 1.97 | 2 | 1074 | 0.26 | 211 |
| MMB-III-16 | 3 | 1.97 | 2 | 1074 | 0.26 | 211 |
| XLI-8TC   | 21.52     | 11.01     | 2.155     | ND        | 4.059     | ND        |
| 6-PBC     | 0.49      | 1.21      | 2.2       | ND        | 2.39      | 1343      |
| YT-III-271 | 32.54 | 1.26 | 2.35 | ND | 103 | ND |
| Ro15-4513 | 3.3       | 2.6       | 2.5       | ND        | 0.26      | 3.8       |
| TG-4-29   | 2.8       | 3.9       | 2.7       | 2.1       | 0.18      | 3.9       |
| TG-II-82  | 1.6       | 2.9       | 2.8       | ND        | 1         | 1000      |
| OMB-18    | 3.9       | 1.2       | 3.4       | 1733      | 0.8       | 5         |
| YT-II     | 6.932     | 0.8712    | 3.518     | ND        | 5.119     | ND        |
## Table 7: Continued.

| Cook code | α₁ | α₂ | α₃ | α₄ | α₅ | α₆ |
|-----------|----|----|----|----|----|----|
| PS-1-34B C20H17N4BrO | ND | 4.198 | 3.928 | ND | ND | ND |
| DMCM | 5.69 | 8.29 | 4 | ND | 1.04 | 134 |
| MSR-I-032 | 6.2 | 18.7 | 4 | ND | 3.3 | 74.9 |
| RY-008 | 3.75 | 7.2 | 4.14 | ND | 1.11 | 44.3 |
| XHE-III-49 | 1.3 | 5.5 | 4.2 | 38.7 | 11.3 | 85.1 |
| JYI-60 (C17H11N2OF) | 3.73 | 1.635 | 4.3 | ND | 1.7 | 5000 |
| FLUNITRAZEPAM | 2.2 | 2.5 | 4.5 | ND | 2.1 | 2000 |
| XLI-317 | 60.24 | 24.05 | 4.562 | ND | 0.295 | ND |
| QH-II-080b | 3 | 3.7 | 4.7 | ND | 24 | 1000 |
| SPH-165 | 0.63 | 2.79 | 4.85 | ND | 10.4 | 1150 |
| SH-I-04 | 7.3 | 6.136 | 5.1 | ND | 7.664 | ND |
| SVO-8-30 | 1.1 | 5.3 | 5.3 | 2.8 | 0.6 | 15 |
| WY-B-99-1 | 4.4 | 4.5 | 5.58 | 2000 | 47 | 2000 |
| YT-III-25 | 2.53 | 5.786 | 5.691 | ND | 0.095 | ND |
| BCCE | 1.2 | 4.9 | 5.7 | ND | 26.8 | 2700 |
| MMB-II-90 | 20 | 24 | 5.7 | 9 | 0.23 | 36 |
| TC-YT-II-76 | 101.1 | 1.897 | 5.816 | ND | 11.99 | ND |
| ZK 93423 | 4.1 | 4.2 | 6 | ND | 4.5 | 1000 |
| RY-061 | 17 | 13 | 6.7 | ND | 0.3 | 31 |
| JYI-54 (C24H15N3O3F4) | 2.89 | 172 | 6.7 | ND | 57 | 1890 |
| TG-4-29 | 8.3 | 10.2 | 6.9 | ND | 0.4 | 7.61 |
| MMB-III-14 | 13 | 13 | 6.9 | 333 | 1.1 | 333 |
| XHE-II-017 | 3.3 | 10 | 7 | 258 | 17 | 294 |
| SHU-1-19 | 4 | 12 | 7 | 48 | 14 | 84 |
| XHE-III-13 | 7.3 | ND | 7.1 | 880 | 1.6 | 311 |
| DM-215 | 6.74 | ND | 7.42 | ND | 0.293 | 8.28 |
| ABECARNIL | 12.4 | 15.3 | 7.5 | ND | 6 | 1000 |
| SVO-8-14 | 8 | 25 | 8 | 6.9 | 0.9 | 14 |
| XHE-III-24 | 0.25 | ND | 8 | 222 | 10 | 328 |
| SH-1-985 | 12.78 | 8.562 | 8.145 | ND | 3.23 | ND |
| PWZ-085 | 4.86 | 13 | 8.5 | ND | 0.55 | 40 |
| XHE-I-093 | 2 | 7.1 | 8.9 | 1107 | 20 | 1162 |
| ZG-168 | 11.2 | 10.7 | 9.2 | ND | 0.47 | 9.4 |
| ZG69A | 6.8 | 16.3 | 9.2 | ND | 0.85 | 54.6 |
| ZG-69a (Ro15-1310) | 6.8 | 16.3 | 9.2 | ND | 0.85 | 54.6 |
| ZG-234 | 7.25 | 22.14 | 9.84 | ND | 0.3 | 5.25 |
| XHE-II-073A (R ENRICHED) | 5.9 | 11 | 10 | 15 | 1.18 | 140 |
| RY-067 | 21 | 12 | 10 | ND | 0.37 | 42 |
| XHE-III-14 | 2.6 | ND | 10 | 13 | 2 | 7 |
| YT-III-272 | 295.9 | 14.98 | 10.77 | ND | 103.3 | ND |
| SPH-38 | 2 | 5.4 | 10.8 | ND | 18.5 | 3000 |
| ZG-208 | 9.7 | 11.2 | 10.9 | ND | 0.38 | 4.6 |
| MLT-II-18 | 3.4 | 11.7 | 11 | ND | 225 | 10000 |
| DM-II-33 (C20H13N3O2BrCl3) | 88.6 | 85 | 11.6 | ND | 26.2 | 5000 |
| JYI-59 (C22H13N3O2F4) | 1.08 | 2.6 | 11.82 | ND | 11.5 | 5000 |
| XHE-II-006b | 3.7 | 15 | 12 | 1897 | 144 | 1000 |
| XHE-II-073B (S-ENRICHED) | 11 | 17 | 12 | 33 | 2.1 | 269 |
| CM-B44 (SS) | 32 | 43 | 12 | 379 | 4.3 | 485 |
| Cook code | α1   | α2   | α3   | α4   | α5   | α6   |
|-----------|------|------|------|------|------|------|
| WYSC1C16H16N2O2 | 1.094 | 5.44 | 12.3 | ND   | 69.8 | 21.2 |
| JYI-48 | 75.59 | 90.68 | 12.78 | ND   | 31.28 | ND   |
| XHE-II-002 | 8.3   | 18   | 13   | 3.9  | 1.5  | 11   |
| RY-076 | 26    | 27   | 13   | ND   | 0.7  | 22   |
| WZ-113 | 19.2  | 13.2 | 13.4 | ND   | 11.5 | 300  |
| SH-I-085 | 11.08 | 4.866 | 13.75 | ND   | 0.24 | ND   |
| CM-E10 | 23    | 26   | 14   | 215  | 0.51 | 96   |
| TJH-IV-51 | 2.39 | 17.4 | 14.5 | ND   | 316  | 10000 |
| YTS-13-38 | 1461  | 18.21 | 14.63 | ND   | 3999 | ND   |
| CM-A87 | 1.62  | 4.54 | 14.73 | 1000 | 4.61 | 1000 |
| diazepam | 14    | 20   | 15   | ND   | 11   | ND   |
| RY-053 | 49    | 29   | 15   | ND   | 1    | 46   |
| YCT-5 | 2.2   | 11.46 | 16.3 | ND   | 200  | 10000 |
| RY-098 | 10.1  | 22.2 | 16.5 | ND   | 1.68 | 100  |
| PWZ-096 | 11.1  | 36   | 16.9 | ND   | 1.07 | 51.5 |
| XLI223C22H20BrN3O2 | 14    | 8.7  | 18   | 1000 | 10   | 2000 |
| XHE-I-065 | 7.2  | 17   | 18   | 500  | 57   | 500  |
| SH-I-02B | 29.82 | 1315 | 18   | ND   | 74.05 | ND   |
| MLI-II-16 | 5.05 | 10.41 | 18.4 | ND   | 260  | 10000 |
| RY-024 C19H19N3O3 | 26.9  | 26.3 | 18.7 | ND   | 0.4  | 5.1  |
| BCCt | 0.72  | 15   | 18.9 | ND   | 110.8 | 5000 |
| LJD-III-15E | 1.93 | 14   | 19   | ND   | 70.8 | 1000 |
| CM-E09b | 20    | 22   | 19   | 55   | 0.45 | 69   |
| RY-I-31 | 10    | 45   | 19   | ND   | 6    | 1000 |
| SH-I-030 | 14.42 | 11.04 | 19.09 | ND   | 1.89 | ND   |
| YTS-11-23 | 19.83 | 23.65 | 19.87 | ND   | 1.105 | ND |
| XHE-II-006a | 4.7  | 4.4  | 20   | 1876 | 89   | 3531 |
| OMB-19 | 22    | 4.6  | 20   | 3333 | 3.5  | 40   |
| XHE-III-06b | 32    | 33   | 20   | 299  | 28.6 | 740  |

\[^a\]Affinity of compounds at GABA\(_{\alpha_i}\)BzR recombinant subtypes was measured by competition for \[^3\text{H}]\text{flunitrazepam} or \[^3\text{H}]\text{Ro15-4513} binding to HEK cell membranes expressing human receptors of compositions \(\alpha_1\beta_3\gamma_2, \alpha_2\beta_3\gamma_2, \alpha_3\beta_3\gamma_2, \alpha_4\beta_3\gamma_2, \alpha_5\beta_3\gamma_2, \alpha_6\beta_3\gamma_2\) [22, 139]. Data represent the average of at least three determinations with a SEM of \(\pm 5\%\). ND: not determined.

Values were converted to logarithmic units for this study. A CoMFA descriptor set was created based on the \(-\log(K_i)\) of over 70 structures. The goal was to derive an alternative three-dimensional shape of the receptor using biological activity of the most selective compounds. Structures were determined by crystal structure where available or by calculation. Charges were provided based on the Gasteiger-Huckel model. Conformations were kept consistent based on previous studies of low energy conformations [110]. It should be noted that this was not a traditional QSAR study as nonselective compounds were excluded. Therefore, \(K_i\) values did not cross 3 log units. This was acceptable since the goal was not to create a predictive QSAR predictive algorithm, rather a map of the receptor based on steric and electrostatics. Hydrogen acceptor radii were set to 3.0 and the hydrogen donor radii were set to 2.6 based on recommendations from Certara (Tripos). Analyses were executed using PLS (partial least squares). The details of modeling will be further discussed in the SI.

For each of the following QSAR models (Figures 49–64), green areas represent desirable sterical bulk and yellow represents undesirable sterical bulk. Positive electrostatic contributions are represented by blue and negative electrostatic contributions are represented by red.

**20. The \(\alpha_1\beta_3\gamma_2\) Receptor Subtype**

See Figures 49–52.

**21. The \(\alpha_2\beta_3\gamma_2\) Receptor Subtype**

See Figures 53–56.
Table 8: Ligands with potent affinity for \( \alpha_4 \); ligands bound with \( K_i \) values <20 nM at this subtype. The structures of these ligands are in the Ph.D. thesis of Clayton (2011) [22].

| Cook code | \( \alpha_1 \) | \( \alpha_2 \) | \( \alpha_3 \) | \( \alpha_4 \) | \( \alpha_5 \) | \( \alpha_6 \) |
|-----------|----------------|----------------|----------------|----------------|----------------|----------------|
| CM-D45 C19H21N3O4 | 90.5 | 65.5 | 30.3 | 0.15 | 1.65 | 0.23 |
| CM-D44 | 34.3 | 56.3 | 20.7 | 0.33 | 0.57 | 0.92 |
| XHE-III-74 | 77 | 105 | 38 | 0.42 | 2.2 | 5.8 |
| TG-4-29 | 2.8 | 3.9 | 2.7 | 2.1 | 0.18 | 3.9 |
| SVO-8-30 | 1.1 | 5.3 | 5.3 | 2.8 | 0.6 | 15 |
| XHE-II-002 | 8.3 | 18 | 13 | 3.9 | 1.5 | 11 |
| XHE-III-06a | 1 | 2 | 1 | 5 | 1.8 | 37 |
| RY-080 C17H15N3O3 | 28.4 | 21.4 | 25.8 | 5.3 | 0.49 | 28.8 |
| TG-4-39 | 1.6 | 34 | 24 | 5.6 | 1.4 | 23 |
| SVO-8-14 | 8 | 25 | 8 | 6.9 | 0.9 | 14 |
| RY-023 C22H27N3O3Si | 197 | 142.6 | 255 | 7.8 | 2.61 | 58.6 |
| MMB-II-90 | 20 | 24 | 5.7 | 9 | 0.25 | 36 |
| XHE-III-14 | 2.6 | 10 | 13 | 2 | 7 |
| XHE-II-024 | 0.09 | 0.18 | 0.32 | 14 | 0.24 | 11 |
| XHE-II-073A (R ENRICHED) | 5.9 | 11 | 10 | 15 | 1.18 | 121 |
| SVO-8-68 | 7 | 41 | 26 | 15 | 2.3 | 191 |
| CM-B3li (ss) | 90 | 184 | 78 | 18 | 4.9 | 121 |
| SVO-8-20 | 11 | 40 | 28 | 19 | 8.6 | 138 |

\( ^a \) Affinity of compounds at GABA\(_A\)/BzR recombinant subtypes was measured by competition for \([^{3}H]flunitrazepam\) or \([^{3}H] \)Ro15-4513 binding to HEK cell membranes expressing human receptors of compositions \( \alpha_1 \beta_3 \gamma_2, \alpha_2 \beta_3 \gamma_2, \alpha_3 \beta_3 \gamma_2, \alpha_4 \beta_3 \gamma_2, \alpha_5 \beta_3 \gamma_2, \) and \( \alpha_6 \beta_3 \gamma_2 \) [22, 139]. Data represent the average of at least three determinations with a SEM of \( \pm 5\% \). ND: not determined.

22. The \( \alpha_3 \beta_3 \gamma_2 \) Receptor Subtype

See Figures 57–60.

23. The \( \alpha_5 \beta_3 \gamma_2 \) Receptor Subtype

From the CoMFA maps several observations (Figure 65) can be made. The yellow steric regions near \( L_3 \) in the \( \alpha_5 \beta_3 \gamma_2 \) map are unique. This illustrated that, in general, benzodiazepines lacking a pendant phenyl are more suited to targeting the \( \alpha_5 \) subtype. The \( L_3 \) region of the \( \alpha_1 \) subtype is most tolerable for compounds with steric interactions in this location while the \( \alpha_3 \) subtype receptor compounds prefer no steric interaction in this location. Negative electrostatics are most preferred by the \( L_3 \) pocket of the \( \alpha_2 \) and \( \alpha_5 \) receptors. In general, the \( \alpha_1 \) subtype receptor prefers molecules without a dipole. It should be noted that none of the analogs are ionic in nature and the charges for this model were provided by the Gasteiger-Huckel model. For this reason more emphasis is placed on the steric relationships which exclude interactions in the pharmacophores. In the future a Q SAR study which includes nonbinding benzodiazepines in the data set along with activity data will permit the creation of a predictive algorithm which will be very useful in lead targeting (see Figures 61–65).

24. Conclusion

Benzodiazepines, \( \beta \)-carbolines, and other classes of compounds readily target the GABA\(_A\) receptors. The difficulty is finding subtype selective ligands, since there is no crystal structure of the Bz/GABA\(_A\)ergic site itself, just one composed of five beta-subunits which has no Bz site to date. The \( \alpha_5 \)-BzR/GABA\(_A\) subunit has recently been shown to be important in the search to treat numerous cognition-based illnesses including Alzheimer’s, schizophrenia, bipolar, and depression, as well as more recently a bronchodilator, potentially important in the treatment of asthma. As an inverse agonist, PWZ-029 was able to counteract the memory impairing effects of scopalamine, a muscarinic antagonist, in both object recognition tests and object retrieval tests in rodents, and was active in primates, as well as samaritan Alzheimer’s rats. The implications of these tests point to a use as a possible treatment for Alzheimer’s disease. The docking of PWZ-029 in the \( \alpha_5 \)y2 GABA\(_A\)-subunit details the interactions between the pharmacophore/receptor model binding site and this important negative allosteric modulator. Furthermore, \( \alpha_5 \)-BzR/GABA\(_A\) positive allosteric modulator, SH-053-2 \( ^3 \)F-R-CH\(_3\), was shown to reverse deleterious effects in the MAM-model of schizophrenia. The recent discovery of \( \alpha_5 \)-GABA\(_A\)R in airway smooth muscle by Emala et al. has also lead to the testing of SH-053-2 \( ^3 \)F-R-CH\(_3\) as a bronchodilator. This SH-053-2 \( ^3 \)F-R-CH\(_3\) was found to be effective in relaxing preconstricted airway smooth muscle, as well as attenuating calcium-ion entry through the plasma membrane. In addition, XLI-093 (an \( \alpha_5 \) receptor antagonist), a potently binding \( \alpha_5 \)-subtype selective bivalent ligand, has been shown to inhibit the \( \alpha_5 \)-cognition deficits effected by diazepam and is a very good \( \alpha_5 \) benzodiazepine receptor site antagonist. It has also been shown to reverse the effects of \( \alpha_5 \) PAMs and NAMs in both rodent and primate models. These findings led to the exploration of the \( \alpha_5 \)-binding pocket in the Milwaukee-based pharmacophore.

New features have been introduced to the unified pharmacophore/receptor model based on many substance classes that act at the diazepam sensitive and diazepam insensitive BzR binding sites of GABA\(_A\) receptors. The major new feature identified for the \( \alpha_5 \beta_3 \gamma_2 \) receptor was a new \( L_4 \) pocket which was found by using pendant 6-phenyl benzodiazepines with a R-CH\(_3\) at the prochiral center at C4. Further enhancement of potency was achieved by addition of 2'-F or 2'-N substituent in the pendant phenyl ring at C-6. While these changes have led to enhanced subtype selective ligands, the overall development guided by this pharmacophore model described here has lead to new agents with varying, fascinating pharmacological profiles, ranging from use in cognition-based diseases such as Alzheimer’s and schizophrenia, to use as a bronchodilator. This research on updating the Milwaukee-based pharmacophore/receptor model can be used in the rational design for improving the selectivity of \( \alpha_5 \) ligands. As the library of compounds increases, the data which follows can then be further evaluated and can lead to more insight to the identification of the possible roles each individual residue may have with the binding pocket.
Table 9: Ligands with potent affinity for $\alpha_5$; ligands bound with $K_i$ values <20 nM at this subtype. The structures of these ligands are in the Ph.D. thesis of Clayton (2011) [22].

| Cook codea | $a_1$ | $a_2$ | $a_3$ | $a_4$ | $a_5$ | $a_6$ |
|------------|------|------|------|------|------|------|
| JYI-57     | 0.076| 0.076| 0.131| ND   | 0.036| ND   |
| YT-II-76   | 95.34| 2.797| 0.056| ND   | 0.04 | ND   |
| QH-II-085  | 0.08 | 0.06 | 0.02 | ND   | 0.08 | ND   |
| YT-III-25  | 2.531| 5.786| 5.691| ND   | 0.095| ND   |
| SH-I-048A  | 0.774| 0.1723| 0.383| ND   | 0.11 | ND   |
| QH-II-077  | 0.06 | 0.08 | 0.05 | ND   | 0.12 | 4    |
| DM-239     | 1.5  | ND   | 0.53 | ND   | 0.14 | 6.89 |
| QH-II-092  | 0.07 | 0.03 | 0.04 | ND   | 0.17 | ND   |
| TG-4-29    | 2.8  | 3.9  | 2.7  | 2.1  | 0.18 | 3.9  |
| SH-I-75    | 1487 | 989.9| 773  | ND   | 0.3825| ND   |
| PWZ-007A   | 0.11 | 0.1  | 0.09 | ND   | 0.2  | 10   |
| XHE-II-024 | 0.09 | 0.18 | 0.32 | 14   | 0.24 | 11   |
| SH-I-085   | 11.08| 4.866| 13.75| ND   | 0.24 | ND   |
| MMB-II-90  | 20   | ND   | 5.7  | 9    | 0.25 | 36   |
| QH-II-090 (CGS-8216) | 0.05 | 0.08 | 0.12 | ND   | 0.25 | 17   |
| JYI-42     | 0.257| 0.146| 0.278| ND   | 0.256| ND   |
| MMB-III-016| 3   | 1.97 | 2    | 1074 | 0.26 | 21   |
| MMB-III-16 | 3   | 1.97 | 2    | 1074 | 0.26 | 21   |
| Rol5-4513  | 3.3  | 2.6  | 2.5  | ND   | 0.26 | 3.8  |
| DM-215     | 6.74 | ND   | 7.42 | ND   | 0.293| 8.28 |
| XLI-317    | 60.24| 24.05| 4.562| ND   | 0.295| ND   |
| RY-061     | 17   | 13   | 6.7  | ND   | 0.3  | 31   |
| ZG-234     | 7.25 | 22.14| 9.84 | ND   | 0.3  | 5.23 |
| PZII-028   | 0.2  | ND   | 0.2  | ND   | 0.32 | 1.9  |
| RY-067     | 21   | 12   | 10   | ND   | 0.37 | 42   |
| DM-III-90 (C17H12N4BrCl) | 0.505| 1    | 0.63 | ND   | 0.37 | 5000 |
| ZG-208     | 9.7  | 11.2 | 10.9 | ND   | 0.38 | 4.6  |
| XHE-III-04 | 1.2  | 2    | 1.1  | 219  | 0.4  | 500  |
| TG-4-29    | 8.3  | 10.2 | 6.9  | ND   | 0.4  | 7.61 |
| KY-024 C19H19N3O3 | 26.9 | 26.3 | 18.7 | ND   | 0.4  | 5.1  |
| JYI-47     | 2.759| 2.282| 0.511| ND   | 0.427| ND   |
| PWZ-0071   | 0.23 | 0.17 | 0.12 | ND   | 0.44 | 17.31|
| CM-E99b    | 20   | 22   | 19   | 55   | 0.45 | 69   |
| ZG-168     | 11.2 | 10.7 | 9.2  | ND   | 0.47 | 9.4  |
| RY-080 C17H15N3O3 | 28.4| 21.4 | 25.8 | 5.3  | 0.49 | 28.8 |
| BRETAZENIL | 0.35 | 0.64 | 0.2  | ND   | 0.5  | 12.7 |
| CM-E10     | 23   | 26   | 14   | 215  | 0.51 | 96   |
| JYI-04 (C21H23N3O3) | 28.3| 16   | ND   | ND   | 0.51 | 1.57 |
| PZII-029   | 0.34 | ND   | 0.79 | ND   | 0.52 | 10   |
| PWZ-085    | 4.86 | 13   | 8.5  | ND   | 0.55 | 40   |
| JYI-70 (C19H13N4F) | 6.3 | 2.1  | ND   | ND   | 0.56 | 5000 |
| CM-D44     | 34.3 | 56.3 | 20.7 | 0.33 | 0.57 | 0.92 |
| SVO-8-30   | 1.1  | 5.3  | 5.3  | 2.8  | 0.6  | 15   |
| Rol5-1788  | 0.8  | 0.9  | 1.05 | ND   | 0.6  | 148  |
| XLI268 C17H13BrN4 | 2.8145| 0.6862| ND | ND | 0.6243| ND |
| ZG-63A     | 17.3 | 21.6 | 29.1 | ND   | 0.65 | 4    |
| SH-053-2F  | 21.99| 12.34| 34.9 | ND   | 0.671| ND   |
| XLI-286    | 0.051| 0.064| 0.188| ND   | 0.684| ND   |
| SH-I-S66   | 22.93| 30.36| 55.26| ND   | 0.69 | ND   |
| Cook code | α1 | α2 | α3 | α4 | α5 | α6 |
|-----------|----|----|----|----|----|----|
| RY-076    | 26 | 27 | 13 | ND | 0.7| 22 |
| DM-173    | 13.1| ND | 38.1| ND | 0.78| 118|
| OMB-18    | 3.9| 1.2| 3.4| ND | 1733| 0.8| 5 |
| HJ-I-037  | 15.07| 8.127| 28.29| ND | 0.818| ND |
| PWZ-009A1 | 1.34| 1.31| 1.26| ND | 0.84| 2.03 |
| ZGl69A    | 6.8| 16.3| 9.2| ND | 0.85| 54.6 |
| ZG-69a (Ro15-1310) | 6.8| 16.3| 9.2| ND | 0.85| 54.6 |
| JYI-64 (C17H12N4FBr) | 0.305| 1.111| 0.62| ND | 0.87| 5000 |
| SVO-8-14  | 8 | 25 | 8 | ND | 6.9 | 0.9 | 14 |
| JYI-03 (C21H21N3O3) | 185.4| 107| ND | ND | 0.954| 3.34 |
| TG-II-82  | 1.6| 2.9| 2.8| ND | 1 | 1000 |
| RY-053    | 49 | 29 | 15 | ND | 1 | 46 |
| YT-6      | 15.31| 87.8| 60.49| ND | 1.039| ND |
| DMCM      | 5.69| 8.29| 4 | ND | 1.04| 134 |
| PWZ-096   | 11.1| 36 | 16.9| ND | 1.07| 51.5 |
| MMB-III-14| 13 | 13 | 6.9 | ND | 333 | 1.1 | 333 |
| YT-III-23 | 19.83| 23.65| 19.87| ND | 1.105| ND |
| RY-008    | 3.75| 7.2| 4.14| ND | 1.11| 44.3 |
| XLI-2TC   | 3.442| 1.673| 44.08| ND | 1.121| ND |
| XHE-II-073A (R ENRICHED) | 5.9| 11 | 10 | 15 | 1.18 | 140 |
| QH-II-075 | 0.18| 0.21| 0.25| ND | 1.3 | 40 |
| RY-054    | 59 | 44 | 27 | ND | 1.3 | 126 |
| TG-4-39   | 1.6| 34 | 24 | 5.6 | 1.4 | 23 |
| XHE-II-002| 8.3| 18 | 13 | 3.9 | 1.5 | 11 |
| RY-031 (RY-10) | 20.4| 27| 26.1| ND | 1.5 | 176 |
| FG8205    | 0.4| 2.08| 1.16| ND | 1.54 | 227 |
| alprazolam| 0.8| 0.59| 1.43| ND | 1.54| 1000 |
| XHE-III-13| 73 | ND | 71 | 880 | 1.6 | 311 |
| CM-D45 C19H21N3O4 | 90.5| 65.5| 30.3| 0.15| 1.65| 0.23 |
| RY-098    | 10.1| 22.2| 16.5| ND | 1.68| 100 |
| YT-5      | 0.421| 0.6034| 36.06| ND | 1.695| ND |
| JYI-60 (C17H11N2OF) | 3.73| 1.635| 4.3| ND | 1.7 | 5000 |
| RY-033    | 14.8| 56 | 25.3| ND | 1.72| 22.9 |
| XHE-III-06a| 1 | 2 | 1 | 5 | 1.8 | 37 |
| SH-I-030  | 14.42| 11.04| 19.09| ND | 1.89| ND |
| XLI352 C18H13CN2O | 1.56| 0.991| ND | ND | 1.957| ND |
| XLI351 C21H21CN2O | 1.507| 0.967| ND | ND | 1.985| ND |
| XHE-III-14| 2.6 | ND | 10 | 13 | 2 | 7 |
| DM-II-72 (C15H10N2BrCl) | 5000| 1.37| ND | ND | 2.02| 5000 |
| XHE-II-073B (S-ENRICHED) | 11 | 17 | 12 | 33 | 2.1 | 269 |
| FLUNITRAZEPAM | 2.2 | 2.5 | 4.5 | ND | 2.1 | 2000 |
| XHE-III-74 | 77 | 105 | 38 | 0.42 | 2.2 | 5.8 |
| SVO-8-67  | 7 | 41 | 26 | 15 | 2.3 | 191 |
| 6-PBC     | 0.49| 1.21| 2.2 | ND | 2.39| 1343 |
| RY-058    | 86 | 40 | 85 | ND | 2.4 | 150 |
| ZG-224    | 171| 33.7| 50 | ND | 2.5 | 31.7 |
| RY-066    | 83 | 60 | 48 | ND | 2.6 | 180 |
| RY-023 C22H27N3O3Si | 197| 142.6| 255| 78 | 2.61| 58.6 |
| XLI350 C17H11CN2O | 1.224| 1.188| ND | ND | 2.9 | ND |
| JYI-32 (C20H15N3O2BrF) | 3.07| 4.96| ND | ND | 2.92| 52.24 |

Table 9: Continued.
| Cook code$^a$ | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\alpha_4$ | $\alpha_5$ | $\alpha_6$ |
|--------------|-----------|-----------|-----------|-----------|-----------|-----------|
| SH-I-89S     | 12.78     | 8.562     | 8.145     | ND        | 3.23      | ND        |
| MSR-I-032    | 6.2       | 18.7      | 4         | ND        | 3.3       | 74.9      |
| OMB-I-19     | 22        | 4.6       | 20        | 3333      | 3.5       | 40        |
| ZG-213       | 12.8      | 49.8      | 30.2      | ND        | 3.5       | 22.5      |
| YT-II-83     | 32.74     | 13.22     | 24.1      | ND        | 3.548     | ND        |
| RY-059       | 89        | 70        | 91        | ND        | 3.7       | 301       |
| SPH-121      | 0.14      | 1.19      | 1.72      | ND        | 4         | 479       |
| RY-047       | 200       | 124       | 79        | ND        | 4         | 340       |
| XLI-8TC      | 21.52     | 11.01     | 2.155     | ND        | 4.059     | ND        |
| YT-I-38      | 945.9     | 326.8     | 245.9     | ND        | 4.07      | ND        |
| DM-I-46      | 6.44      | ND        | 148       | ND        | 4.23      | 247       |
| CM-B44 (ss)  | 32        | 43        | 12        | 379       | 4.3       | 485       |
| KM-B47       | 32        | 63        | 34        | 2007      | 4.4       | 717       |
| XLI-JY-DMH ANX3 | 3.3  | 0.58     | 1.9       | ND        | 4.4       | 5000      |
| WY-B-15      | 0.92      | 0.83      | 0.58      | 2080      | 4.42      | 646       |
| ZK 93423     | 4.1       | 4.2       | 6         | ND        | 4.5       | 1000      |
| JYI-12 (C19H16N3O3F3) | 91  | 39       | ND        | ND        | 4.5       | 6.8       |
| CM-A87       | 1.62      | 4.54      | 14.73     | 1000      | 4.61      | 1000      |
| DM-III-01 (C18H12N3O2Br) | 5000 | 12 | ND | ND | 4.73 | 5000 |
| RY-057       | 73        | 85        | 97        | ND        | 4.8       | 333       |
| JYI-15 (C19H14N3O3F3) | 205 | 812      | ND        | ND        | 4.8       | 22        |
| CM-B31i (ss) | 90        | 184       | 78        | 18        | 4.9       | 121       |
| RY-079       | 121.1     | 141.9     | 198.4     | 159       | 5         | 113.7     |
| JCY208 C15H10N2OS | 22.42 | 18.89   | ND        | ND        | 5.039     | ND        |
| YT-II        | 6.932     | 0.8712    | 3.518     | ND        | 5.119     | ND        |
| XLI270 C19H14N4 | 36.39 | 25.81   | ND        | ND        | 5.291     | ND        |
| XHE-I-051    | 35        | 39        | 42        | ND        | 5.3       | 979       |
| MMB-II-87    | 205       | 812       | ND        | ND        | 4.8       | 22        |
| XLI-210      | 231       | 661       | 2666      | ND        | 5.4       | 54.22     |
| XHE-II-053-ACID | 50.35 | 11.8     | 44        | ND        | 5.9       | 5000      |
| ABECARNIL    | 12.4      | 15.3      | 7.5       | ND        | 6         | 1000      |
| RY-I-31      | 10        | 45        | 19        | ND        | 6         | 1000      |
| QH-II-082    | 1.7       | 1.8       | 1.6       | ND        | 6.1       | 100       |
| SH-TSC-1 (PWZ-029) | 362.4 | 180.3   | 328.2     | ND        | 6.185     | ND        |
| XHE-II-065   | 1000      | 499       | 216       | 37        | 6.4       | 175       |
| JYI-49 (C20H12N3O2F4Br) | 1.87 | 2.38   | ND        | ND        | 6.7       | 3390      |
| JCI84 C13H9BrN2OS | 9.606 | 10.5     | ND        | ND        | 6.709     | ND        |
| QH-II-066    | 76.3      | 42.1      | 47.4      | 2000      | 6.8       | 3000      |
| XLI-381      | 619.9     | 285.6     | 3639      | ND        | 7.051     | ND        |
| RY-071       | 19        | 56        | 91        | ND        | 7.2       | 266       |
| RY-1-28      | 283       | 318       | 102       | ND        | 7.2       | 61        |
| CM-A82a      | 2.78      | 8.93      | 24.51     | 1000      | 7.49      | 1000      |
| YT-III-31    | 36.39     | 67.85     | 129.7     | ND        | 7.59      | ND        |
| SH-I-04      | 7.3       | 6.136     | 5.1       | ND        | 7.664     | ND        |
| QH-146       | 0.49      | ND        | 0.76      | ND        | 7.7       | 1000      |
| QH-II-063    | 9.4       | 9.3       | 31        | ND        | 7.7       | 3000      |
| JC221 ANXI    | 106.175   | 49.405    | 182       | ND        | 7.7495    | 362       |
| DM-II-30 (C20H13N3O2BrF3) | 176  | 13.4     | 28.51     | ND        | 7.8       | 5000      |
| SH-TS-CH3    | 107.2     | 50.09     | 20.95     | ND        | 8.068     | ND        |
| RY-073       | 156       | 88        | 122       | ND        | 8.5       | 267       |
Table 9: Continued.

| Cook code | α1 | α2 | α3 | α4 | α5 | α6 |
|-----------|----|----|----|----|----|----|
| SVO-8-20  | 11 | 40 | 28 | 19 | 8.6 | 138 |
| SHU-221-1 | 66 | 41 | 43 | 3000 | 9 | 3000 |
| YT-III-231| 51.09 | 61.46 | 26.34 | ND | 9.124 | ND |
| CM-E09a   | 176 | 192 | 122 | 490 | 9.2 | 718 |
| DM-139    | 5.8 | ND | 169 | ND | 9.25 | 325 |
| YT-III-42 | 382.9 | 16.83 | 44.04 | ND | 9.77 | ND |
| CD-214    | 16.4 | 48.2 | 42.5 | ND | 9.8 | 168 |
| XHE-III-24| 0.25 | ND | 8 | 222 | 10 | 328 |
| XLI223 C22H20BrN3O2 | 14 | 8.7 | 18 | 1000 | 10 | 2000 |
| SPH-165   | 0.63 | 2.79 | 4.85 | ND | 10.4 | 1150 |
| JYI-01 (C19H20N3O3Br) | 59.2 | 159 | 96 | ND | 10.6 | 2.88 |
| diazepam  | 14 | 20 | 15 | ND | 11 | ND |
| XHE-III-49| 1.3 | 5.5 | 4.2 | 38.7 | 11.3 | 85.1 |
| WZ-113    | 19.2 | 13.2 | 13.4 | ND | 11.5 | 300 |
| JYI-59 (C22H13N3O2F4) | 1.08 | 2.6 | 11.82 | ND | 11.5 | 5000 |
| JYI-72 (C22H14N4SF) | 48.5 | 18.5 | ND | ND | 11.5 | 5000 |
| TC-YT-II-76| 101.1 | 1.897 | 5.816 | ND | 11.99 | ND |
| JYI-10 (C17H13N3O3F3Br) | 5000 | 368 | ND | ND | 12.3 | 23 |
| WZ-069    | 40 | 30.5 | 38.5 | ND | 12.6 | 1000 |
| JYI-06 (C23H23N3O4) | 16.5 | 5.48 | 5000 | ND | 12.6 | 5000 |
| RY-072    | 220 | 150 | 184 | ND | 12.7 | 361 |
| JYI-14 (C17H4N3O3F3) | 32 | 25 | ND | ND | 13 | 565 |
| XHE-II-053| 287 | 45 | 96 | 1504 | 13.8 | 1000 |
| XLI-347 C34H28N6O7 | 828.05 | 690.2 | ND | ND | 13.87 | ND |
| SHU-1-19 | 4 | 12 | 7 | 48 | 14 | 84 |
| CM-C28 (SR) | 176 | 752 | 244 | 290 | 14 | 141 |
| CM-E11    | 333 | 308 | 161 | 394 | 14 | 750 |
| XHE-II-012| 49 | 24 | 31 | 1042 | 14 | 2038 |
| MMB-III-018| 117 | 140 | 78 | 3500 | 14 | 976 |
| MMB-III-18| 117 | 140 | 78 | 3500 | 14 | 976 |
| CM-B31c (ss) | 118 | 319 | 173 | 37 | 15 | 137 |
| CM-B45    | 230 | 557 | 336 | 265 | 15 | 230 |
| XLI-093   | 1000 | 1000 | 858 | 1550 | 15 | 2000 |
| DM-II-20 (C22H14N3O2F3) | 54.3 | 2714 | 35.68 | ND | 15.35 | 5000 |
| XLI269 C22H22N4Si | 221.8 | 154.2 | ND | ND | 15.51 | ND |
| SH-O53-S-CH3-2′F | 350 | 141 | 1237 | ND | 16 | 5000 |
| JYI-13 (C21H16N3O4F3) | 5000 | 63.7 | ND | ND | 16 | 8.38 |
| CM-B34    | 472 | 451 | 223 | 114 | 17 | 175 |
| XHE-II-017| 3.3 | 10 | 7 | 258 | 17 | 294 |
| JC222 C16H12N2OS | 86.7 | 45.11 | ND | ND | 17.63 | ND |
| SPH-38    | 2 | 5.4 | 10.8 | ND | 18.5 | 3000 |
| WZ-070    | 72.7 | 30.7 | 53.2 | ND | 18.6 | 300 |
| RY-069    | 692 | 622 | 506 | ND | 19 | 1000 |
| SH-O53-2′F-S-CH3 | 468.2 | 33.27 | 291.5 | ND | 19.2 | ND |
| XHE-1-093 | 2 | 7.1 | 8.9 | 1107 | 20 | 1162 |

*Affinity of compounds at GABA_A/BzR recombinant subtypes was measured by competition for [3H]flunitrazepam or [3H] Ro15-4513 binding to HEK cell membranes expressing human receptors of compositions α1β3γ2, α2β3γ2, α3β3γ2, α4β3γ2, α5β3γ2, and α6β3γ2 [22, 139]. Data represent the average of at least three determinations with a SEM of ±5%. ND: not determined.
The X-ray structure determination of the α5β3y2 GABA(A) receptor is eagerly awaited, while that with five β3-subunits has been reported recently (Miller and Aricescu, Nature 2014). It is hoped that the proposed orientation may be used by others to gain additional insight into the potential mechanisms underlying binding and modulation at the Bz site, all of which will lead to a better understanding of the structure and function of GABA(A) receptors, ultimately targeted toward treatment of diseases.

25. Synthesis of Ligands with α5 BzR Subtype Selectivity

Briefly, bromoacetyl bromide was added to 2-aminobenzophenone 44, followed by treatment with methanol, which had been saturated with ammonia (g) under the cooling of an ice-water bath. The benzodiazepine, 45, was brominated to provide 46 and then reacted with ethyl isocyanocacetate to generate the imidazobenzodiazepine, 47. A much better one-pot process has now been devised using KtBuO at −30°C [140]. The bromide 48 was subjected to a Stille-type coupling to give DM-1-81 (9) [126]. This route (Scheme 1) can be executed on several hundred gram scales.

The benzodiazepine monomers were prepared by the method of Fryer and Gu [89, 141]. The isatoic anhydride was heated with sarcosine in dimethyl sulfoxide to provide amide 49. Bromination of 49 in a mixture of acetic acid, bromine, and sodium acetate afforded the corresponding monosubstituted bromide 50 in good yield. Deprotonation of 50 with lithium disopropyl amide (LDA) in THF was followed by treatment with diethyl chlorophosphate to provide the intermediate enol phosphate. The enol phosphate was stirred with a solution of ethyl isocyanocacetate and LDA to yield the imidazo congener. Again, a better one-pot procedure has been developed using KtBuO at −30°C in place of LDA at 0°C. A Heck type coupling reaction was employed with the bromide 51 with bis(aceate)bis(triphenylphosphine)palladium(II) to provide the TMS-acetylene 52. Treatment of 52 with Bu4NF removed the trimethylsilyl group. Hydrolysis of the ester function of 53 provided the acid 54 in excellent yield and this material was dried scrupulously and subjected to a standard CDI-mediated coupling reaction to furnish bivalent ligand XLI-093 (4). The imidazobenzodiazepine diethyl diester XLI-356 (10) was obtained from XLI-093 (Scheme 2) in high yield via catalytic hydrogenation (Pd/C, H2).

26. Synthesis of Bivalents

Inverse agonist 53 was synthesized via the reported procedure. Hydrolysis of the ester function of 53 provided the acid 54 in excellent yield. This material was dried scrupulously and was subjected to a standard CDI-mediated coupling reaction to furnish bivalent ligands 4, 55, and 56 in 60% yield (Scheme 3) [13].

The acid 57, obtained from the ester 47, which was available from the literature [13], was stirred with CDI in DMF, followed by stirring with the required diol and DBU to provide bromide substituted dimers 58 or 59, respectively. They were converted into the trimethylsilylacetylenyl 60 or 61, respectively, under standard conditions (Pd-mediated, Heck-type coupling) [142]. The bisacetylene 62 or 63 (individually) was easily obtained by treatment of the trimethylsilyl ligand 60 or 61 with fluoride anion, as shown in Scheme 4.

Table 10: Ligands with potent affinity for α6; ligands bound with Kᵢ values < 20 nM at this subtype. The structures of these ligands are in the Ph.D. thesis of Clayton (2011) [22].

| Cook code | α1 | α2 | α3 | α4 | α5 | α6 |
|-----------|----|----|----|----|----|----|
| CM-D45 C19H21N3O4 | 90.5 | 65.5 | 30.3 | 0.15 | 1.65 | 0.23 |
| CM-D44 | 34.3 | 56.3 | 20.7 | 0.33 | 0.57 | 0.92 |
| JYI-04 (C2H23N3O3) | 28.3 | 16 | ND | ND | 0.51 | 1.57 |
| PZII-028 | 0.2 | ND | 0.2 | ND | 0.32 | 1.9 |
| PWZ-009A | 1.34 | 1.31 | 1.26 | ND | 0.84 | 2.03 |
| JYI-01 (C19H20N3O3Br) | 59.2 | 159 | 96 | ND | 10.6 | 2.88 |
| JYI-03 (C2H21N3O3) | 185.4 | 107 | ND | ND | 0.95 | 3.34 |
| Ro15-4513 | 3.3 | 2.6 | 2.5 | ND | 0.26 | 3.8 |
| TG-4-29 | 2.8 | 3.9 | 2.7 | 2.1 | 0.18 | 3.9 |
| JYI-11 (C2H22N3O3F3Si) | 5000 | 5000 | ND | ND | 648 | 3.97 |
| QH-II-077 | 0.06 | 0.08 | 0.05 | ND | 0.12 | 4 |
| ZG-63A | 17.3 | 21.6 | 29.1 | ND | 0.65 | 4 |
| XHE-III-74 | 91 | 39 | ND | ND | 4.5 | 6.8 |
| DM-239 | 1.5 | ND | 0.53 | ND | 0.14 | 6.89 |
| XHE-III-14 | 2.6 | ND | 10 | 13 | 2 | 7 |
| TG-4-29 | 8.3 | 10.2 | 6.9 | ND | 0.4 | 7.61 |
| DM-215 | 6.74 | ND | 7.42 | ND | 0.293 | 8.28 |
| JYI-13 (C2H16N3O4F3) | 5000 | 63.7 | ND | ND | 16 | 8.38 |
| CGS9895 | 0.21 | ND | ND | ND | 9.3 |
| ZG-168 | 11.2 | 10.7 | 9.2 | ND | 0.47 | 9.4 |
| PWZ-007A | 0.11 | 0.1 | 0.09 | ND | 0.2 | 10 |
| PZII-029 | 0.34 | ND | 0.79 | ND | 0.52 | 10 |
| XHE-II-024 | 0.09 | 0.18 | 0.32 | 14 | 0.24 | 11 |
| XHE-II-002 | 8.3 | 18 | 3.9 | ND | 1.5 | 11 |
| BRETAZENIL | 0.35 | 0.64 | 0.2 | ND | 0.5 | 12.7 |
| JYI-19 (C2H23N3O3S) | 2.176 | 205 | ND | ND | 34 | 12.7 |
| SVO-8-14 | 8 | 25 | 8 | 6.9 | 0.9 | 14 |
| SVO-8-30 | 1.1 | 5.3 | 2.8 | 0.6 | 15 |
| WYS10 C14H9F3N2O2 | 0.88 | 36 | 25.6 | ND | 548.7 | 15.3 |
| QH-II-090 (CGS-8216) | 0.05 | 0.08 | 0.12 | ND | 0.25 | 17 |
| PWZ-0071 | 0.23 | 0.17 | 0.12 | ND | 0.44 | 17.31 |

*The affinity of compounds at GABA_A/BzR recombinant subtypes was measured by competition for [3H]flunitrazepam binding to HEK cell membranes expressing human receptors of compositions α1β2γ2, α2β3γ2, α3β3γ2, α4β3γ2, and α6β3γ2 [139]. Data represent the average of at least three determinations with a SEM of ±5%. ND: not determined.
Figure 34: Overlay of selected compounds for α1β3γ2 subtype from Table 5.

Figure 35: Updated α1β3γ2 subtype (blue solid) overlaid with the previous model (red wire). Overlap identified where wire and solid overlap.

Figure 36: Overlay of compounds selective for α2β3γ2 subtype.

Figure 37: Updated α2β3γ2 subtype (solid) overlaid with the previous model (red wire). Overlap identified where wire and solid overlap.

Figure 38: Overlay of compounds selective for α3β3γ2 subtype.

Figure 39: Updated α3β3γ2 subtype (blue solid) overlaid with the previous model (red wire). Overlap identified where wire and solid overlap.

Figure 40: Overlay of selected compounds selective for α4β3γ2 subtype.

Figure 41: Updated α4β3γ2 subtype (blue solid) overlaid with the previous model (yellow wire). Overlap identified where wire and solid overlap.
E. M. Laboratories. Melting points were taken on a Thomas-Hoover melting point apparatus or an Electrothermal Model IA8100 digital melting point apparatus and are reported uncorrected. NMR spectra were recorded on a Bruker 300 or 500 MHz multiple-probe spectrometer. Infrared spectra were recorded on a Nicolet DX FTIR BX V5.07 spectrometer or a Mattson Polaris IR-10400 instrument. Low-resolution mass spectral data (EI/CI) were obtained on a Hewlett-Packard 5985B GC-mass spectrometer, while high resolution mass spectral data were taken on a VG autospectrometer (Double Focusing High Resolution GC/Mass Spectrometer, UK). Microanalyses were performed on a CE Elantech EA1110 elemental analyzer. Methods of specific experiments can be found in corresponding cited works.

27. Materials, Methods, and Experimental

27.1. Materials and General Instrumentation. Chemicals were purchased from Aldrich Chemical Co. or Tokyo Chemical Industries and were used without further purification except where otherwise noted. Anhydrous THF was distilled from sodium/benzophenone ketyl. TLC analyses were carried out on Merck Kieselgel 60 F$_{254}$, and flash column chromatography was performed on silica gel 60b purchased from E.M. Laboratories. Melting points were taken on a Thomas-Hoover melting point apparatus or an Electrothermal Model IA8100 digital melting point apparatus and are reported uncorrected. NMR spectra were recorded on a Bruker 300 or 500 MHz multiple-probe spectrometer. Infrared spectra were recorded on a Nicolet DX FTIR BX V5.07 spectrometer or a Mattson Polaris IR-10400 instrument. Low-resolution mass spectral data (EI/CI) were obtained on a Hewlett-Packard 5985B GC-mass spectrometer, while high resolution mass spectral data were taken on a VG autospectrometer (Double Focusing High Resolution GC/Mass Spectrometer, UK). Microanalyses were performed on a CE Elantech EA1110 elemental analyzer. Methods of specific experiments can be found in corresponding cited works.

27.2. Competition Binding Assays. Competition binding assays were performed in a total volume of 0.5 mL of a 50 mM Tris-acetate at 4° degree centigrade for 1 hour using [³H]flunitrazepam as the radioligand. For these binding assays, 20–50 mg of membrane protein harvested with hypotonic buffer (50 mM Tris-acetate pH 7.4 at 4 degree) was incubated with the radiolabel as previously described [139, 143]. Nonspecific binding was defined as radioactivity bound in the presence of 100 μM diazepam and represented less than 20% of total binding. Membranes were harvested with a Brandel cell harvester followed by three ice-cold washes onto polyethyleneimine-pretreated (0.3%) Whatman GF/C filters. Filters were dried overnight and then soaked in Ecoscint A liquid scintillation cocktail (National Diagnostics; Atlanta, GA). Bound radioactivity was quantified by liquid scintillation counting. Membrane protein concentrations were determined using an assay kit from Bio-Rad (Hercules, CA) with bovine serum albumin as the standard.

27.3. Radioligand Binding Assays (Drs. McKernan and Atack) [12]. In brief, the affinity of compounds for human recombinant GABA(A) receptors was measured by competition binding using 0.5 nM [³H]flunitrazepam. Transfected HEK cells (beta2 gamma2 and desired alpha subtype) were harvested into phosphate-buffered saline, centrifuged at 3,000 g, and stored at −70°C until required. On the day of the assay, pellets were thawed and resuspended in sufficient volume of 50 mM Tris/acetate (pH 7.4 at 4°C) to give a total binding of approximately 1500–2000 dpm. Nonspecific binding was defined in the presence of 100 mM (final concentration) diazepam. Test compounds were dissolved in DMSO at a concentration of 10 mM and diluted in assay buffer to give an appropriate concentration range in the assay, such that the final DMSO concentration in the assay was always less than 1%. Total assay volume was 0.5 mL and assays were carried out in 96-well plates and incubation time started by the addition of 0.1 mL of resuspended cell membranes. Following incubation for 1 hour at 4°C, assays were terminated by filtration through GF/B filters, washed with 10 mL ice cold buffer, dried, and then counted using a liquid scintillation counter. The percentage of inhibition of [³H]flunitrazepam binding, the IC$_{50}$, and the Kᵢ values were calculated using the Activity Base Software Package (ID Business Solutions,
Guildford, UK) according to the Cheng-Prusoff equation [143]. We have previously reported the synthesis of the following.

1,3-Bis(8-acetyleno-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxy) propyl diester 4 (XLi-093) (Procedure A), experimental details previously reported [17].

1,5-Bis(8-acetyleno-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxy) pentyl diester 56 (XLi-210), experimental details previously reported [17].
Figure 48: The previous benzodiazepine subtype selective receptor pharmacophore models [23]. (1) The $L_2$ region in the $\alpha_5$ subtype is larger than the $\alpha_1$ subtypes. This is a key result. It is the principle difference between $\alpha_5$ subtypes compared to $\alpha_2$ and $\alpha_3$ subtypes, but especially in regard to $\alpha_1$ subtypes ($L_2$ smaller in $\alpha_1$). (2) The $L_3$ region is larger in the $\alpha_5$ subtype as compared to the $\alpha_1, \alpha_2, \alpha_3, \alpha_4$, and $\alpha_6$ BzR sites. R analogs of benzodiazepines with pendant phenyls had increased affinity to $\alpha_5$ supporting the larger $L_3$ pocket in this receptor subtype, while S isomers bound to $\alpha_2, \alpha_3$, and $\alpha_5$ subtypes because of different conformational constraints.

Figure 49: Steric (left) and electrostatic maps of the $\alpha_1\beta_3\gamma_2$ receptor subtype shown in the transparent mode as seen from the classic perspective.

Figure 50: Steric (left) and electrostatic maps of the $\alpha_1\beta_3\gamma_2$ receptor subtype shown in the transparent mode as seen from the classic perspective (Figure 45) rotated 90°.

1,3-Bis(8-ethyl-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxy) propyl diester 10 (Xli-356), experimental previously published [144].

Bis(8-acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5a][1,4]benzodiazepine-3-carboxy) dimethyl glycol dies-
ter 55 (Xli-374), experimental details previously reported [17].

8-Bromo-6-phenyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylic acid 57, experimental details previously reported [17].
Figure 60: Steric (left) and electrostatic maps of the $\alpha_3\beta_3\gamma_2$ receptor subtype shown in line mode as seen from the classic perspective rotated $90^\circ$.

Figure 61: Steric (left) and electrostatic maps of the $\alpha_5\beta_3\gamma_2$ receptor subtype shown in the transparent mode as seen from the classic perspective.

Figure 62: Steric (left) and electrostatic maps of the $\alpha_5\beta_3\gamma_2$ receptor subtype shown in the transparent mode as seen from the classic perspective rotated $90^\circ$.

Figure 63: Steric (left) and electrostatic maps of the $\alpha_5\beta_3\gamma_2$ receptor subtype shown in line mode as seen from the classic perspective.

Figure 64: Steric (left) and electrostatic maps of the $\alpha_5\beta_3\gamma_2$ receptor subtype shown in line mode as seen from the classic perspective rotated $90^\circ$. 
Figure 65: Clockwise from the top left, line maps of the $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_3$, $\alpha_3\beta_3\gamma_2$, and $\alpha_5\beta_3\gamma_2$ CoMFA.

Scheme 1: Synthesis of 8-substituted imidazobenzodiazepines following chemistry earlier developed by Sternbach, Fryer et al. Reagents and Conditions. (a) Bromoacetyl bromide, sodium bicarbonate, and chloroform; (b) ammonia (anhydrous), methanol, and reflux; (c) bromine, sulfuric acid, and acetic acid; (d) sodium hydride, diethyl chlorophosphite, and tetrahydrofuran; (e) sodium hydride, ethyl isocyanoacetate, and tetrahydrofuran, $-30^\circ$C to r.t.; (f) tributyl(phenyl)stannane, Pd($\text{PPh}_3)_4$.

1,3-Bis(8-bromo-6-phenyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxy) propyl diester 59 (DMH-D-070) (Procedure B), experimental details previously reported [17]. 1,3-Bis(8-trimethylsilylacetylenyl-6-phenyl-4H-benzo[f]imidazo[1,5-a][1,4]-diazepine-3-carboxy) propyl diester 61 (DMH-D-048) (Procedure C), experimental details previously reported [17].

1,3-Bis(8-acetylenyl-6-phenyl-4H-benzo[f]imidazo[1,5-a][1,4]-diazepine-3-carboxy) propyl diester 63 (DMH-D-053): experimental details previously reported [17].

Bis(8-bromo-6-phenyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxy) diethylene glycol diester 58 (DM-III-93), experimental details previously reported [17].

Bis(8-acetylenyl-6-phenyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxy) diethylene glycol diester 62 (DM-III-96), experimental details previously reported [17].

Abbreviations

- APD: Antipsychotic drug
- ASM: Airway smooth muscle
- BS: Binding site
- BZD, Bz: Benzodiazepine
- BzR: Benzodiazepine receptor
- DA: Dopamine
- DAPI: 4',6-Diamidino-2-phenylindole
SCHEME 2: Synthesis of 8-substituted imidazobenzodiazepine bivalent ligands. Reagents and Conditions. (a) DMSO, 180°C, 90%; (b) bromine, sodium acetate, and acetic acid, r.t., 80%; (c) LDA, THF, and diethyl chlorophosphate, 0°C; (d) LDA, THF, and ethyl isocyanoacetate; (e) trimethylsilylacetylene, Pd(OAc)$_2$(PPh$_3$)$_2$, triethylamine, acetonitrile, and reflux, 80%; (f) tetrabutylammonium fluoride, THF, and H$_2$O, r.t., 88%; (g) 2NaOH and ethanol, 70°C, 90%; (h) CDI, DMF, HO(CH$_2$)$_3$OH, and DBU, 60%; (i) Pd/C, H$_2$, ethanol, and DCM, 90%.

SCHEME 3: Synthesis of bivalent analogs of XLi-093 (4). Reagents and Conditions. (a) 2M NaOH, EtOH, 70°C; (b) 10% aq HCl; (c) CDI, DMF; (d) diol, DBU.
Scheme 4: Synthesis of bivalent analogues of DMH-D-053 (63). Reagents and Conditions. (a) 2 N NaOH, EtOH, and reflux; (b) 10% aq. HCl; (c) CDI, DMF; (d) diol, DBU; (e) trimethylsilylacetylene, Pd(OAc)$_2$(PPH$_3$)$_2$, Et$_3$N, CH$_3$CN, and reflux; (f) TBAF*0.5H$_2$O, THF, $-78^\circ$C.

GABA: Gamma amino butyric acid
GABA$_A$ : Gamma amino butyric acid A
GABA$_A$ R: Gamma amino butyric acid A receptor
HAL: Haloperidol
HEK: Human embryonic kidney
HPC: Hippocampal
LTK: Leukocyte tyrosine kinase
MAM: Methylazoxymethanol
NAM: Negative allosteric modulator
QSAR: Quantitative structure-activity relationship
PAM: Positive allosteric modulator
PV: Parvalbumin
SAL: Saline
SH-053: SH-053-2'F-R-CH$_3$
SMA: Smooth muscle actin
TTX: Tetrodotoxin
VTA: Ventral tegmental area.

Conflict of Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution
Terry Clayton and Michael M. Poe contributed equally to this work.

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