THE SYNTHESIS OF ALPHA 5 SUBTYPE SELECTIVE GABA(A) /BENZODIAZEPINE RECEPTORS LIGANDS

by

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A Thesis Submitted in
Partial Fulfillment of the
Requirements for the Degree of
Master of Science
in Chemistry
at
The University of Wisconsin-Milwaukee

August 2014
GABA_A complexes are a class of receptors that respond to the neurotransmitter GABA, the chief inhibitory neurotransmitter in the vertebrate CNS. A widely accepted pharmacological target for enhancing cognition is the benzodiazepine-binding site on the gamma-aminobutyric acid type A (GABA_A) receptor complex. Inverse agonists acting at α5 subunits containing GABA_A receptors are thought to act as cognitive enhancers while eliminating unwanted side effects associated with non-selective compounds. From the recent work of Rowlett, Cook et al. it was demonstrated that the novel α5 selective inverse agonist PWZ-029 (20) was active as a cognitive enhancer in rhesus monkeys in the CANTAB paradigm. This ligand (PWZ-029) is about 60-fold more selective for the α5 subunit compared to α1, α2 and α3 GABA(A)ergic receptors. It is found that PWZ-029 significantly attenuated scopolamine-induced impairment of contextual memory in rodents and primates. In the object retrieval task, PWZ-029 showed only a modest trend for enhancement of performance, but when task difficulty was increased by testing with difficult trials only, PWZ-029 robustly increased performance. In addition, PWZ-029 enhanced performance in the DNMS (Delayed non-matching to sample) task using the 10 minute delay with distracters. This ligand also exhibited anxiolytic activity in some
primates and was an orally active anticonvulsant in rats (James Stables, NINDS). GABA<sub>\text{A}</sub> receptor complexes that contain subunits are abundantly expressed in the hippocampus and therefore considered to be a therapeutic target for treating cognitive disorders, like Alzheimer’s and ADHD. In order to search for better agents, a number of analogs of PWZ-029 were prepared in this research and have been sent out for biological testing. In order to do this a key benzylic bromide was prepared and purified which is a key to future work, stability issues with this bromide were solved which is important to success in this research.
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I would like to acknowledge all those who made this thesis possible. I would like to thank my advisor Professor James M. Cook, for his guidance, support, enthusiastic supervision and patience throughout the course this work.

I would like to thank the members of my committee, Professor’s Arnold and Pacheco for their assistance, suggestions and instructive discussions during my graduate studies at UW-Milwaukee. I am also very grateful to Dr. Föersterling for his instruction and guidance for the NMR studies. I greatly appreciated Mr. Neal Korfhage for his expertise in glassblowing which enabled me to set up equipment that may have otherwise been impossible. Thanks to Elise Nicks, Mary Eckert and Wendy Grober for all their help during my graduate studies at UW-Milwaukee.

I would like to thank my parents Hari Ram Biawat and Bimla Biawat for their unequivocal support needed in order to continually push myself to succeed. Without their love, support and guidance this achievement would not have been accomplished. Special thanks to my brother Sandeep Biawat who has always encouraged and supported me during this endeavor. I am indebted to him who have always been my pillar of strength. Without his great love and support it would have been impossible for me to reach this milestone. I would like to thank my friend Vikram Paul Singh Mehmi for all his help and also for his advice. His beliefs in me sustained me throughout my graduate studies, and his friendship have graced my life.

I would also like to thank my colleagues Mr. Toufiqur Rahman, Mr. Christopher Witzigman, Mr. Michael Poe and Mr. Phanibabu Tiruveedula for providing a stimulating
environment in which to learn and grow. I would like thank Toufiquur Rahman for all his help with NMR corrections. I would especially like to thank Dr. Sundari Rallapalli, Dr. Ranjit Verma, Dr. Rahul Edwanker, Dr. Chitra edwanker, Dr. Ojas and Dr. German for their help and many inspiring discussions during initial years of my masters program. I wish to extend a special thanks to Dr. Ranjit Verma for his support and encouragement in my studies. His guidance throughout my program will never be forgotten. I would also like to acknowledge the financial, academic and technical support of the Department of Chemistry at UW-Milwaukee, the Graduate School at UW-Milwaukee, and the NIMH.
1. Introduction

1.1. Molecular composition of GABA/benzodiazepine receptors

The GABA<sub>A</sub>/benzodiazepine receptors are ligand-gated chloride channels which comprise the major inhibitory neurotransmitter in the central nervous system.<sup>1</sup> The receptors are heteroligomeric membrane–bound protein complexes which play a central role in the molecular mechanisms in the CNS and are composed of several subunits and termed type A, type B, type C. In the case of type A receptors, the inhibitory effects of GABA mediated by these receptors can be modulated by a number of pharmacological agents that selectively bind to allosteric sites on these ion channels.<sup>2-5</sup> GABA<sub>A</sub> receptors are heteropentameric assemblies of proteins derived from a family of subunits (6α3β3γ1δ1π1θ1ε and 3ρ)<sup>9,13</sup> which form the chloride ion channel (Figure 1). The most common form of native GABA<sub>A</sub> receptors in the CNS are benzodiazepine sensitive and contain α, β, and γ subunits in a 2:2:1 stoichiometry.<sup>5,9,7</sup> They are required to construct a fully functional recombinant GABA<sub>A</sub>/BzR chloride channel which closely mimic the biological, electrophysiological and pharmacological properties of native GABA<sub>A</sub> receptors.<sup>5,7,9</sup> The benzodiazepine binding site is located at the interface between the α and γ subunits.<sup>10</sup> While it is clear the γ subunit is required for benzodiazepine binding,<sup>5,7</sup> the fact that most native GABA<sub>A</sub> receptors contain an α subunit is in agreement with the α subunit as the key determinant of benzodiazepine binding and efficacy.<sup>5,7</sup> However, it is clear the gamma subunit is also required for benzodiazepine binding.<sup>5,7</sup>
Studies in molecular biology have identified 21 subunits which comprise a series of GABA_A/BzR subtypes (ion channels); the principle ones of interest here are α1β3γ2 (α1 = ω1 = Bz1), α2β3γ2 (α2 = ω2 = Bz2), α3β3γ2, α4β3γ2, α5β3γ2 and α6β3γ2. Subtype assemblies which contain an α1 subunit (α1β3γ2) are present in most areas of the brain and are thought to account for 40-50% of GABA_A receptors in the rat CNS. Subtype assemblies which contain α2 and α3 subunits, respectively, are thought to account for about 25% and 17% of the GABA_A/BzR receptors in the rat, respectively. The GABA_A/BzR receptors which contain the α5 subunit are of minor abundance (5%) in the whole brain, but are significantly expressed in the hippocampus (Figure 2), where they comprise 15-20% of the diazepam-sensitive GABA_A receptor population and are predominantly co-assembled with β3 and γ2 subunits. The assembly of α1-6β3γ2 receptors in the rat are very similar to those in the human CNS.
Figure 2: Distribution of α5 receptors in the hippocampus. Reprinted with permission of the author.

The majority of Bz1 GABA_A receptors are composed of 2α, 2β and 1γ subunits. Each subunit has per convention a “plus” and a “minus” side (Figure 3). Consequentely, the subunit interfaces consist of the plus and minus sides of neighboring subunits. The modulatory benzodiazepine binding site is located at the α⁺γ⁻ subunit interface and is larger than, but homologous to the two agonist (GABA) binding sites, which are located at the β⁺α⁻ subunit interfaces. The absolute subunit configuration for the α₁β₂γ₂ GABA_A receptor appears to be γβαβα when viewed counterclockwise from the synaptic cleft.
Figure 3. Absolute subunit arrangement of the α12βγ2 GABA<sub>A</sub> receptors when viewed from the synaptic cleft.<sup>18,19</sup>

1.2. **History**

Although the BZs were first introduced into clinical practice in the early 1960s, it was not until 1975 that these drugs were shown to act by potentiating the inhibitory actions of GABA in the brain.<sup>1,20</sup> The presence of high affinity, specific binding sites for BZs in the mammalian brain was then demonstrated by Squires, Haefely et al.<sup>3,21,22</sup> Converging lines of evidence established that these sites were contained in the same macromolecule as the GABA sites and the chloride channel, moreover that all three elements were coupled allosterically.<sup>23-25</sup> The term “GABA/BZ receptor” came into use for this complex (and is still encountered). Until recently, progress in this field was driven by the synthesis of a vast range of BZs and BZ-like drugs, which acted at the allosteric BZ sites of brain GABA<sub>A</sub> receptors and exhibited clinical anxiolytic or sedative properties which correlated to their binding-potencies in the CNS.<sup>26</sup> The structure of some ligands which act at the BZ site are shown in Figure 4.
1.3. **Pharmacology**

Overall, the design, synthesis and biological evaluation of αβγ (x1-6) subtype selective ligands will provide the pharmacological tools necessary to determine which GABA/BzR subtype mediates with physiological response. This will also provide entry into potential therapeutic agents to treat anxiety disorders, sleep disorders, epilepsy as well as enhance cognition in age-associated memory impairment in the absence of deleterious side effects. Ligands that bind to the BZ site can influence the binding of GABA to its receptor and thereby alter the flux of chloride ions through the ion channel. Ligands at the BZ site are categorized as agonist (positive allosteric modulator), inverse agonist (negative allosteric
modulator), or antagonist, all of which can bind with high affinity. Agonists enhance the effects of GABA by increasing the frequency of channel opening to provide a net hyperpolarization of the neuron and a decreased excitability. BZ inverse agonists have the opposite effect and decrease the flow of chloride ions (negative modulators, NAM) which results in a depolarization and an increased neuronal excitability. Between the two efficacy extremes, there is a continuum of partial agonists and partial inverse agonists as well as antagonists, the latter of which do not alter chloride flow and are functionally silent. These different efficacies are reflected in different behavioral effects of BzR site ligands in mammalian species.

The benzodiazepine binding site is the most explored of the GABA\textsubscript{A} receptor modulatory sites and this central role carries with it a direct influence on many diseases of the central nervous system.\textsuperscript{29-31} The interaction with benzodiazepines has been a major influence in studies on GABA receptors because of the long history of the therapeutic application of benzodiazepines as anxiolytics, anticonvulsants, sedative-hypnotics, and muscle relaxants.\textsuperscript{5,32} It has been found that certain disease states, as well as tolerance and dependence are related to the up regulation or down regulation of specific subunits.\textsuperscript{33} The sedative properties of diazepam appear to be due to the interaction at the $\alpha1$ subtype, whereas the anxiolytic effects originate from $\alpha2$ and/or $\alpha3$ subtypes (Table 1). In contrast, it has been reported that $\alpha1$ subtypes play a significant role in the sedative-hypnotic, ataxic, amnestic and addictive effects of benzodiazepines.
Table 1. Action of Benzodiazepine at GABA_\text{A} \alpha (1-6)\beta3\gamma2 Receptor Subtypes.\textsuperscript{33}

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Associated Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha_1)</td>
<td>Sedation, anterograde amnesia, some anticonvulsant action, ataxia and addiction</td>
</tr>
<tr>
<td>(\alpha_2)</td>
<td>Major anxiolytic, hypnotic (EEG) at higher doses maybe some muscle relaxation at high doses, some anticonvulsant action</td>
</tr>
<tr>
<td>(\alpha_3)</td>
<td>Some anxiolytic action, maybe some muscle relaxation at high doses</td>
</tr>
<tr>
<td>(\alpha_4)</td>
<td>Diazepam-insensitive site</td>
</tr>
<tr>
<td>(\alpha_5)</td>
<td>Cognition, temporal and spatial memory</td>
</tr>
<tr>
<td>(\alpha_6)</td>
<td>Diazepam-insensitive site</td>
</tr>
</tbody>
</table>

Modified in 2007 by Clayton, Cook et al. from a talk by Ruth McKerman at the ACNP meeting in Dec 2004.\textsuperscript{17}

The hippocampus is relatively enriched in \(\alpha_5\) containing GABA_\text{A} receptors compared to other brain areas.\textsuperscript{34} Interest in BzR/GABA_\text{A} \(\alpha_5\) subtypes has been stimulated by the report of Mohler et al.\textsuperscript{35} on \(\alpha_5\) “knock in” mice. This group has provided strong evidence that hippocampal extrasynaptic \(\alpha_5\) GABA_\text{A} receptors play a critical role in associative learning and memory.\textsuperscript{35} In addition, an \(\alpha_5\) subtype selective inverse agonist was shown by Bailey, Helmstetter, Cook et al.\textsuperscript{36} to be important in acquisition of fear conditioning and provided further evidence for the involvement of hippocampal GABA_\text{A}/benzodiazepine receptors in
learning and anxiety. A selective α5 inverse agonist might have therapeutic utility as an agent to enhance cognition without the unwanted side effects associated with activity at other receptor subtypes. Most drugs currently used in the treatment of cognitive deficiency act through the cholingric system and have moderate clinical efficacy. GABA_A α5 subtype selective inverse agonists may offer an alternative mechanism for the symptomatic treatment of memory impairment associated with Alzheimer’s disease and related dementias. This is, in part, because cholingeric neurons are destroyed rapidly in Alzheimers, while GABA(A) receptors remain functional throughout the disease or at least until the very end (McKernan et al.).

1.4. Molecular modeling

A simple unified pharmacophore receptor model for the agonist, antagonist and inverse agonists at the benzodiazepine binding site of GABA_A receptors has been developed in Milwaukee and is shown in Figure 5 using the techniques of chemical synthesis, radioligand binding and receptor mapping based on the synthesis of rigid ligands. The experimental data of recent and past years have been evaluated, and definite trends with regards to the orientation of the regions of the protein relative to the descriptors of the pharmacophore receptor model have been identified and are employed in this work by using this ligand-based pharmacophore receptor model and our α1β2γ2 GABA_A receptor model. The important points of the model include three anchor points which were termed L1, H1, and H2; region L1 represents a center of lipophilic interaction with the ligands, while H1, H2 and A2 represent two hydrogen bond donor sites and one hydrogen bond acceptor site on the receptor protein, respectively, in the receptor binding domain. Three additional lipophilic regions were also proposed, termed L2, L3 and LDi, which are
important for binding affinity and selectivity as well as efficacy of the ligands. It is noteworthy to mention that lipophilic interactions between a ligand and the receptor include Vander Waals interactions, as well as potential π-π and p-π stacking between the aromatic moieties of the ligand and groups on the receptor protein.

**Figure 5.** Diazepam in the Pharmacophore/Receptor Model for the BzR Site.

Pyrazolo[3,4-c]quinolin-3-one CGS-9896 (dotted line), a diazadiindole (thin line), and diazepam (thick line) fitted to the inclusive pharmacophore model for the BzR. Sites H1 and H2 represent hydrogen bond donor sites on the receptor protein complex, while A2 represents a hydrogen bond acceptor site necessary for potent inverse activity in vivo. L1, L2, L3 and LDi are four lipophilic regions in the binding pharmacophore. Descriptors S1, S2, and S3 are regions of negative steric repulsion.

Areas of negative steric repulsion with the protein are described as S1, S2, and S3. Examination of the included volumes indicated that the shapes of binding pockets for α1, α2 and α3 subtypes are very similar to each other. Region L2 for the α5 containing subtype
appeared to be larger in size than the analogous region of the other receptor subtypes. The LDi region in contrast appeared to be larger in the $\alpha_1$ subtype than in the other subtypes.\(^ {49}\)

The receptor subtype descriptor termed the DI site is related to $\alpha_4$ and $\alpha_6$ BzR, which is devoid of/or has a very small lipophilic pocket L\(_3\) in comparison to the other four ($\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_5$) subtypes. The DI region appears to be near the outside of the receptor binding cleft and may be important as well for interactions at the $\alpha_1$ subtype which results sometimes in increased $\alpha_1$ subtype selective affinity. With the aid of these models, several series of ligands have been synthesized and evaluated pharmacologically to determine the interaction with the GABA/benzodiazepine receptor subtype selective ligands as well as the physiologically related response.\(^ {17}\)

2. Objective of the Research

To employ PWZ-029 (19) as a template and prepare analogs related to this lead compound. The analogs must be similar in structure and electron density or the entire $\alpha_5$ subtype selectivity will change.\(^ {17}\) The goal is to make analogs that have 40-50% negative efficacy (in oocytes) as compared to 20% exhibited by PWZ-029, while maintaining little or no efficacy at $\alpha_1$, $\alpha_2$ and $\alpha_3$ subtypes.

3. Results and Discussion

3.1 Synthesis of 8-Substituted Imidazobenzodiazepines

As mentioned above, the $\alpha_5$ subtypes are primarily found in the hippocampus\(^ {34}\) which is an important brain region for memory and learning.\(^ {36}\) Studies carried out by Mohler et al.\(^ {35}\) on $\alpha_5$ knockin mice, as pointed out previously, provided strong evidence that hippocampal extrasynaptic $\alpha_5$ GABAA receptors play a critical role in associative learning.\(^ {35}\) The 8-
substituted imidazobenzodiazepines such as RY-23 and RY-24 exhibited a greater affinity and selectivity at the α5 subtype than ligands previously reported.\textsuperscript{36} Previously, the subtype selective inverse agonist activity\textsuperscript{49-53,54} of a series of α5 subtype selective ligands [(6, RY-023), (7, RY-024), (8, RY-079) and (9, RY-080)] was reported. The design was based on the structure of 5 (Ro 15-4513) and, in addition, McKernan, Atack et al. (see 10) reported several related ligands as well.\textsuperscript{55,56} These ligands are BzR inverse agonists \textit{in vivo} and have been shown to enhance cognition.\textsuperscript{13-16} One of these ligands was shown by Bailey et al.\textsuperscript{36} to be important in the acquisition of fear conditioning and provided further evidence for the involvement of hippocampal GABAA/benzodiazepine receptors in learning and anxiety. This was supported by the work of DeLorey et al.\textsuperscript{57} in a memory model with a ligand closely related to α5 subtype selective inverse agonists 6 and 7.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure6}
\caption{Ro 15-4513 (5), RY-023 (6), RY-024 (7), RY-079 (8), RY-80 (9) and the Merck compound (10).}
\end{figure}
The ligands 6-9 and 10 depicted in Figure 6 were 40-70 fold more selective for α5 subtypes in comparison to α1 subtypes; however, better subtype selectivity remains a goal of paramount importance today. In collaboration with Sieghart *et al.* the efficacy of inverse agonist RY 24 (7) was determined. It was demonstrated that RY 24 (7) was a potent inverse agonist at α5 subtypes in oocytes with a much weaker efficacy at the other subtypes.

**Figure 7.** Subtype selective efficacy of RY-24 (7); dose response curves for RY-24 in oocytes expressing different subunit combinations of GABA<sub>A</sub> receptors. Subtype combinations are indicated in legends. cRNA–injected Xenopus oocytes were held at −60 mV under two-electrode voltage clamp. Increasing concentrations of RY-24 were superfused together with a GABA concentration eliciting ~ 20% of the maximal current amplitude. RY-24 was pre-applied for 30 sec before the addition of GABA, which was co-applied with the drugs until a peak response was observed. Data were normalized for each curve assuming 100% for the response in the absence of RY-24. RY-24 was made up and diluted as a stock solution in DMSO. Final DMSO concentrations perfusing the oocyte were 0.1%. Values are presented as mean ± SD of at least 4 oocytes from at least 2 batches.
The lipophilic substituent at R₈ of RY-24 7 and RY-80 9 decreased the affinity for α₁, α₂ and α₃ subtypes; it retained affinity for α₅ subtypes. This data again supports the importance of the occupation of the lipophilic pocket L₂ for potent selectivity at the α₅ subtype.

**Table 2. In Vitro Receptor Binding Data of α₅ Ligands.** Binding Affinity at αxβ3γ2 GABA₅/BzR Receptor Subtypes (Values Reported in nM)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>R₈</th>
<th>R₃</th>
<th>α₁</th>
<th>α₂</th>
<th>α₃</th>
<th>α₅</th>
<th>α₆</th>
</tr>
</thead>
<tbody>
<tr>
<td>RY-24 (21)</td>
<td>H</td>
<td>t-Bu</td>
<td>26.9</td>
<td>26.3</td>
<td>18.7</td>
<td>0.4</td>
<td>5.1</td>
</tr>
<tr>
<td>RY-23 (20)</td>
<td>TMS</td>
<td>t-Bu</td>
<td>197</td>
<td>143</td>
<td>255</td>
<td>2.61</td>
<td>58.6</td>
</tr>
<tr>
<td>RY-80 (23)</td>
<td>H</td>
<td>Et</td>
<td>28.4</td>
<td>21.4</td>
<td>25.8</td>
<td>0.49</td>
<td>28.8</td>
</tr>
<tr>
<td>RY-79 (22)</td>
<td>TMS</td>
<td>Et</td>
<td>121</td>
<td>142</td>
<td>198</td>
<td>5.0</td>
<td>114</td>
</tr>
</tbody>
</table>


As previously stated, the hippocampus contains the greatest concentration of α₅-containing receptors in the CNS³⁴,⁵⁸ and it is possible that these hippocampal α₅ receptors may also regulate alcohol-motivated responding following systemic administration of an α₅-selective agent. Furthermore, RY-24 also antagonized the motor-impairing and sedative effects of ethanol in Long-Evans rats. Combined with additional studies within the ventral
pallidum (VP), it has been proposed that the GABAergic systems within the VP and hippocampal pathways may represent new extensions of the mesolimbic ethanol reward circuitry. Although these data do not strongly support a direct role for the modulatory influences of intrinsic efficacy in the behaviors examined, the synthesis of α5 subtype selective ligands provides researchers a unique opportunity to explore the role of this subtype in the neurobehavioral effects of alcohol.\textsuperscript{50,59} However, the partial efficacy (inverse agonist, negative modulation) of RY-23 and RY-24 at α1 and α2 subtypes precludes potential clinical uses of such agents for both agents are proconvulsant and RY-24 is also convulsant.

Additional behavioral studies on RY-24 were performed by Helmhstetter \textit{et al.} and provided further support for the role of the hippocampus in anxiety and learning.\textsuperscript{36} Moreover, the data suggested that Bz BSs within the hippocampus are important for the acquisition of fear conditioning. Although this subtype selective ligand has been shown to be an inverse agonist at the α5 subtype,\textsuperscript{51,60} this study suggested that RY-24 may act as an agonist at other alpha subtypes because larger doses of RY-24 were not as anxiogenic as the smaller doses and resulted in decreased learning. Consistent with the studies of Stephens \textit{et al.} using α5 knock-out mice\textsuperscript{61} and the efficacy studies of Lüddens, June and Cook \textit{et al.},\textsuperscript{62} these findings support the concept that the pharmacology observed depends upon the dose, behavioral paradigm employed and subunit composition activated. Ligands such as RY-24 have proven to be valuable in the study of the biochemical and pharmacological properties of GABA\textsubscript{A} receptors and have permitted insight into the role this protein plays in anxiety and learning. In order to repeat and further investigate the physiological processes mediated by RY-24 at α5 subtypes, a large scale synthesis was executed. In brief, isatoic anhydride
11 and sarcosine were heated in DMSO to form the benzodiazepine 12 in 84% yield. Benzodiazepine 12 was treated with bromine in the presence of CH$_3$COOH and H$_2$SO$_4$ to provide the bromide 13 in 80% yield, as illustrated in Scheme 1. Deprotonation of 13 with KOrBu in THF followed by treatment with diethyl chlorophosphate at -30 °C provided the intermediate enol phosphate. The enol phosphate was stirred with a solution of t-butyl isocyanate and KOrBu at -30 °C to yield the imidazobenzodiazepine 14 in 70% yield. This bromide was converted into 6 by a Heck-type coupling reaction using bis(acetate) bis(triphenylphosphine) palladium(II) to provide TMS analog RY 23 (6) and the silyl group was removed in high yield on treatment with TBAF/THF to provide gram quantities of RY 24 (7).

Scheme 1: Large Scale Synthesis of RY-23(6) and RY-24(7)
4. Synthesis of α5 subtype selective inverse agonist PWZ-029 on multigram scale

PWZ-029 (19) was effective in improving memory and cognition, especially in models with baseline cognitive deficits. The high specificity of PWZ-029 (19) for α5-containing GABA_A receptors has successfully negated adverse side effects of seizures and sedation, which are commonly observed with typical negative modulators (BZ) and nonselective inverse agonists of the GABA_A complex. The novel ligand PWZ-029, which was synthesized on large scale and characterized electrophysiologically, possesses in vitro binding selectivity and moderate inverse agonist functional selectivity at α5-containing GABA_A receptors (20%). This ligand has also been examined in rats in the passive and active avoidance, spontaneous locomotor activity, elevated plus maze and grip strength tests, primarily predictive of the effects on memory acquisition, basal locomotor activity, anxiety level and muscle tone, respectively. The improvement of task learning was detected at the dose of 5 mg/kg in the passive (hippocampal-driven), but not the active avoidance test. The inverse agonist PWZ-029 had no effect on anxiety nor muscle tone, whereas at higher doses (10 and 20 mg/kg) it decreased locomotor activity in this one paradigm. This effect was antagonized by flumazenil and also by the lower (but not the higher) dose of an agonist (SH-053-2’F-R-CH3) selective for GABA_A receptors containing the α5 subunit. The hypolocomotor effect of PWZ-029 was not antagonized by the antagonist β-CCt exhibiting a preferential affinity for α1-subunit containing receptors. These data suggest that moderate negative modulation at GABA_A receptors containing the α5 subunit is a sufficient condition for eliciting enhanced encoding/consolidation of declarative memory,
while the influence of higher doses of modulators at these receptors on motor activity shows an intricate pattern whose relevance and mechanism wait to be defined.

The effect on motor activity is often times confounded by stereotypical behavior. In all other studies PWZ-029 in rodents and primates, no sedation was observed. One desirable property in this regard is the pro-amnesic activity of BZ site inverse agonists, repeatedly reported in animal models,\(^{64,65}\) as well as in human volunteers.\(^{66,67}\) However, this desirable effect is confounded by different concomitant psychomotor effects (increased vigilance, anxiogenic and/or proconvulsant state), some of which have been described in memory studies with non-selective inverse agonists in humans, urging their early termination.\(^{66}\)

Point mutated mice could not be used to identify the receptor subtypes mediating the promnesic activity of inverse agonists because an unexplained switch to the agonist mode of action occurs when an inverse agonist at wild type diazepam-sensitive recombinant \(\text{GABA}_{\text{A}}\) receptors is altered in the respective point-mutated receptors.\(^{68,69}\) Thus, inverse agonists exerted agonistic-like sedative and anticonvulsant effects in mice with the point-mutated \(\alpha 1\) subunits,\(^{69}\) while the corresponding experiments in models of learning and memory were not performed.\(^{69}\) Nevertheless, behavioral examination of genetically modified animals conducted, to date, has indicated the \(\alpha 1\) and \(\alpha 5\) subunit-containing \(\text{GABA}_{\text{A}}\) receptors comprise the ‘memory-modulating’ population of these receptors.\(^{70,71}\) It is notable, as mentioned, that \(\text{GABA}_{\text{A}}\) receptors containing the \(\alpha 5\) subunit are abundantly expressed in the hippocampus,\(^{7,72,73}\) the structure substantially involved in memory formation.\(^{74}\) Recent evidence from animal studies with affinity-selective\(^{75}\) or efficacy-selective ligands\(^{76-78}\) has confirmed that the \(\alpha 5\) subunit was significantly involved in cognition enhancement mediated by the negative modulation of \(\text{GABA}_{\text{A}}\) receptor
functions. Moreover, it was shown in humans that pre-treatment with an α5 efficacy-selective inverse agonist significantly reduces the amnesic effect of alcohol on learning a word list. However, the affinity- or efficacy-selectivity of the ligands, as well as the diversity of the behavioral tasks used in their characterization to date, were of limited extent, which necessitated screening of newer BZ site negative modulatory ligands, to determine the putative therapeutic role of such compounds in various disorders with diminished cognitive capabilities in humans.

In this regard, the BZ site ligand PWZ-029 was synthesized (Scheme 2) and prepared on large scale. A mixture of 5-chloroisatoic anhydride and sarcosine in DMSO was heated to reflux to furnish benzodiazepine in 84% yield. Potassium t-butoxide was added to a solution of in THF at 0 °C and stirred for 20 min. The reaction was cooled to -35°C and diethylchlorophosphate was added slowly. After stirring at 0 °C for 30 minutes, the reaction mixture was cooled to -78 °C and ethyl isocyanoacetate was added followed by potassium t-butoxide to form in 89% yield. A solution of imidazobenzodiazepine can be reduced with LiBH4 to furnish alcohol in 67% yield as colorless crystals. To a slurry of KOH in DMSO at room temperature were added alcohol and CH3I to give PWZ-029 (19), as an off-white powder in 95% yield on 1-15 gram scale.
Electrophysiology experiments in *Xenopus* oocytes demonstrated that PWZ-029, at a concentration of 100 nM, significantly reduced GABA initiated control currents, thereby indicating a partial inverse agonist effect of PWZ-029 on GABA(A) receptors possessing the α5 isoform (Figure 8). Illustrated in Figure 8, are the concentration–effect curves for modulation of GABA_A elicited currents by PWZ-029 on *Xenopus* oocytes expressing GABA_A receptor subtypes α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2, concentrations of GABA_A that elicit 3% of the maximum GABA_A-triggered current of the respective cells were applied alone (EC₃) and with various concentrations of PWZ-029. Control currents represent responses in the absence of PWZ-029. Data points represent means±SEM from 4 oocytes from ≥2 batches [1μM PWZ-029 resulted in 114±4%, 105±8%, 118±5% and
80±4% of control current (at GABAA EC3) in α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors, respectively]. All these values except the one for α2β3γ2 receptors were significantly different from that of the respective control currents (p<0.01, Student's t-test).

Note that 100 nM is the pharmacologically relevant concentration.

**Table 3. In Vitro Binding Affinities of PWZ-029 at GABAA/BzR subtypes**

<table>
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<td>&gt;300</td>
<td>&gt;300</td>
<td>ND</td>
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<td>38.8</td>
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<tr>
<td>Moltech</td>
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<td>920</td>
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<td>ND</td>
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<tr>
<td>UNC-Roth</td>
<td>291.73</td>
<td>362.4</td>
<td>180.330</td>
<td>328.2</td>
<td>ND</td>
<td>6.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

**Figure 8.** Oocyte data for PWZ-029 by Sieghart et al.82
This ligand as mentioned, has also been examined in rats in the passive and active avoidance, spontaneous locomotor activity, elevated plus maze and grip strength tests, primarily predictive of the effects on the memory acquisition, basal locomotor activity, anxiety level and muscle tone, respectively. The improvement of task learning was detected at the dose of 5 mg/kg in the passive avoidance task (Figure 9). The passive avoidance task is a one trial fear-motivated avoidance task in which the mouse learns to refrain from stepping through a door to an apparently safer but previously punished dark compartment. The latency to refrain from crossing into the punished compartment serves as an index of the ability to avoid, and allows memory to be assessed.

![Fear conditioned contextual memory](image)

**Figure 9.** Fear conditioned contextual memory of PWZ-029

The PWZ-029 at 5 mg/kg, administered before the acquisition session, significantly increased retention session latency relative to the control group. The inverse agonist PWZ-029 (47) had no effect on anxiety or muscle tone, whereas at higher doses (10 and
20 mg/kg) it decreased locomotor activity. This effect was antagonized by flumazenil and also by the lower (but not the higher) dose of an agonist (SH-053-R-CH3-2'F) selective for GABA\textsubscript{A} receptors containing the \(\alpha5\) subunit. The hypolocomotor effect of PWZ-029 (47) was not antagonized by the antagonist BCCt, which exhibits a preferential affinity for \(\alpha1\)-subunit-containing receptors. These data suggest that moderate negative modulation at GABA\textsubscript{A} receptors containing the \(\alpha5\) subunit is a sufficient condition for eliciting enhanced encoding/consolidation of declarative memory, while the influence of higher doses of modulators at these receptors on motor activity shows an intricate pattern whose relevance and mechanism await to be defined.\textsuperscript{82} In fact, in the conflict paradigm in Rowlett’s lab in rhesus monkeys, PWZ-029 (19) was a weak anxiolytic with no sedation observed in either the suppressed or nonsuppressed portion of the test. Recently, Stables et al. at NINDS has shown PWZ029 to be a weak anticonvulsant. Therefore PWZ-029 (47) an \(\alpha5\) inverse agonist cannot be proconvulsant or convulsant, and, consequently, will not exhibit the side effects of other inverse agonists (NAM).

![Figure 10. Passive avoidance task\textsuperscript{82}](image-url)
The effects of DMCM (0.2 mg/kg) and PWZ-029 (2, 5 and 10 mg/kg) on retention performance in a passive avoidance task (*p<0.05 compared to solvent (SOL) group). Number of animals per treatment group: 10.

**CANTAB Approach (Rowlett, and Cook et al.)**

Recent studies by Rowlett, and Cook et al. on PWZ-029 showed that this ligand acted as an inverse agonist at α5/GABA_A receptor subtypes which enhanced memory. Rowlett has used several primate models, including the Cambridge Neuropsychological Test Automated Battery, or CANTAB. A clear strength of the CANTAB approach is that its tasks are based on corresponding procedures developed for human patients, which greatly facilitate the ultimate goal of translating findings from monkeys to humans.

Because PWZ-029 has been shown to be safe and to have cognitive-enhancing effects in rodents, experiments were initiated with this compound in rhesus monkeys as mentioned. Initial results, shown in Figure (11), are quite provocative and exciting: PWZ-029 enhanced performance in the DNMS (Delayed non-matching to sample) task using the 10-min delay with distracters (Figure 11A) and although it had no effect alone, PWZ-029 completely reversed the scopolamine-induced deficit in the ORD (Object Retrieval with Detours) task (Figure 11B). Interestingly, PWZ-029 induced anti-conflict (anxiolytic) effects in monkeys without the concomitant response rate suppressing effects characteristic of BZ-type drugs such as diazepam (Figure 11C). It is felt that these latter effects are due to PWZ-029’s partial agonist effects at α2/3GABA_A receptor subtypes, whereas the cognition-enhancing effects of this compound are due to inverse agonist (NAM) action at α5/GABA_A receptors. The lack of negative efficacy in PWZ-029 at α1, α2 and α3 subtypes as compared to proconvulsant/convulsant RY-24 is responsible for the safe profile of PWZ-029.
PWZ-029 (19) provides an important lead compound based not only on potential effectiveness against cognitive impairment, but also as a potential treatment for anxiety and agitation in older patients with dementias, similar to the anxiolytic effects of classical BZs (Meehan et al. 2002). Studies will continue with PWZ-029 and expand them to include comparative studies with non-selective agonists and α5GABA_\text{A}-preferring agonists; as well as with pre-treatments with α5GABA_\text{A}-preferring antagonists such as XLi-093. In addition, in our initial screens compounds will be sought with similar, and hopefully improved, efficacy profiles. For example, a compound that is an α5GABA_\text{A} partial inverse agonist, an α2/3GABA_\text{A}-preferring partial agonist, but ineffective at α1GABA_\text{A} receptors is both desirable and feasible in our synthesis/screening program.
Figure 11: Preliminary profile of cognitive enhancing and anxiolytic-like effects of PWZ-029 in monkeys. Data are from n=2 monkeys (A & C) and n=4 monkeys (B). Red arrow indicates the dose (0.01mg/kg, i.v.) of PWZ-029 tested in A & B Importantly, in the ORD task, PWZ-029 showed only a modest trend for enhancement of performance (Fig. 12A), but when task difficulty was increased by testing with difficult trials only, PWZ-029 robustly increased performance (Fig. 12B). This enhancement was reversed by administration of the α5GABA<sub>A</sub>-preferring antagonist XLI-093, and this antagonism, in turn, was reversed by increasing the dose of PWZ-029 (Fig. 12C). Finally, PWZ-029 completely reversed deficits in performance induced by the memory-impairing anti-cholinergic scopolamine (Fig. 12D).

Figure 12. Cognitive-enhancing effects of PWZ-029 in the rhesus monkey Object Retrieval with Detours (ORD) task (N=5 monkeys). A. Effects of PWZ-029 on ORD tests
consisting of both easy and difficult trials. No effect of the compound was observed (mean % correct first reaches, p>0.05, repeated measures ANOVA). B. PWZ-029 enhanced performance in the ORD task when tested with difficult trials only (*p<0.05 vs. vehicle, V, Bonferroni t-tests). C. Enhancement of ORD performance by 0.018 mg/kg of PWZ-029 was attenuated by the α5GABA_A-preferring antagonist XLi-093, and this antagonism was surmountable by increasing the PWZ-029 dose (*p<0.05 vs. vehicle, **p<0.05 vs. 0.018 mg/kg PWZ-029, Bonferroni t-tests). D. PWZ-029 reversed performance impaired by 0.01 mg/kg of scopolamine. (*p<0.05 vs. vehicle; **p<0.05 vs. scopolamine alone, Bonferroni t-tests).

5. Synthesis of PWZ-029 analogs

In order to obtain ligands with potentially a better efficacy profile (oocytes) than PWZ-029, negative modulation of 40% to 50% as compared to 20% is a goal of these studies. The approach is in agreement with our PWZ-029 template and by a recent study on compounds (NAM) patented by Roche. Several new analogs have been proposed here and docked in the Milwaukee-based pharmacophore by Clayton et al.

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Affinities for $\alpha_x\beta_3\gamma_2 (x=1-6)$ benzodiazepine receptors isoforms

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<td>328.2</td>
<td>ND</td>
<td>6.2</td>
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</tbody>
</table>

**Figure 13:** Receptor binding affinity data of PWZ-029 (19) determined by Merck, Moltech and Roth for Cook et al. and the Roche difluoro ligand 28. The new analogs of PWZ-029 are of strong interest due to the recent results on contextual memory impairment data wherein doses of scopolamine decreased memory while the $\alpha_5$ ligand reversed this, as described above.

**Synthesis of PB-I-052**

18 (1.5 gram)

21
Synthesis of PB-I-054

\[
\begin{align*}
\text{Cl} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{Cl} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{N} \\
\text{Br} & \quad \text{Br} & \quad \text{Br} & \quad \text{Br} \\
\text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{N} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} \\
\text{Cl} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{Cl} \\
\end{align*}
\]

\[\text{NaH, CH}_3\text{CH}_2\text{SH} \quad \text{DMF, rt, 80\%} \]

21 (1 gram) \rightarrow 22

Synthesis of PB-I-056

\[
\begin{align*}
\text{Cl} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{Cl} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{N} \\
\text{Br} & \quad \text{Br} & \quad \text{Br} & \quad \text{Br} \\
\text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 & \quad \text{CH}_3 \\
\text{N} & \quad \text{N} & \quad \text{N} & \quad \text{N} \\
\text{O} & \quad \text{O} & \quad \text{O} & \quad \text{O} \\
\text{Cl} & \quad \text{Cl} & \quad \text{Cl} & \quad \text{Cl} \\
\end{align*}
\]

\[\text{NaH, NaSMe} \quad \text{DMF, rt, 82\%} \]

21 (1 gram) \rightarrow 23

\[\text{N} \quad \text{N} \quad \text{N} \quad \text{O} \quad \text{Cl} \quad \text{CH}_3 \quad \text{Br} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{O} \quad \text{Cl} \quad \text{CH}_3 \quad \text{S} \quad \text{NaH}, \text{CH}_3\text{CH}_2\text{SH} \quad \text{DMF, rt, 80\%} \]

\[\text{N} \quad \text{N} \quad \text{N} \quad \text{O} \quad \text{Cl} \quad \text{CH}_3 \quad \text{Br} \quad \text{N} \quad \text{N} \quad \text{N} \quad \text{O} \quad \text{Cl} \quad \text{CH}_3 \quad \text{S} \quad \text{NaH}, \text{NaSMe} \quad \text{DMF, rt, 82\%} \]

Figure 14. Oocyte data of PB-I-056
Synthesis of PB-I-059

\[
\begin{align*}
\text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{O} & \quad \text{C}_3
\\
\text{N} & \quad \text{C}_3
\\
\end{align*}
\]

\[\begin{array}{c}
\text{SOCl}_2, \text{CH}_2\text{Cl}_2 \\
\text{reflux 2h;} \\
\text{CH}_2\text{Cl}_2, 0^\circ\text{C} \\
\text{CH}_2\text{CH}_2\text{SH}, \text{Et}_3\text{N} \\
70\%
\end{array}\]

\[\begin{array}{c}
\text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{O} & \quad \text{C}_3
\\
\text{N} & \quad \text{C}_3
\\
\end{array}\]

Synthesis of PB-I-060

\[
\begin{align*}
\text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{O} & \quad \text{C}_3
\\
\text{N} & \quad \text{C}_3
\\
\end{align*}
\]

\[\begin{array}{c}
\text{SOCl}_2, \text{CH}_2\text{Cl}_2 \\
\text{reflux 2h;} \\
\text{CH}_2\text{Cl}_2, 0^\circ\text{C} \\
\text{NaSMe, 75\%}
\end{array}\]

\[\begin{array}{c}
\text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{O} & \quad \text{C}_3
\\
\text{N} & \quad \text{C}_3
\\
\end{array}\]

Synthesis of PB-I-061

\[
\begin{align*}
\text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{Br} & \quad \text{C}_3
\\
\text{N} & \quad \text{C}_3
\\
\end{align*}
\]

\[\begin{array}{c}
\text{NaH, } \\
\text{rt, 3hrs 70\%}
\end{array}\]

\[\begin{array}{c}
\text{Cl} & \quad \text{N} & \quad \text{N} & \quad \text{O} & \quad \text{C}_3
\\
\text{N} & \quad \text{C}_3
\\
\end{array}\]
Synthesis of PB-I-063

From the recent work of Rowlett, it was demonstrated that novel α5 selective inverse agonist PWZ-029 (19) enhanced cognition in rhesus monkeys in the CANTAB paradigm. This ligand had the ability to reverse cholinergic deficits in performance induced by the antimuscarinic scopolamine under mixed trial conditions. In the ORD task, PWZ-029 showed only a modest trend for enhancement of performance, but when task difficulty was increased by testing with difficult trials only, PWZ-029 robustly increased performance. This enhancement was reversed by administration of the α5 GABA (A) subtype selective antagonist XLi-093 and this antagonism in turn was reversed by increasing the dose of PWZ-029. In addition, PWZ-029 enhanced performance in the DNMS task using the 10
minute delay with distracters. This ligand also exhibited anxiolytic activity in some primates and was a weakly active anticonvulsant in rats (NINDS, Jim Stables et al). These findings are consistent with a role for PWZ-029 (21) at α5 GABA<sub>A</sub> receptors in the treatment of age associated memory impairment and Alzheimer’s disease. It is the subject of an SBIR I grant submitted December 5th, 2012 by Physiogenix which was funded by NIH.

7. General Experimental Details

All reactions were carried out under an argon atmosphere with dry solvents using anhydrous conditions unless otherwise stated. Tetrahydrofuran (THF) and diethyl ether were freshly distilled from Na/benzophenone ketyl prior to use. Dichloromethane was distilled from calcium hydride prior to use. Methanol was distilled over magnesium sulfate prior to use. Benzene and toluene were distilled over sodium and acetonitrile was distilled over CaH<sub>2</sub> prior to use. Reagents were purchased of the highest commercial quality and used without further purification unless otherwise stated. Thin layer chromatography (TLC) was performed using Dynamic Adsorbents Inc. UV active silica gel, 200 µm, plastic backed plates on Dynamic Adsorbents Inc. UV active alumina N, 200 µm, F254 plastic backed plates. Flash and gravity chromatography were performed using silica gel P60A, 40-63 µm purchased from Silicycle. Proton (1H NMR) and carbon high resolution nuclear magnetic resonance spectra (13C NMR) were obtained on a Bruker 300-MHz or a GE 500-MHz NMR spectrometer. 1H NMR data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, dt = doublet of triplets, ddd = doublet of doublet of doublet, td = triplet of doublets, qd = quartet of doublets, sex = sextet, m = multiplet), integration, and coupling constants (Hz). 13C NMR
data are reported in parts per million (ppm) on the δ scale. The low resolution mass spectra (LRMS) were obtained as electron impact (EI, 70eV), which were recorded on a Hewlett-Packard 5985B gas chromatography-mass spectrometer, while high resolution mass spectra (HRMS) were recorded on a VG Autospec (Manchester, England) mass spectrometer. HRMS recorded by electrospray ionization (ESI) methods were performed at the Laboratory for Biological Mass Spectrometry at Texas A&M University on an API QStar Pulsar model, manufactured by MDS Sciex.

4-Methyl-3,4-dihydro-1H- benzo[e][1,4]diazepine-2,5-dione(12): A mixture of isatoic anhydride 11 (20g, 101 mmol) and sarcosine (9.02g, 101 mmol) in DMSO (160mL) was heated at 150°C for 5 hr, after which the solution was cooled to rt and poured into ice water (750mL) to furnish a light brown solid. This solid was collected by filtration, washed with water (3x200mL) and dried. The benzodiazepine 12 was obtained as a light brown solid (19g, 84%). This material was used directly in the next experiment.

7-Bromo-4-methyl-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione(13):

Benzodiazepine 12 (32.4g, 0.17mol) was added to a solution of acetic acid (400mL) in a 3-neck reaction flask. Sodium acetate (27.9g, 0.34mol) was added to this solution. Bromine (13.1mL, 0.255mol) was added using an addition funnel and the solution which resulted was allowed to stir for 18h and then poured into ice water (200mL) to provide a white solid. This solid was collected by filtration and then washed (3x200mL) and dried (Na$_2$SO$_4$). The bromobenzodiazepine 13 (36.6g, 80%) was used directly in the next experiment. mp 240-242 °C; 1H NMR (DMSO-d6, 300MHz) δ10.54 (br s, 1H), 7.81 (d, 1 H, J = 2.2 Hz), 7.67 (dd, 1 H, J = 2.4,8.6 Hz), 7.04 (d,l H,J = 8.7 Hz),3.87 (s,2 H),3.09 (s,3 H); MS (CI) m/z 269 (M + H).
**tert-Butyl-8-bromo-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (14):** The t-BuOK (7.50 g, 66.91 mmol) was added to a solution of benzodiazepine 13 (15 g, 55.76 mmol) in anhydrous THF (1500 mL) at 0°C and the mixture which resulted was stirred for 20 min. The reaction mixture was cooled to −35 °C and diethyl chlorophosphate (10.47 mL, 72.48 mmol) was added slowly. After stirring at 0°C for 30 min the mixture was cooled to −78°C and t-butyl isocyanooacetate (8.926 mL, 61.33 mmol) was added, and this was followed by the addition of t-BuOK (6.88g, 61.33 mmol) at −78°C. After stirring at rt for 4 h the reaction was quenched with a saturated aq NaHCO₃ solution (500 mL) and extracted with EtOAc (3 × 1000 mL). The combined organic layers were dried (Na₂SO₄) and concentrated to give a solid residue. This solid residue was triturated with Et₂O (250 mL) and the ester 14 precipitated as an off-white solid. The mother liquor was further purified by flash chromatography on silica gel (gradient elution 40–60% EtOAc in hexane) to afford additional ester 14 in a combined yield of (15.3g, 70%). **mp** 180-183 °C; **1H NMR** (CDCl₃, 300MHz) δ 8.33 (s, 1H), 7.90 (s, 1H), 7.62 (d, 1H, J =10.8 Hz), 7.03 (d, 1H, J = 8.6 Hz), 4.71 (br, s, 1H), 4.13 (br, s, 1H), 3.10 (s, 3H), 1.56 (s, 9H); **MS (EI) m/e** 394 (M+, 100).

**tert-Butyl-8-(trimethylsilyl)-acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine3-carboxylate (6):** A mixture of imidazobenzodiazepine 14 (7.5g, 19.12mmol) was dissolved in dry triethyl amine (223mL ) and acetonitrile (149mL; 3:2 ratio). This mixture was degassed under argon three times. To this degassed mixture, bis (triphenylphosphone)-palladium (II) acetate (1g, 7 mol %) was added. The mixture was again degassed (with vacuum) three times under argon. Trimethylsilyl acetylene (5.52mL, 38.24mmol) was added and this reaction mixture was heated to reflux for 8h under argon.
The reaction mixture was cooled to rt and the solvents were concentrated under reduced pressure. This concentrated mixture was dissolved in ethyl acetate and passed through a bed of celite. After the removal of solvent under reduced pressure the residue was purified by flash chromatography (silica gel, EtOAc) to afford a yellow solid of **RY-23** (6) (6.5g, 83%). **mp** 199-200 °C. **IR** (KBr) 2971, 2156, 1723, 1662, 1500 cm⁻¹; **1H NMR** (CDCl₃, 300MHz) δ 8.13 (s, 1H), 7.84 (s, 1H), 7.64 (d, 1H, J = 8.2 Hz), 7.32 (d, 1H, J = 8.2 Hz), 5.11 (br, 1H), 4.31 (br, 1H), 1.63 (s, 9H), 0.25 (s, 9H); **MS** (EI) m/e 409 (M+, 4), 354 (19), 353 (82), 335 (45), 307 (100), 279 (23), 251 (16), 143 (13), 160 (21), 107 (14); **Anal. Calcd for C₂₂H₂₇N₃O₃Si:** C, 64.52; H, 6.64; N, 10.26; Found C, 64.57; H, 6.70; N, 10.46. The spectral data for 6 were in excellent agreement with the literature.⁵⁴

**tert-Butyl-8-acetylene-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate (7):** A solution of 20 (1.2g, 2.93 mmol) in THF (360mL) was treated with a solution of TBAF solution (1.0M in THF; 3.52mL,3.51mmol) at -30°C. The mixture which resulted was allowed to stir for 30 min at rt, after which the mixture was added to H₂O (50mL) and extracted with EtOAc (3x100mL). The combined organic extracts were washed with brine (50mL) and dried (Na₂SO₄). After removal of the solvent under reduced pressure, the residue was purified by a wash column (silica gel, EtOAc) to give 7 (RY-24) as a yellow solid (0.89g, 90%): **mp** 213-214 °C. **1H NMR** (CDCl₃, 300MHz) δ 8.16 (s, 1H), 7.85 (s, 1H), 7.69 (d, 1H, J = 8.3 Hz), 7.35 (d, 1H, J = 8.3 Hz), 5.15 (br, 1H), 4.32 (br, 1H), 3.23 (s, 3H), 3.20 (s, 1H), 1.63 (s, 9H); **MS** (EI) m/e 337 (M+, 2), 282(15), 281 (78), 264 (31), 263(56), 235 (100), 229(22), 207 (36), 138 (16), 101(19); **Anal. Calcd for C₁₉H₁₉N₃O₃:** C, 67.64; H, 5.68; N, 12.45; Found C, 67.50; H,
5.78; N, 12.41. The spectral data for 7 were in excellent agreement with the reported values.\textsuperscript{54}

**7-Chloro-4-methyl-3,4-dihydro-1H-benzo[e][1,4]diazepine-2,5-dione (16):** A mixture of 5-chloroisatoic anhydride 15 (20g, 101mmol) and sarcosine (9.02g, 101mmol) in DMSO (160mL) was heated at 150°C for 5 hr, after which it was cooled to rt and poured into ice water (750mL) to furnish a light brown solid. This solid was collected by filtration, washed with water (3x200mL) and dried (Na\textsubscript{2}SO\textsubscript{4}). The benzodiazepine 16 was obtained as a light brown solid (19g, 84%). This material was used directly in the next experiment.

**Ethyl-8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5][1,4]benzodiazepine3-carboxylate (17):** t-BuOK (8.99 g, 80.14 mmol) was added to a solution of amide 16 (15 g, 66.77 mmol) in anhydrous THF (1500 mL) at 0 °C and the solution allowed to stir for 20 min. The reaction mixture which resulted was cooled to −35 °C and diethyl chlorophosphate (12.48 mL, 86.80 mmol) was added slowly. After stirring at 0 °C for 30 min, the mixture was cooled to −78 °C and ethyl isocyanoacetate (8.03 mL, 73.44 mmol) was added and this was followed by the addition of t-BuOK (8.24g, 73.44mmol). After stirring at rt for 4 h, the reaction mixture was quenched with a saturated aq solution of NaHCO\textsubscript{3} (500 mL) and extracted with EtOAc (3 × 1000 mL). The combined organic layers were dried (Na\textsubscript{2}SO\textsubscript{4}) and concentrated to give a solid residue. This solid residue was treated with Et\textsubscript{2}O (250 mL) and the ester 17 was precipitated as an off-white solid. The mother liquor was further purified by flash chromatography on silica gel (gradient elution, 40–60%, EtOAc in hexane) to afford additional ester 17 with an overall yield of (19g, 89%). mp 192-193 °C; 1H NMR (CDCl\textsubscript{3}, 300MHz) δ 8.1 (d, 1H, J = 2.4 Hz), 7.90 (s, 1H), 7.62 (dd, 1H, J = 8.7, 2.5 Hz), 7.40 (d, 1H, J = 8.6 Hz), 5.23 (br, s, 1H), 4.46 (q, 2H, J=7.12Hz),
4.13 (br, s, 1H), 3.27 (s, 3H), 1.47 (t, 3H, J=7.12Hz); MS (EI) m/e 319 (M+, 100). The data for this material were identical to the published values.\textsuperscript{14}

\textbf{8-Chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4]benzodiazepine-3-methyl alcohol (18).} A solution of imidazobenzodiazepine \textbf{17} (5 g, 15.6 mmol) in a mixture of ethyl ether (50 mL), and THF (50 mL) was stirred with LiBH\textsubscript{4} (2.0 M in THF, 9 mL, 18 mmol). The mixture which resulted, was then heated to reflux for 30 min, after which it was cooled to rt and treated with a saturated aq solution of NaHCO\textsubscript{3} (5 mL). The solvent was concentrated and the residue was taken up in EtOAc (100 mL). The organic layer was washed with water (2 × 20 mL), brine (20 mL) and dried (MgSO\textsubscript{4}). After removal of the solvent under reduced pressure the residue was purified by flash chromatography (silica gel, EtOAc) to afford alcohol \textbf{18} as colorless crystals (2.9 g, 67\%): mp 252-253 °C; \textbf{IR} (KBr) 3500 (br, OH), 3100, 1667, 1612, 823 cm\textsuperscript{-1}; \textbf{1H NMR} (CDCl\textsubscript{3}, 300MHz) δ 8.00 (d, 1H, J = 2.4 Hz), 7.80 (s, 1H), 7.55 (dd, 1H, J = 8.7, 2.4 Hz), 7.30 (d, 1H, J = 8.6 Hz), 4.70 (d, 2H, J = 4.2 Hz), 4.40 (s, 2H), 3.20 (s, 3H); MS (EI) m/e 279 (M+, 41), 277 (M+, 100), 259 (84), 246 (55), 231(41). The data for this material were identical to the published values.\textsuperscript{15}

\textbf{Methyl(8-chloro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-α][1,4] benzodiazepin-3-yl)methyl ether (19).} The alcohol 18 (3g, 11 mmol) was dissolved in DMSO (56 mL) and KOH (2.41g, 44 mmol) was added to it. The mixture which resulted was stirred for 2-3 minutes and then excess CH\textsubscript{3}I added to it. Then the reaction mixture was stirred for 5-10 min and poured into ice water (100 mL) after which it was extracted with EtOAc (3 × 100 mL). The combined organic extracts were washed with brine (50mL) and dried (MgSO\textsubscript{4}). After the removal of solvent under reduced pressure, the residue was purified by a wash
column on silica gel (EtOAc) to give ether 19 (PWZ-029) as an off-white powder (2.83g, 90%): mp 193-194 °C; IR (KBr) 3122, 2973, 1632, 1611, 811 cm⁻¹; 1H NMR (CDCl₃, 300MHz) δ 8.00 (d, 1H, J = 2.4 Hz), 7.80 (s, 1H), 7.55 (dd, 1H, J = 8.7, 2.5 Hz), 7.30 (d, 1H, J = 8.6 Hz), 4.55 (br, 2H), 4.38 (s, 2H), 3.42 (s, 3H), 3.18 (s, 3H). The spectral data for this material were identical to the published values.

8-Chloro-5-methyl-6-Oxo-5,6-dihydro-4H-benzo-[f]-imidazo-[1,5-a]-[1,4]-diazepine-3-carboxylic acid (20). The ester 17 (2 g, 6 mmol) was dissolved in EtOH (150 mL) to form a solution and then 10% aq sodium hydroxide solution (40 mL) was added to the mixture. The mixture was heated to reflux for 0.5 h and the EtOH was removed under reduced pressure, after which the solution was allowed to cool. The pH was then adjusted to 4 by adding cold 10% aq HCl dropwise. The solid which precipitated was filtered and the solid acid which was isolated was washed with water and ether. The solid was dried to provide acid 20: mp >260°C; IR (neat) 3100, 2424, 1661, 1642, 1562 cm⁻¹; 1H NMR (300 MHz, DMSO) δ 8.35 (s, 1H), 7.77-7.88 (m, 3H), 5.28 (br s, 2H), 4.55 (br s, 2H), 3.09 (s, 3H); MS (EI) m/e (relative intensity) 291 (M+, 44), 273 (61), 245 (100), 230 (47), 217 (62), 75 (37). Anal. Calcd. for C₁₃H₁₀ClN₃O₃: C, 50.57; H, 3.37; N, 13.32. Found: C, 50.64; H, 3.25; N, 13.12.

3-(Bromomethyl)-8-chloro-5-methyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-6(5H)-one (21). The alcohol 18 (500mg, 1.80mmol) was dissolved in anhydrous CH₂Cl₂ (100mL). To this mixture, a solution of PBr₃ (0.378 mL, 3.61mmol) was added at °C and the reaction was allowed to stirred for 2h. The reaction mixture was then quenched with a saturated aq solution of Na₂CO₃ and extracted with CH₂Cl₂. The combined organic layers were washed with brine and dried (MgSO₄). The solvent was removed under reduced
pressure to afford bromide analog (21) as a white powder 80%. 1H NMR (CDCl₃, 300MHz) δ 8.05 (d, 1H, J = 2.3Hz), 7.83 (s, 1H), 7.59 (dd, 1H, J = 2.39 Hz, 6.23 Hz), 7.35 (d, 1H, J = 8.25Hz), 4.60 (br, s, 2H), 4.38 (br, s, 2H), 3.10 (s, 3H). 13C NMR (75 MHz, CDCl₃): δ 165.38, 134.79, 134.54, 134.15, 132.75, 132.61, 131.11, 130.14, 127.59, 123.11, 42.32, 36.12, 24.26. HRMS (ES+) calculated for C₁₃H₁₁ClN₃O: 260.0591; found: 260.0586. This material was used in a later step.

8-chloro-3-((ethylthio)methyl)-5-methyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-6(5H)-one (22). The bromide 21 (100mg, 2.96 mmol) was dissolved in DMF (1.5mL) at rt. In a separate flask, NaH (70mg, 2.9 mmol) was dissolved in CH₃CH₂SH (220mg, 3.54 mmol). The solution of NaH and CH₃CH₂SH which resulted was added to the bromide and the reaction mixture was stir overnight. The reaction mixture was then quenched with water and extracted with ethyl acetate. The combined organic layers were washed with brine and dried (MgSO₄). The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (gradient elution 40-60% EtOAc : hexane) to afford 22 with an overall yield of 80%. 1H NMR (CDCl₃, 300MHz) δ 8.40 (d, 1H, J = 2.4Hz), 7.80 (s, 1H), 7.55 (dd, 1H, J = 8.3 Hz, 2.39 Hz), 7.34 (d, 1H, J = 8.6Hz), 4.40 (s, 2H), 3.82 (s, 3H), 2.62 (m, 2H), 1.32(t,3H). 13C NMR (75 MHz, CDCl₃): δ 164.89, 135.80, 133.86, 133.73, 132.61, 132.52, 131.42, 130.08, 126.38, 123.08, 42.40, 35.97, 27.65, 26.01, 14.46 HRMS (ES+) calculated for C₁₅H₁₇ClN₃OS: 322.0781, Found Mass: 322.0770

8-Chloro-5-methyl-3-((methylthio)methyl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-6(5H)-one (23). The bromide 21 (200mg, 0.587mmol) was dissolved in DMF (20ml). To this mixture, NaSCH₃ (411mg, 5.86mmol) was added and the reaction mixture was allowed
to stir overnight. The reaction mixture was then quenched with water and extracted with ethyl acetate. The combined organic layers were washed with brine and dried (MgSO₄). The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (gradient elution 3:2 EtOAc : hexane) to afford 23 (PB-I-056) with an overall yield of 85%. 1H NMR (CDCl₃, 300MHz) δ 8.04 (d, 1H, J = 2.4Hz), 7.89 (s, 1H), 7.58 (d, 1H, J = 8.4, 2.4 Hz), 7.35 (d, 1H, J = 8.4Hz), 4.45 (s, 2H), 3.8 (s, 2H), 3.28 (s, 3H), 2.23(s, 3H). 13C NMR (75 MHz, CDCl₃): δ 165.60, 135.59, 133.86, 133.73, 132.61, 132.52, 131.47, 130.08, 126.38, 123.08, 42.36, 35.92, 29.98, 15.54. HRMS (ES+) calculated for C₁₄H₁₅ClN₃OS: 308.0624, Found Mass: 308.0620.

S-ethyl-8-chloro-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5a][1,4]diazepine-3-carbothioate (24). The acid 20 (100mg, 0.297mmol) was dissolved in dry CH₂Cl₂ (20mL) at room temperature, excess SOCl₂ was added in one portion. The reaction mixture was heated to reflux for 2h until analysis of the mixture by TLC indicated the absence of starting material. The reaction mixture was concentrated under reduced pressure on a rotavapor a few times with CH₂Cl₂ to remove the excess SOCl₂ and dried under vaccum. The solid which resulted was stir in dry CH₂Cl₂ (20 mL) at 0°C. To this reaction mixture, Et₃N (0.5) and CH₃CH₂SH (0.185mg, 2.97mmol) were added and allowed to stir overnight. The mixture which resulted was then quenched with H₂O. The combined organic layers were washed with brine and dried MgSO₄. After the removal of the solvent under reduced pressure, the residue was purified by flash chromatography on silica gel (gradient elution 3:2 EtOAc : hexane) to afford 24 (PB-I-059) in an overall yield of 70%. 1H NMR CDCl₃, 300MHz) δ 8.07 (d, 1H, J = 2.19Hz), 7.88 (s, 1H), 7.62 (d, 1H, J = 2.4, 6.2 Hz), 7.39 (d, 1H, J = 8.5Hz), 5.19 (br, s,1H), 4.38 (br,s, 1H), 3.25 (s, 3H), 3.07(m, 2H),
1.38(t, 3H). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 188.33, 165.18, 134.91, 134.52, 132.79, 132.69, 131.41, 130.54, 130.17, 123.21, 42.03, 36.17, 22.64, 14.66. HRMS (ES+) calculated for C$_{15}$H$_{15}$ClN$_3$O$_2$: 336.0574, Found Mass: 336.059.

**S-Methyl8-chloro-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carbothioate** (25). The acid 20 (100mg, 0.297mmol) was dissolved in dry CH$_2$Cl$_2$ (20mL) at room temperature, excess SOCl$_2$ was added in one portion. The reaction mixture was heated to reflux for 2h until analysis of the mixture by TLC indicated the absence of starting material. The reaction mixture was concentrated under reduced pressure on a rotavapor a few times with CH$_2$Cl$_2$ to remove the excess SOCl$_2$ and dried under vaccum. The solid which resulted was stir in dry CH$_2$Cl$_2$ (20 mL) at 0°C. To this reaction mixture, NaSCH$_3$ (82mg, 1.2mmol) was added and allowed to stir overnight. The mixture which resulted was then quenched with H$_2$O. The combined organic layers were washed with brine and dried MgSO$_4$. After the removal of the solvent under reduced pressure, the residue was purified by flash chromatography on silica gel (gradient elution 3:2 EtOAc : hexane) to afford 25 (PB-I-060) in an overall yield of 75%. 1H NMR (CDCl$_3$, 300MHz) δ 8.08 (d, 1H, J = 2.1Hz), 7.90 (s, 1H), 7.63 (d, 1H, J = 8.03 Hz), 7.40 (d, 1H, J = 8.45Hz), 5.20 (br, s, 1H), 4.37 (br, s, 1H), 3.26 (s, 3H), 2.48 (S, 2H). $^{13}$C NMR (75 MHz, CDCl$_3$): δ 188.71, 165.19, 134.93, 134.58, 132.79, 132.71, 131.34, 130.55, 123.19, 42.02, 36.18, 11.03. HRMS (ES+) calculated for C$_{14}$H$_{13}$ClN$_3$O$_2$: 322.0417, Found Mass: 322.0446.

**8-Chloro-3-(isopropoxymethyl)-5-methyl-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-6(5H)-one** (26). To a slurry of NaH (84mg, 3.5mmol) in 2-propanol (4.5 ml) at rt was added to bromide 21 (100mg, .304mmol). The reaction mixture was allowed to stir for 3hr
at rt and then quenched with water. The combined organic layers were washed with brine and dried MgSO₄. After the removal of the solvent under reduced pressure, the residue was purified by flash chromatography on silica gel (gradient elution 4:1 EtOAc: hexane) to afford 26 (PB-I-061) with an overall yield of 75%. 1H NMR (CDCl₃, 300MHz) ) δ 8.04 (d, 1H, J = 2.39Hz), 7.81 (s, 1H), 7.56 (d, 1H, J = 8.4,2.4 Hz), 7.35 (d, 1H, J = 8.4Hz), 4.61 (s, 2H), 3.8 (m, 1H), 3.28 (s, 3H), 1.267(d, 9H, J = 5.8) 13C NMR (75 MHz, CDCl₃): δ165.61, 135.92, 133.94, 133.61, 132.52, 132.43, 131.47, 130.24, 127.02, 123.05, 71.70, 64.23, 42.42, 35.53, 29.68, 22.13. HRMS (ES+) calculated for C₁₆H₁₉ClN₃O₂: 320.1166, Found Mass: 320.1189.

8-Chloro-5-methyl-3-((methylselanyl)methyl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepin-6(5H)-one (27). The bromide 21 (100mg, 0.30mmol) was dissolved in THF (10mL). In a separate flask, NaBH₄ (70mg, 2.9mmol) was dissolved in CH₃SeCH₃ (55mg, .293mmol). The solution which resulted was added to the bromide and the reaction mixture was stir overnight. The reaction mixture was then quenched with water and extracted with ethyl acetate. The combined organic layers were washed with brine and dried (MgSO₄). The solvent was removed under reduced pressure and the residue was purified by flash chromatography on silica gel (gradient elution 40-60% EtOAc : hexane) to afford selenium analog 27 in an overall yield of 60%. 1H NMR (CDCl₃, 300MHz) ) δ 8.08 (d, 1H, J = 2.0Hz), 7.98 (s, 1H), 7.66 (d, 1H, J = 6.60, 1.88 Hz), 7.42 (d, 1H, J = 8.4Hz), 5.164 (s, 2H), 4.46 (s, 1H), 3.25 (s, 3H), 2.19 (s, 2H) , 1.27 (S,3H). 13C NMR (75 MHz, CDCl₃): δ188.13, 165.14, 135.62, 135.16, 132.87, 132.76, 130.63, 129.90, 123.23, 41.77, 36.26, 30.92, 29.70. HRMS (ES+) calculated for C₁₄H₁₅ClN₃OSe: 356.0069, Found Mass: 356.0071.
8-Chloro-N-cyclopropyl-5-methyl-6-oxo-5,6-dihydro-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxamide (28). The acid 20 (100mg, 0.297mmol) was dissolved in dry CH$_2$Cl$_2$ (20mL) at room temperature and excess SOCl$_2$ was added in one portion. The reaction mixture was heated to reflux for 2h until analysis of the mixture by TLC indicated the absence of starting material. The reaction mixture was concentrated under reduced pressure on a rotavapor and flash evaporated a few times with CH$_2$Cl$_2$ to remove the excess SOCl$_2$ and dried under vacuum. The solid which resulted was stirred in dry CH$_2$Cl$_2$ (20 mL) at 0°C. To this reaction mixture, cyclopropylamine (1ml, 4.45mmol) and triethylamine (0.5ml) were added and the mixture allowed to stir overnight. The mixture which resulted was then quenched with CH$_3$Cl$_2$. The combined organic layers were washed with brine and dried MgSO$_4$. After the removal of the solvent under reduced pressure, the residue was purified by flash chromatography on silica gel (gradient elution 3:2 EtOAc: hexane) to afford 27 (PB-I-076) with an overall yield of 92%. 1H NMR (CDCl$_3$, 300MHz) δ 8.054 (d, 1H, J = 2.4Hz), 7.832 (s, 1H), 7.59 (dd, 1H, J = 8.7,2.4 Hz), 7.80 (d, 1H, J = 2.1Hz), 5.42 (br, s,1H), 4.33 (br,s, 1H), 3.20 (s, 3H), 2.88(m, 1H), 0.86(m,2H), 6.75(m,2H) 13C NMR (75 MHz, CDCl$_3$): δ 165.31, 163.79, 134.52, 133.44, 132.63, 132.59, 132.36, 130.99, 130.65, 130.50, 123.04, 42.05, 36.10, 22.12, 6.49 HRMS (ES+) calculated for C$_{15}$H$_{16}$ClN$_4$O$_2$: 319.0962, Found Mass: 319.0847.
8. Computer Modeling Methods

The core structures of the ligands were taken from available X-ray crystallographic coordinates or generated using the SYBYL fragment library. The structures which resulted were energy minimized using MM2 (molecular mechanics program 2) or MMFF (Merck molecular force field), and the subsequent Monte Carlo conformational searches were carried out on MacroModel 6.0 on a Silicon Graphics Personal Iris 4D/35 workstation or a Silicon Graphics Octane SI 2P 175 R10000 workstation, respectively. The low energy conformations were then fully optimized via molecular orbital calculations at the 3-21G basis set with torsional angles fixed. The structures which resulted were further calibrated with 6-31G* single point calculations at an “SCF=TIGHT” convergence criteria via Gaussian 92165 on a Silicon Graphics Indigo R4400 workstation, or Gaussian 94166 on a Silicon Graphics Octane SI2P175R10000 workstation.

9. Competition Binding Assays (With Dr. Majumder and Dr. Roth).

Competition binding assays were performed in a total volume of 0.5 mL at 4 °C for 1 h using [3H] flunitrazepam as the radiolabel. A total of 6 μg of cloned human GABAA receptor DNA containing the desired α subtype along with the β2 and γ2 subunits were used for transfecting the HEK 293T cell line using Fugene 6 (Roche Diagnostic) transfecting reagent. Cells were harvested 48 h after transfection, washed with Tris-HCl buffer (pH 7.0) and Tris Acetate buffer (pH 7.4) and the resulting pellets were stored at -80 °C until assayed. On the day of the assay, pellets containing 20-50 μg of GABAA receptor protein were resuspended in (50 mM Tris-acetate pH 7.4 at 4 °C) and incubated with the radiolabel as previously described. 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.

10. **CANTAB work (Rowlett and Cook et al.)**: Preferential inverse agonist action at α5GABAA receptors resulted in enhancement of performance on memory tasks.

10.1 **Subjects**: Experiments were conducted with adult male rhesus monkeys (*Macaca mulatta*), aged 10-15 years (corresponding roughly to “middle aged” monkeys), born and reared at the New England Primate Research Center breeding facility. The monkeys were experimentally naïve at the beginning of the study. Monkeys were prepared with a non-allergic nylon vest (Lomir) and seated in primate chairs (Crisp Instruments) enclosed in ventilated, sound-attenuating chambers (Med Associates); or prepared with the non-allergic nylon vest attached to a flexible, stainless steel tether (Lomir) connected to the top of the monkey’s home cage. The restraint chair system was used in CANTAB studies, and the tether system was used in ORD, observational and MAP studies. All monkeys were housed in a temperature- and humidity-controlled housing room and maintained on a 12-hr on/12-hr off light/dark schedule (lights on at 0600 hr). Water was available continuously in the home cage.
10.2 Food availability: The protocol for food restriction developed by Taffe (2004) was used. Briefly, the monkeys in all studies were weighed weekly, and these weights were used to calculate food (LabDiet 5038) allotments based on metabolic energy requirements for rhesus monkeys. The calculation was based on the equation: \( y = 15.504x^{-0.8219} \), in which \( y = \text{food biscuits/kg/day} \) and \( x = \text{body weight in kg} \) (Taffe 2004). Monkeys were maintained at 100% of this allotment for at least 3 weeks or until weights were stable (no upward or downward trend over three weight determinations). Once stable, the monkeys’ food allotments were gradually reduced to 70 to 85% (note that this was not a reduction in weight, rather a reduction in food allotment), an amount shown to result in stable and accurate performance in CANTAB tasks. Weekly weighing occurred throughout the studies to adjust feeding (within the 70-85% range) based on a combination of weight, performance, and overall health (as determined by routine physical exams conducted by the Clinical Veterinary Staff).

10.3 Surgery and drug delivery: All compounds were administered via the intravenous (i.v.) route. We used this route in order to minimize pharmacokinetic variables and to allow direct comparisons with other procedures at Harvard Medical School (NERPC) designed to study BZ effects in rhesus monkeys (e.g., i.v. self-administration models of abuse potential). Monkeys were implanted with venous catheters using the surgical procedures described by Platt et al. (2005). Surgery was conducted under aseptic conditions using isoflurane/oxygen anesthesia. Catheters were passed by way of a jugular, femoral or brachial vein to the level of the right atrium. The distal end of the catheter was passed subcutaneously to a mid-scapular exit point. Catheters were flushed routinely with saline containing Heparin (100-150 U/ml), and physical exams were conducted that
occasionally included contrast-dye infusion into the catheter, followed by X-ray to verify catheter patency and proper placement. Using these procedures, catheters can remain patent for up to 3 years.

10.4 General design and data analyses: Separate groups of 6-8 monkeys were used in the CANTAB vs. the ORD/MAP/observation studies, with each monkey in a group receiving all treatments whenever possible. Dependent measures for the studies depended on the individual tasks, as described below. The studies employed within-subjects design, in which each subject serves as its own control. This design permits scientifically meaningful results to be obtained with fewer animals than would be required using most other approaches. For individual subject analysis, data during test sessions were compared to data from control sessions and significance of effects were determined on the basis of 95% confidence intervals established under baseline conditions. For comparison of doses within individual tasks, repeated-measures ANOVAs were used to evaluate statistical reliability when warranted. For all studies, multiple comparisons were assessed for statistical reliability using a priori Bonferroni t-tests, when applicable. Comparisons of drug potencies were conducted by computing ED50 values (dose engendering 50% of the maximum effect). The ED50s were computed using non-linear regression analysis techniques.

10.5 CANTAB Tasks in Monkeys.

10.5.1 Apparatus: As described above, monkeys were trained to sit in primate chairs that were be placed in a sound-attenuating chamber. Inside this chamber were a computer-controlled touch-screen monitor (Campden or Lafayette Instruments, Inc.), equipped with
a dispenser to deliver 190-mg flavored food pellets (BioServe, Inc.). Experimental events were controlled by the CANTAB software system on a computer in an adjacent room.

10.5.2 Delayed non-matching to sample (DNMS). The DNMS task was a short-term recognition memory task involving sets of visual discriminations. A sample stimulus is presented in the center of the screen, and the animal must touch this stimulus within 30 sec. After a touch, the screen was blanked and following a variable retention interval, two stimuli were presented on the lower left and right of the screen. The retention interval consisted of 0, 1, 3, or 10 min; if performance was highly variable, the delays were adjusted for individual monkeys based on performance such that short, medium, and long delays were tested. Of the two “matching” stimuli, one stimulus was identical to the sample stimulus (“matching” stimulus) and the other was novel (“non-matching” stimulus). A touch directed to the non-matching stimulus was followed by reinforcer delivery. In addition to the four retention interval conditions, a simultaneous condition was included in which the sample stimulus remained present while the matching and non-matching stimuli were present. A session consisted of 10 trials of each retention interval, presented in a randomly intermixed fashion (total trials=50). The CANTAB software used 469 shapes and 7 colors to ensure that discriminations were unique for approximately 120,000 trials. The primary dependent measured for the DNMS task are the percent correct responses at each delay and the latency to respond at each delay. The number of trials completed also was recorded as a measure of motivation and/or ability to perform the task.

10.5.3 Paired associated learning (PAL). Large colored abstract stimuli were displayed in one of 4 positions (top center, bottom center, left middle, right middle). The subject was required to touch the sample stimulus, which then disappeared. After a 1-sec
delay, the same pattern reappears (choice phase) in two or more locations on the screen (the original location plus one or more novel locations). The subject was required to touch the stimulus that was presented in the same location as the sample item to obtain a food pellet. Task difficulty was increased by increasing the number of stimulus-location associations required on each trial. Training was initiated with sessions that present 25 1-stimulus trials and 25 2-stimulus trials until performance averaged >50% correct trials on the 2-stimulus trials. Next, monkeys received sessions of 25 2-stimulus trials and 25 3-stimulus trials until performance averaged >25% correct trials on the 3-stimulus trials. This part of the PAL task measured memory for stimulus-location associations. Next, a learning component is introduced in which a trial repeats if a mistake was made in attempting to complete this trial (i.e. the monkey gets 0-2 of 3 stimulus-locations correct in a 3-stimulus trial). Up to 6 attempts at a given trial were allowed. Finally, 4-stimulus trials were added when performance of 3-stimulus trials exceeded 50% correct (thus, the PAL task measured both memory and learning of stimulus-location associations). The dependent measures were the percent correct at each difficulty level and the latencies to respond.

10.5.4 Intradimensional/extradimensional set shifting (ID/ED). The ID/ED shift task evaluated the ability of a subject to attend to specific attributes of a stimulus, as well as the ability to shift attention to other attributes when required. This task consisted of a series of eight discrimination learning stages wherein touching only one of two stimuli presented on the screen resulted in food pellet delivery. Within any given stage of the task, a pair of stimuli was presented and the same stimulus was associated with reinforcement (S+ stimulus) until the performance criteria were met (18 of 20 consecutive trials correct). Correct choices must be made within 30 sec, and following a correct choice the screen was
blanked for 5 sec while an incorrect choice (S- stimulus) resulted in a 0.2 sec tone and a 9-sec period of blank screen. The task consisted of 4 levels of stimulus sets, each progressively more difficult. In the first stimulus set (Level 1), two distinctly shaped stimuli were presented, with touches on one shape resulting in food pellet delivery. This level (and all subsequent levels) included a stimulus reversal, in which the same two shapes are retained but pressing the S+ stimulus now does not result in reinforcement, whereas touching the former S- stimulus did result in food pellet delivery. In Level 2, a compound discrimination was presented. For this discrimination, the two shapes from Level 1 are present, but additional stimuli, consisted of lines, were superimposed onto the existing shapes. Because the shape discrimination from the previous stage did not change, the lines are irrelevant to this discrimination. Level 3 consisted of the intra-dimensional (ID) shift stage. For this discrimination, two new shapes with new lines were presented. This was considered an ID shift due to the fact that despite new shapes and lines being introduced, the shape remained the relevant dimension for the discrimination. In the final level, the extra-dimensional (ED) shift will be introduced. Thus, in Level 4, new shapes and lines were presented; however, the lines—not the shape—were the relevant discriminative stimulus. Performance was determined as the number of errors at each stage, and the data were subjected to a square root transformation to achieve normal distributions.

**10.5.5 Progressive-Ratio (PR).** PR procedures consisted of response requirements that progressively increased across a session until responding ceases. The last response requirement completed, termed “break point”, provided a quantitative measure of the reinforcing effectiveness of a stimulus. For these studies, a session was initiated by a colored box appearing in the middle of the screen. Touching the box once resulted in food
pellet delivery, and the response requirement were progressively increased following each reinforcer by an incremental value beginning at 1 and doubling after 8 response requirements were completed successfully. Thus, the response requirement sequences consisted of: Increment= 1, response requirements= 1, 2, 3, 4, 5, 6, 7, 8; Increment = 2, response requirements = 10, 12, 14, 16, 18, 20, 22, 24; Increment = 4, response requirements = 28, 32, 36, 40, 44, 48, 52, 56; and so on. Sessions were a maximum of 30 min, and were terminated if 3 min elapses without a response. Performance was measured as the break point and number of food pellets delivered per session. This procedure provided a measure of the ability of the animal to respond, as well as an assessment of “motivation” to perform.

10.5.6 Training. Initial training began with shaping the basic response of touching the screen. A large colored square was displayed that occupied most of the screen. Touching the screen inside the box was a “correct” response and results in food reinforcement. With each correct response, the size of the square decreased until the monkey readily pressed a 1-in box in any location on the screen. This initial training typically required up to 5 sessions. Currently 4 monkeys were trained at various stages of the DNMS and ID/ED task. These monkeys initiated training on PAL (a task that requires a lengthy training period, Taffe et al. 2004) with “reminder” trials for DNMS and ID/ED scheduled one day per week. We anticipated approximately 5-6 more months of training for PAL performance to reach acceptable performance.

For new monkeys, CANTAB training was initiated by gradually introducing different tasks, and also incorporate training on the ORD task. In the first phase of training, the PAL task was introduced 5 days/week. After approximately 6 months, the DNMS task was
introduced on days 1-4 of the week, with PAL training continuing on day 5. In our experience, DNMS training typically took up to 3 months, once criterion performance was met, DNMS training was initiated on days 1-3, with PAL training scheduled for days 4 and 5, respectively. PAL training may take up to and over 12 months, therefore, after 9 months the ID/ED task was introduced into the sequence once or twice per week on randomly chosen days (this task did not require special training, Weed et al., 1999). In the final phase of training, two tasks per day were introduced and the PR schedule introduced according to the schedule: day 1: PR/PAL; day 2: DNMS/PR; day 3: PAL; day 4: DNMS/PR; day 5: ID/ED/PR.

10.5.7 Testing. Once training criteria were reached on all tasks, the 8-day cycles shown in Table 4 were initiated. The two cycles shown in the Table allowed a dose of test compound and vehicle to be determined twice in all tasks. For each compound, vehicle plus at least 4 doses were evaluated. All doses of a compound (or dose of compound plus antagonist) were tested in irregular order, with a compound finished prior to moving to the next compound. A test session consisted of i.v. injections of compound(s) at an appropriate pretreatment time prior to the session. Based on the scheduling shown in Table 20, for CANTAB a dose-response function was required approximately 10 weeks to complete.

Table 4: Schedule of CANTAB tests for a single dose of compound plus vehicle

<table>
<thead>
<tr>
<th>Cycle 1</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose A</td>
<td>PAL</td>
<td>ID/ED</td>
<td>ORD</td>
<td>DNMS/PR</td>
<td>DNMS/PR</td>
<td>ORD</td>
<td>ID/ED</td>
<td>PAL</td>
</tr>
<tr>
<td>vehicle</td>
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<td>vehicle</td>
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</table>

<table>
<thead>
<tr>
<th>Cycle 2</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNMS/PR</td>
<td>ORD</td>
<td>ID/ED</td>
<td>PAL</td>
<td>PAL</td>
<td>ID/ED</td>
<td>ORD</td>
<td>DNMS/PR</td>
<td></td>
</tr>
<tr>
<td>Dose A</td>
<td>vehicle</td>
<td>vehicle</td>
<td>vehicle</td>
<td>vehicle</td>
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</tbody>
</table>

To augment CANTAB-based testing, parallel assessments were conducted using the ORD Task, which was a manual test sensitive to frontostriatal deficits (Taylor et al. 1990; Jentsch et al. 1999) and has been validated in pharmacological studies at NERPC. The task required the monkey to retrieve a small piece of palatable food from a transparent box that was open on one side only. The box was attached to a tray that can be positioned within easy reach of the monkey. Different levels of task complexity were achieved by varying the orientation of the open side of the box, the position of the box on the tray, and the position of the food relative to the opening. The level of cognitive complexity and motor difficulty of each task was rated *a priori* using the criteria of Taylor et al. (1990). The 12 task configurations varied from those characterized by high cognitive complexity/low motor difficulty to low cognitive complexity/high motor difficulty. Training on the ORD task typically required 14-21 sessions, and tests were scheduled as described above in Table 4. The primary dependent measure for the ORD task was “percent success”, which was the total number of reaches minus “incorrect” (i.e., a reach in which the food item was not obtained) and “barrier” reaches (i.e., a reach in which the monkey contacted a closed side of the box), divided by total reaches and then multiplied by 100. Barrier reaches were also analyzed separately as a measure of perseverative behavior, and reach latencies (i.e., time to obtain food or maximum trial length of 20 seconds) were recorded as a measure of motor impairment.
12. Assessment of Motor Coordination and Behavioral Effects in Monkeys.

**MAP apparatus and procedure.** To evaluate fine motor coordination, monkeys were trained using the movement assessment panel (MAP) system developed by Gash and colleagues (Gash et al. 1999). The MAP (Quanteon, Inc.) consists of a Lexan® box attached to a clear Lexan® panel that was mounted on to home cage (Figure 14). Portals in the front panel and on the sides of the food receptacle allowed monkeys to retrieve small food objects (e.g., miniature marshmallows or raisins). Arrays of photocells are attached to the portals on the front panel (Portal A) and food receptacle (Portal B). The amount of time that the photobeams for Portal A were broken was considered a measure of gross motor coordination, whereas the amount of time that the photobeams for Portal B were broken was considered a measure of fine motor coordination (Gash et al. 1999). MAP studies occurred in the home cage of monkeys prepared with the tether system. The MAP apparatus fit the cage door, and monkeys received initial training by placing food treats in different parts of the apparatus until they consistently retrieved treats from the food receptacle.
Training occurred 5 days per week until latencies retrieved the treat are stable (no upward or downward trends over 3 consecutive sessions) and testing occurred on day 2 and day 5 of this 5-day cycle.

**Observation studies.** Observers were trained in the use of the behavioral scoring instrument developed in previous studies at NERPC. Each observer underwent at least 20 hrs of observational training until they reached a > 90% reliability criterion based on percent agreement scores. The scoring system included 19 categories encompassing a wide range of species-typical and drug-induced behaviors including locomotion, environmental manipulation, foraging, self-grooming, scratching, stereotypic behavior such as self-grasping, back flipping, and hair pulling, ataxia, sleep posture, moderate and deep sedation. These behavioral categories have proven to be useful for characterization of the sedative and motoric effects of BZ-type compounds in previous research at NERPC. Observation
studies occurred in the same monkeys that were trained on the MAP task. Observation studies were conducted in these monkeys in alternate weeks, on the 2nd and 3rd day of the 5-day cycle (i.e., MAP testing occurred one week, observation tests occurred the following week, and so on). Video cameras were used to record the monkeys’ behavior during observation sessions. This provided an archival record of experimental sessions, permitted subsequent scoring of videotapes by independent observers, and eliminated the possibility that an observer’s presence disturbed the monkeys’ behavior. All behaviors were scored during 5-min sampling periods as described above. Each 5-min period was subdivided into twenty 15-s intervals, and frequency scores were calculated as the proportion of 15-s intervals in which a particular behavior occurred. Data was analyzed separately in individual subjects and for the group when appropriate used the statistical approach described earlier at NERPC.

13. Experimental procedures for the electrophysiology (with Dr.R. Furtmüller and Dr. W. Sieghart)\textsuperscript{91-94}

Preparation of cloned mRNA

Cloning of GABAA receptor subunits α1, β3 and γ2 into pCDM8 expression vectors (Invitrogen, CA) has been described elsewhere.\textsuperscript{96} GABAA receptor subunit α4 was cloned in an analogous way. cDNAs for subunits α2, α3 and α5 were gifts from P. Malherbe and were subcloned into a pCI-vector. cDNA for subunit α6 was a gift from P. Seeburg and was subcloned into the vector pGEM-3Z (Promega). After linearizing the cDNA vectors with appropriate restriction endonucleases, capped transcripts were produced using the mMMessage mMMachine T7 transcription kit (Ambion, TX). The capped transcripts were
polyadenylated using yeast poly (A) polymerase (USB, OH) and were diluted and stored in diethylpyrocarbonate-treated water at –70 °C.

Functional expression of GABA<sub>A</sub> receptors

The methods used for isolating, culturing, injecting and defolliculating of the oocytes were identical to those as described previously. Briefly, mature female *Xenopus laevis* (Nasco, WI) were anaesthetized in a bath of ice-cold 0.17 % Tricain (Ethyl-m-aminobenzoate, Sigma, MO) before decapitation and removal of the frog ovary. Stage 5 to 6 oocytes with the follicle cell layer around them were singled out of the ovary using a platinum wire loop. Oocytes were stored and incubated at 18 °C in modified Barths medium (MB, containing 88 mM NaCl, 10 mM HEPES-NaOH (pH 7.4), 2.4 mM NaHCO<sub>3</sub>, 1 mM KCl, 0.82 mM MgSO<sub>4</sub>, 0.41 mM CaCl<sub>2</sub>, 0.34 mM Ca(NO<sub>3</sub>)<sub>2</sub>) that was supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. Oocytes with follicle cell layers still around them were injected with a total of 2.25 ng of cRNA. This solution contained the transcripts for the different α subunits and the β 3 subunit at a concentration of 0.0065 ng/nL as well as the transcript for the γ2 subunit at 0.032 ng/nL. After injection of the cRNA, oocytes were incubated for at least 36 h before the enveloping follicle cell layers were removed. To this end, oocytes were incubated for 20 min at 37 °C in MB that contained 1 mg/mL collagenase type IA and 0.1 mg/mL trypsin inhibitor I-S (both Sigma). This was followed by osmotic shrinkage of the oocytes in doubly concentrated MB medium supplied with 4 mM Na-EGTA. Finally, the oocytes were transferred to a culture dish containing MB and were gently pushed away from the follicle cell layer which stuck to the surface of the dish. After removal of the follicle cell layer, oocytes were allowed to recover for at least 4 h before being used in electrophysiological experiments.
Electrophysiological experiment.  

For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of *Xenopus* Ringer solution (XR, containing 90 mM NaCl, 5 mM HEPES NaOH (pH 7.4), 1 mM MgCl2, 1 mM KCl and 1 mM CaCl2). The oocytes were constantly washed by a flow of 6 mL/min XR which could be switched to XR containing GABA and/or drugs. Drugs were diluted into XR from DMSO solutions resulting in a final concentration of 0.1 % DMSO perfusing the oocytes. Drugs were preapplied for 30 sec before the addition of GABA, which was coapplied with the drugs until a peak response was observed. Between the two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. For current measurements the oocytes were impaled with two microelectrodes (2–3 mΩ) which were filled with 2 mM KCl. All recordings were performed at rt at a holding potential of ~60 mV using a Warner OC-725C two-electrode voltage clamp (Warner Instruments, Hamden, CT). Data were digitized, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA). Results of concentration response experiments were fitted using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA). The equation used for fitting the concentration response curves was $Y=\text{Bottom} + \frac{(\text{Top}-\text{Bottom})}{(1+10^{(\log \text{EC50} - X) \times \text{HillSlope}})}$; $X$ represents the logarithm of concentration, $Y$ represents the response; $Y$ starts at Bottom and goes to Top with a sigmoid shape.

14. Behavioral experiments (with Savic et al.)

Experiments were carried out on male Wistar rats (Military Farm, Belgrade, Serbia), weighing 220-250 g. All procedures in the study conformed to EEC Directive 86/609 and
were approved by the Ethical Committee on Animal Experimentation of the Medical Faculty in Belgrade. The rats were housed in transparent plastic cages, six animals per cage, and had free access to pelleted food and tap water. The temperature of the animal room was 22±1°C, the relative humidity 40-70%, the illumination 120 lux, and the 12/12 h light/dark period (light on at 6:00 h). All handling and testing took place during the light phase of the diurnal cycle. Throughout the study the animals were used only once, with the exception of the grip strength measurement, which was done immediately after the tracking of behavior on the elevated plus maze. Spontaneous locomotor activity and elevated plus maze behavior were analyzed by the ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA). All drugs were dissolved/suspended with the aid of sonication in a solvent containing 85% distilled water, 14% propylene glycol, and 1% Tween 80, and were administered intraperitoneally in a volume of 1 mL/kg, 15 min before behavioral testing (for active and passive avoidance test, before the acquisition session). Time of administration, as well as, the doses of DMCM in various tests were chosen based on our previous studies (Savić et al., 2005a; 2005b; 2006). For interaction studies in the locomotor activity assay, the first treatment indicated in combination was administered into the lower left quadrant of the peritoneum 20 minutes before testing, and the second treatment 5 minutes later into the lower right quadrant of the peritoneum.

**Passive Avoidance (PA) Paradigm**

The experiments were performed in an adapted Automatic reflex conditioner (Ugo Basile, Milan, Italy, Model 7051), as described earlier. In short, the apparatus consisted of a shuttle-box, equipped with a grid floor and divided with a sliding door into a lit and a dark compartment, and a programming unit. The animals were submitted to two, 24 hour –
separated sessions. The acquisition session started by placing individual subjects in the illuminated compartment. After 30 seconds, the entrance to the dark compartment was opened, and as soon as the rat had entered it with all four paws, the footshock (2 s, 0.3 mA) was delivered. Immediately afterwards, the rat was returned to its home cage. The same procedure was repeated 24 hours later (retention session), without footshock. A cut-off time of 180 seconds was used on the training day, whereas, on the retention trial, a ceiling of 300 seconds was imposed.

15. Measurement of Locomotor Activity (with Savic et al.)

Activity of single rats in a clear Plexiglas chamber (40 x 25 x 35 cm) under dim red light (20 lux) was recorded for a total of 30 minutes, without any habituation period, using ANY-maze software (as described above). For purposes of improving data analysis, the central 20% of the chamber (200 cm2) was virtually set as a central zone. The minimum percentage of animal that must have been in the zone for an entry to occur was set at 70%, and 50% of the animal must have remained in the zone for an exit not to occur.

16. Behavior on the Elevated Plus Maze (EPM) (with Savic et al.)

The apparatus consisted of two open (50 x 10 cm, with a ledge of 0.3 cm) and two enclosed arms (50 x 10 x 40 cm), connected by a junction area (10 x 10 cm). The illumination in the experimental room was 10 lux on the surface of the arms. At the beginning of the experiment, single rats were placed in the center of the maze, facing one of the enclosed arms, and their behavior was recorded for 5 min. An entry into an open or closed arm was scored when 90% of the animal crossed the virtual line separating the central square of the
maze from the arm, whereas an exit occurred when more than 90% of the animal left the respective arm. After each trial, the maze was cleaned with dry and wet towels.

17. Grip Strength Test (with Savic et al.)

Muscle strength was assessed by the grip strength meter (Ugo Basile, Milan, Italy, model 47105). When pulled by the tail, the rat grasps the trapeze connected to a force transducer, and the apparatus measures the peak force of experimenter’s pull (in g) necessary to overcome the strength of the animal’s forelimbs grip. Each animal was given three consecutive trials, and the median value was used for further statistics.

18. Statistical analysis (with Dr. Miroslav Savic)

All numerical data presented in the figures were given as the mean ± SEM, except for results from the PA test (median latency with 25th, 75th interquartile range; data were non-parametric because the procedure involved a cut-off). For electrophysiological data Student’s t-test was used for statistical analysis. Data from PA test were assessed by a Kruskal-Wallis non-parametric ANOVA, with post-hoc comparison relative to solvent control by a Dunn's test (α=0.05). Data from the AA, EPM, grip strength and activity assay were assessed by a one-way ANOVA. If the ANOVA was significant, each treatment condition was compared with control by a Dunnett's test (α=0.05). Where appropriate, the assessment of the antagonist influence on the inverse agonist effect was conducted by a student’s t-test. Statistical analyses were performed with ANY-maze Video Tracking System software (Stoelting Co., Wood Dale, IL, USA) and SigmaStat 2.0 (SPSS, Inc., Chicago, IL, USA).
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II. Appendix I: Proton and Carbon Spectrum of PB-I-052
III. Appendix II: Proton and Carbon Spectrum of PB-I-054
IV. Appendix III: Proton and Carbon Spectrum of PB-I-056
V. Appendix IV: Proton and Carbon Spectrum of PB-I-059
VI. Appendix V: Proton and Carbon Spectrum of PB-I-060
VII. Appendix VI: Proton and Carbon Spectrum of PB-I-061
VIII. Appendix VII: Proton and Carbon Spectrum of PB-I-063
IX. Appendix VIII: Proton and Carbon Spectrum of PB-I-076