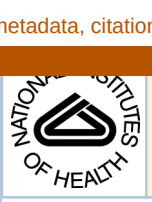

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Search for α3β$_{2/3}$γ2 Subtype Selective Ligands That are Stable on Human Liver Microsomes

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Abstract

Selective modulation of specific benzodiazepine receptor (BzR) gamma amino butyric acid-A ($GABA_A$) receptor ion channels has been identified as an important method for separating out the variety of pharmacological effects elicited by BzR-related drugs. Importantly, it has been demonstrated that both α2β$_{(2/3)}$γ2 (α2BzR) and α3BzR (and/or α2/α3) BzR subtype selective ligands exhibit anxiolytic effects with little or no sedation. Previously we have identified several such ligands; however, three of our parent ligands exhibited significant metabolic liability in rodents in the form of a labile ester group. Here eight analogs are reported which were designed to circumvent this liability by utilizing a rational replacement of the ester moiety based on medicinal chemistry precedents. In a metabolic stability study using human liver microsomes, four compounds were found to undergo slower metabolic transformation, as compared to their corresponding ester analogs. These compounds were also evaluated in *in vitro* binding as well as efficacy assays. Additionally, bioisostere **11** was evaluated in a rodent model of anxiety. It exhibited anxiolytic activity at doses of 10 and 100 mg/kg and was devoid of sedative properties.

1. Introduction

Gamma amino butyric acid-A ($GABA_A$) receptors are the major inhibitory neurotransmitter receptors of the central nervous system (CNS) and the site of action of a variety of pharmacologically and clinically important drugs. Thus, benzodiazepines, barbiturates, neuroactive steroids, anesthetics and convulsants exert their action by modulating the function of these receptors.[1] From the action of these drugs it is clear that $GABA_A$ receptors

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regulate numerous neurological functions including convulsions, anxiety, sedation, ataxia and sleep activity, as well as memory and learning processes.[2–5] $GABA_A$ receptors are composed of 5 subunits that form a central chloride channel and can belong to different subunit classes. A total of 19 subunits (6α, 3β, 3γ, 1δ, 1ε, 1π, 1θ, 3ρ) of the $GABA_A$ receptor have been cloned and sequenced from the mammalian nervous system.[6, 7] The homology within each subunit class is about 60–80 %, while the homology between the subunit classes is about 30–40 %.

The majority of $GABA_A$ receptors is composed of 1γ, 2α, and 2β subunits. The classical benzodiazepines, such as diazepam or flunitrazepam, bind at the extracellular domain of the α+γ2− interface of these receptors[8] and exhibit a high affinity for receptors composed of $\alpha1\beta_{(2/3)}\gamma2$, $\alpha2\beta_{(2/3)}\gamma2$, $\alpha3\beta_{(2/3)}\gamma2$ or $\alpha5\beta_{(2/3)}\gamma2$ subunits (diazepam sensitive receptors). Different receptor subtypes reside within anatomically distinct regions of the brain and are responsible for different physiological and pathological processes.[9, 10] Data from knock in mice indicate that the α1 containing receptors mediate the sedative, anticonvulsant, ataxic effects, anterograde amnesia and abuse liability,[11, 12] while the α2 and α3[13, 14] receptor subtypes mediate anxiolytic activity.[15–17] The α5 containing $GABA_A$ receptors have been implicated in memory and learning processes,[18,19] but seem neither to influence anxiolysis nor motor effects,[9, 19] although some reports of a decrease in locomoter effects due to α5 BzR subtype specific ligands have also been reported.[20] It has been shown that a substantial amount of agonist activity at the α1 BzR is required for effects on spatial learning and memory impairments, while much weaker, but simultaneous activity at α1 and α5 together is sufficient for eliciting sedation.[21] Tolerance to some of the effects of benzodiazepines, most notably the anticonvulsant and analgesic actions are mediated by the simultaneous action of α1 and α5 BzR.[22, 23]

Agents selective for specific BzR subtypes should permit one to separate out the pharmacological activities of these different isoforms and reduce the chances of tolerance and abuse potential.[12, 23–27] The $GABA_A$/α1-selective positive allosteric modulators alpidem and zolpidem are clinically prescribed as hypnotic agents suggesting that at least much of the sedation associated with known anxiolytic drugs which act at the BzR binding site is mediated through $GABA_A$ receptors containing the α1 subunit, as mentioned. Consequently, novel benzodiazepine-like drugs that have pharmacological selectivity for α2 $GABA_A$ and/or α3 $GABA_A$ receptors and low receptor efficacy at α1$GABA_A$ and α5$GABA_A$ receptors may be particularly useful as anxiolytics lacking sedative, ataxic, and amnestic side effects as well as little or no abuse potential.[11, 12]

Recently, with the help of $GABA_A$ receptor point mutated mice, it was also demonstrated that α2 and/or α3 receptor subtypes are largely responsible for the spinal antihyperalgesic actions of classical benzodiazepines, while α1 receptors do not contribute.[28] Interestingly, in α1-$GABA_A$ receptor point-mutated mice, which are protected from the sedative effects of diazepam, pronounced analgesia against formalin-induced pain has also been reported after systemic treatment with diazepam.[29] These results suggest that α1 sparing (non-sedative) benzodiazepine-site agonists should exert a genuine analgesic effect after systemic treatment.[29]

Through the iterative process of computational modeling and synthesis, benzodiazepine derivatives, XHe-II-053 (**1**), HZ-166 (**2**), and JY-XHe-053 (**3**) have been synthesized in Milwaukee (Figure 1). All three compounds **1**-**3** were exceptionally selective (hence non-sedating) $GABA_A$ α2/α3 agonists in *in vitro* electrophysiological experiments (Figure 2).[21, 30] In a rhesus monkey conflict procedure, over the dose range tested (0.1 to 10 mg/kg), **1** and **2** produced an anti-conflict effect without producing diazepam-like alterations in the absence of response-contingent electric shock (non-suppressed responding). Both the

compounds were anxiolytic, but lacked sedative, ataxic, muscle relaxant and amnestic side effects.[30] The 2′-F ligand **3** also produced a robust anti-conflict effect; however, it also produced some reduction in response rates at the highest dose tested. This was, presumably, due to its greater efficacy at $GABA_A$ α1 receptors as compared to α2 or α3 BzR ligands, clearly illustrating the subtle differences that structures have on efficacy of ligands at $GABA_A$ α1 BzR.

In another study in mouse models of neuropathic and inflammatory pain, α2/α3 ligand **2** exhibited a dose-dependent antihyperalgesic effect and was as efficacious as gabapentin.[31] This antihyperalgesic activity was antagonized by flumazenil and hence mediated via the benzodiazepine-binding site of $GABA_A$ receptors. At doses producing maximal antihyperalgesia, ligand **2** was devoid of sedation and motor impairment, and showed no loss of analgesic activity during a 9-day chronic treatment period (i.e. no tolerance development) versus acute treatment.[23]

In preclinical studies **1** was shown to be safe and effective (anxiolytic but not sedating, not ataxic, and had reduced abuse potential in rodents and was anxiolytic in non-human primate models).[30] Because of its encouraging results in pre-clinical testing, **1** had been taken into Phase I studies in humans for anxiety disorders (Bristol-Myers Squibb, unpublished results of Phase I trials). Ligand **1** was found to be safe in humans. It was found to be stable in human blood, plasma, brain and kidney (Mithridion, unpublished results). However, in human liver, ligand **1** was largely transformed into the inactive metabolite XHe-II-053-acid (**4**) via hepatic enzymes, resulting in sub-optimal pharmacokinetics (unpublished results). The related analog mining was an effort to circumvent the metabolic liability of these compounds in human liver microsomes vs the control anxiolytic **1**, in an effort to develop nonsedating anxiolytics with longer half lives in humans.

A metabolic stability study of these compounds was undertaken at SRI International by Dr. Ng and coworkers as a collaborative project funded by National Institute of Mental Health (NIMH). In this study the test compounds were incubated with pooled human liver microsomes and the aliquots were analyzed at various time points using LC-MS/MS. Illustrated in Table 1 are the results from this study. Significant metabolic lability was observed at both 1 and 10 μM for **1** (less than 14% remaining at 30 min) and **3** (less than 20% remaining at 15 min), while the key ligand **2** underwent minimal metabolism (80% remaining at 60 min). The corresponding carboxylic acid controls of **1** and **2**, i.e. acids **4** and SR-II-54 (**5**), did not exhibit any metabolism during the test period. Additionally, all the compounds when incubated with heat-inactivated human liver microsomes underwent no significant change in the % remaining of any of the compounds, suggesting that the compounds were stable under the control incubation conditions. These results indicated that the main site of metabolism in human liver was due to the pendant ethyl ester moiety, which was not unexpected but designed as a method of clearance (metabolic switching).

2. Design of novel ligands

Based on these findings it was decided to design novel analogs with bioisosteric replacement of the labile ethyl ester moiety of the lead analogs (Figure 1). Amides are well known bioisosteric replacements for substituted esters. Hence compounds **6**-**8** were synthesized (Figure 2). The N,N-dimethylamide ligand **9** was included to determine the importance of a H-bond donor at that position as compared to amide **7**. Previously, in the case of $GABA_A$ modulators in the benzodiazepine series of compounds, it has been demonstrated that replacement of the ethyl ester at the 3-position by a 1,2,4-oxadiazole moiety resulted in higher intrinsic efficacy at benzodiazepine receptors, as compared to the corresponding ethyl esters.[32] Substituted 1,2,4-oxadiazoles are also metabolically stable and slightly less

lipophilic than the corresponding ester derivatives.[33] Hence compounds **10**-**13** were synthesized based on molecular modeling to limit the number of compounds.[34, 35]

3. Synthesis

Ligands **6**-**8** were prepared by heating **1**-**3** with methylamine at 50 °C in ethanol in a sealed vessel. Ligand **9** was synthesized by heating acyl chloride of **5** (obtained by treatment of **5** with thionyl chloride at room temperature) with dimethyl amine at reflux in the presence of triethylamine. Ligands **10** and **11** were prepared by treatment of **1** with sodium hydride and *N*-hydroxy-propanimidamide or *N*-hydroxy-2-methyl-propanimidamide, respectively, in anhydrous THF at reflux in the presence of 4 Å molecular sieves. Ligands **12** and **13** were prepared from **2** and **3**, respectively, by treatment with sodium hydride followed by *N*-hydroxy-2-methyl-propanimidamide in anhydrous THF at reflux in the presence of molecular sieves (4 Å).

4. Results and discussion

4.1 In vitro metabolic stability of ligands 6-12

BzR ligands **6**-**8**, **11** and **12** were evaluated for metabolic stability (Table 2). Bioisosteric replacement of the ester moiety with an amide improved the metabolic stability of amides **6**-**8** in human microsomes. Monomethyl amide **6** (YT-III-31) exhibited a decreased rate of metabolism, as compared to the corresponding ethyl ester analog **1** in liver microsomes; however, over the period of 60 minutes almost 95% of the compound was metabolized. However the N-methyl amide analog **7** (HJ-I-40) of anxiolytic/analgesic **2** was not metabolized during the 60 minutes of incubation and exhibited improved metabolic stability as compared to the ethyl ester analog **2** (HZ-166). Amide **8** (HJ-I-37, approximately 92% remaining at the end of 60 min) was significantly more stable than the corresponding ethyl ester analog **3**, which had underwent biotransformation almost quantitatively. Substituted 1,2,4-oxadiazole analogs **11** (EMJ-I-026) and **12** (ZJW-II-40) were significantly more stable than **1** and **2** respectively, as expected, based on medicinal chemistry precedents.

Further assessment of these compounds in regard to agonist efficacy at BzR was carried out by *in vitro* electrophysiological studies on recombinant $GABA_A$ receptor subtypes expressed in *Xenopus* oocytes.

4.1.2. *In vitro* Electrophysiological Studies on 6-8 and 11-13 for Efficacy at Bz/GABAergic Receptor Subtypes—The dose-response curves for the stimulation of GABA-induced currents by BDZs **6**-**8** and **11**-**13** in oocytes, which expressed $GABA_A$ receptors of the subtypes $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$, $\alpha3\beta3\gamma2$, and $\alpha5\beta3\gamma2$, are illustrated in Figures 3–8. Amide **6** exhibited the most selective efficacy at $\alpha3\beta3\gamma2$ receptor subtypes. At 100 nM concentration it exhibited very weak agonistic effects at $\alpha1\beta3\gamma2$, $\alpha2\beta3\gamma2$ and $\alpha5\beta3\gamma2$; while acting as an antagonist at $\alpha1\beta3\gamma2$ and $\alpha5\beta3\gamma2$ at higher concentration, which is extremely desirable. This indicates that **6** is a potential nonsedating anxiolytic. Amides **7** and **8** exhibited selective efficacy at $\alpha3\beta3\gamma2$ BzR at higher concentration. Ligand **7**, however was relatively less potent than **8**. Dose-response curves for **11** indicated a maximum separation between efficacies at $\alpha1\beta3\gamma2$ and those at the other subtypes at physiologically relevant concentrations (100 nM). Oxadiazole **12** exhibited selective efficacy at $\alpha3\beta3\gamma2$, however it was far less potent than **6** and **11**. Oxadiazole **13**, at lower concentration, selectively modulated $\alpha5\beta3\gamma2$ and $\alpha3\beta3\gamma2$ receptor subtypes. At higher concentration this compound modulated $\alpha3\beta3\gamma2$ receptors with higher efficacy than $\alpha5\beta3\gamma2$ receptors. In general, all of the ligands exhibited the desired selective efficacy at the $\alpha3\beta3\gamma2$ Bz receptor subtype. Bioisostere **11** (EMJ-I-026) was selected for *in vivo* assessment for three reasons – it exhibited relatively more selective efficacy at $\alpha3\beta3\gamma2$ receptor subtypes at

physiologically relevant concentrations, it was metabolically stable as indicated in the liver microsomal assays, and it is a novel 1,2,4-oxadiazole analog.

4.1.3. Behavioral studies

Anxiolytic effect of 11 (EMJ-I-026) in the mouse model - The Light/Dark Cycle Test and Locomotor Activity Test: "Anxiolytic-like behavior" of subtype selective and metabolically stable oxadiazole **11** (EMJ-I-026) was tested in rodents using the light/dark cycle test (Figure 9). Increased time spent in the lighted area compared to vehicle indicates anti-anxiety effects. Diazepam was used as a positive control. Diazepam was significantly anxiolytic as compared to vehicle, as expected. Oxadiazole **11** exhibited significant anxiolytic activity at 10 and 100 mg/kg. Amphetamine-induced locomotor activity was a measure of the animal's activity related to sedation. Although this is not a direct measure of sedation, it gives some idea about the sedative nature of a compound. Diazepam was used as a positive control. Diazepam significantly reduced locomotor activity over vehicle i.e. an indicator of sedation, while α3 subtype selective oxadiazole **11** at doses of 10 and 100 mg/kg significantly increased locomotor activity. From this data, it was clear that α3 ligand **11** did not decrease locomotor activity. In these mice, there appeared to be no effects from either ataxia or sedation. This indicated in this paradigm that **11** (EMJ-I-026) was a potential nonsedating anxiolytic.

5. Summary

In this report, eight bioisosteric analogs of XHe-II-053 were designed in order to circumvent any potential metabolic liability in humans of the previously described ligand. In fact, the 2′N-analog nonsedating anxiolytic HZ-166 (**2**) was nearly 80-fold more stable than XHe-II-053 (**1**) after one hour (see table 1). Interestingly the 2′-F analog JY-XHe-053 (**3**) was not very stable. Importantly, the 5 bioisosteric ligands tested comprised of amides (**6**-**9**) and oxadiazoles (**10**-**13**) were much more stable on human liver microsomes than **1** again indicating these bioisosteres (**6**-**13**) are potential nonsedating anxiolytics as well as HZ-166 (**2**) useful for treatment of anxiety disorders in human populations. Gratifyingly, ligands **6**, **7**, and **11** were clearly α3 Bz/GABAergic receptor subtype selective ligands at pharmacologically relevant doses (approximately 100 nM) and, presumably, provide agents to study physiologically processes mediated by α3 subtypes including anxiety and in addition were much more stable on human liver microsomes than **1**. In this regard α3 subtype selective ligand oxadiazole **11** (EMJ-I-026) has been evaluated in the light dark paradigm and clearly is a nonsedating anxiolytic, wherein this ligand was anxiolytic with no sedative properties, *in vivo*, as compared to diazepam in this paradigm. This study indicated that the ester function in these molecules can be replaced with a metabolically more stable ester bioisostere and still retain anxiolytic activity. The in depth study of these ligands in animal models and other receptor systems is underway and will be reported in due course.

7. Experimental

7.1. General methods for organic synthesis

Proton and carbon nuclear magnetic resonance spectra were obtained on a Bruker 300-MHz spectrometer. Chemical shifts are reported as parts per million (δ) using an internal standard of residual $CHCl_3$ (7.26 ppm). The low resolution mass spectra (LRMS) were obtained on an electron impact (EI, 70 eV) mass spectrometer, 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. Infra-red spectra were recorded on a Thermo Nicolet Nexus 870 FT-IR or a Perkin Elmer 1600 series FT-IR spectrometer. Elemental analyses were performed on a Carlo Erba model

EA-1110 carbon, hydrogen, and nitrogen analyzer. All samples submitted for CHN analyses were first dried under high vacuum for a minimum of six hours using a drying pistol with isopropyl alcohol as the solvent with potassium hydroxide pellets in the drying bulb. Melting points were taken on an Electrothermal model IA8100 digital melting point apparatus and are uncorrected. Analytical thin layer chromatography plates employed were Dynamic Adsorbents Inc UV active silica gel on plastic, while silica gel 60A, grade 60 for flash and gravity chromatography, were purchased from E. M. Science. Tetrahydrofuran (THF) was dried by distillation from sodium-benzophenone ketyl. Dichloromethane was distilled from calcium hydride. Acetonitrile was used directly as received.

7.1.1. 8-Ethynyl-N-methyl-6-phenyl-4H-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-carboxamide (6)[36]—Ester **1** (355 mg, 1 mmol) was treated with methylamine (33% wt solution in ethanol, 10 mL). The suspension which resulted was stirred in a sealed vessel at 45–50 °C for 24 h during which time it became a clear solution. After removal of the ethanol and methylamine under reduced pressure, the residue was purified by a wash column (silica gel, gradient elution CH_2Cl_2-0.5% MeOH in CH_2Cl_2) to afford amide **6** as a white powder (238 mg, 0.7 mmol, 70%): mp 237–238°C; IR (KBr) 3284, 1651, 1607, 1567, 1525, 1409, 1303, 1256, 1208, 1010, 942, 826, 782, 765, 743, 698 cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.99-2.97(d, 3H, J = 5.04 Hz), 3.17(s, 1H), 4.09-4.05(d, 1H, J = 12.1 Hz), 6.29-6.26 (d, 1H, J = 10.7 Hz), 7.09-7.08 (t, 1H), 7.59-7.36 (m, 7H), 7.78-7.75 (dd, 1H, J = 1.8 Hz and 1.6 Hz), 7.85 (s, 1H); MS (EI) m/e(relative intensity) 340 (M^+, 100). HRMS(TOF) Calcd for $C_{21}H_{16}N_4ONa$ $(M+Na)^+$ 363.1222, found: 363.1202. CHN Analysis: Anal. Calcd. for $C_{21}H_{16}N_4O$•0.95 H_2O: C, 70.56; H, 5.05; N, 15.67. Found: C, 70.58; H, 4.82; N, 15.20.

7.1.2. 8-Ethynyl-*N*-methyl-6-(pyridine-2-yl)-4*H*-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-carboxamide (7)—Target **7** was prepared in 80% yield from **2**[30] using the procedure as described above. **7**: mp 215–216 °C; ^1H NMR ($CDCl_3$) δ 2.97–2.99 (d, 3H, J = 4.9 Hz), 3.16 (s, 1H), 4.12 (bs, 1H), 6.29 (bs, 1H), 7.14–7.15 (bs, 1H), 7.33–7.37 (t, 1H), 7.51–7.57 (m, 2H), 7.71–7.84 (m, 3H), 8.12–8.15 (d, 1H), 8.55–8.57 (d, 1H). $C_{20}H_{15}N_5O$; MS (EI) m/e (relative intensity) 341(M^+, 100), 342 (22). HRMS(TOF) Calcd for $C_{20}H_{16}N_5O(M+H)^+$ 342.1355, found: 342.1365.

7.1.3. 8-Ethynyl-*N*-methyl-6-(2-fluorophenyl)-4*H*-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-carboxamide (8)—Amide **8** was prepared in 75% yield from **3**[31] using the procedure analogous to the one used for the synthesis of **6**. Compound **8**: mp 235–236 °C; ^1H NMR ($CDCl_3$) δ 2.96–2.98 (d, 3H, J = 4.57 Hz), 3.16 (s, 1H), 4.10 (bs, 1H), 6.29 (bs, 1H), 6.96–7.02 (t, 1H), 7.14 (bs, 1H), 7.21–7.26 (t, 1H), 7.39–7.46 (m, 2H), 7.52–7.55 (d, 1H), 7.65–7.73 (m, 2H), 7.86 (s, 1H). $C_{21}H_{15}FN_4O$; MS (EI) m/e (relative intensity) 358 (M^+, 100), 359 (23). HRMS(TOF) Calcd for $C_{20}H_{15}FN_4ONa$ $(M+Na)^+$ 381.1128, found: 381.1140. CHN Analysis: Anal. Calcd. for $C_{21}H_{15}FN_4O$•0.17 CH_2Cl_2: C, 68.21; H, 4.15; N, 15.03. Found: C, 68.29; H, 4.28; N, 14.71.

7.1.4. 8-Ethynyl-*N, N*-dimethyl-6-(pyridine-2-yl)-4*H*-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-carboxamide (9)[36]—Acid **5** (328 mg, 1 mmol) was dissolved in CH_2Cl_2 (5 mL). To the suspension which resulted was added $SOCl_2$ (0.5 mL) and the mixture was stirred at rt for 1 h during which time it became a clear solution. After removing the solvent and $SOCl_2$ under reduced pressure, the residue was redissolved in CH_2Cl_2 (5 mL) and was treated with Et_3N (0.2 mL) and Me_2NH (0.2 mL). After stirring at reflux for 8 h, the reaction was quenched with water (5 mL). The organic layer was washed with brine (5 mL) and dried (Na_2SO_4). After removal of the solvent under reduced pressure, the residue was purified by a wash column (silica gel, gradient elution CH_2Cl_2-0.5% MeOH

in CH_2Cl_2) to afford amide **9** (178 mg, 0.5 mmol, 50%) as a light yellow powder: mp 193–194°C. ^{1}H NMR ($CDCl_3$) δ 3.10(s, 3H), 3.16(s, 1H), 3.35(s, 1H), 4.17–4.21(d, 1H, *J* = 12.09 Hz), 5.89–5.93(d, 1H, *J* = 12 Hz), 7.35–7.39(m, 1H), 7.52–7.55(m, 2H), 7.73–7.86(m, 3H), 8.10-8.07(d, 1H, *J* = 7.8 Hz), 8.58–8.59(d, 1H, *J* = 4.8 Hz); ^{13}C NMR ($CDCl_3$) δ 35.9, 39.1, 45.2, 79.3, 81.8, 120.9, 122.7, 124, 124.7, 127, 132.6, 133.3, 135.2, 135.8, 136.2, 136.8, 136.9, 148.7, 156.7, 164.5, 167.4. HRMS (ESI) m/z calcd for $C_{21}H_{18}N_5O$ $(M+H)^+$ 356.1511, found: 356.1528.

7.1.5. 5-(8-Ethynyl-6-phenyl-4H-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-ethyl-1,2,4-oxadiazole (10)[23]—Ethyl amido oxime (59.5 mg, 0.676 mmol) was added to a stirred suspension of powdered 4 Å molecular sieves (75 mg) in anhydrous THF (15 mL) under argon. After the mixture was allowed to stir at rt for 10 min, NaH (60 % dispersion in mineral oil; 0.676 mmol) was added to the mixture. After the mixture had stirred for another 30 min, a solution of the forgoing ester **1** (120 mg, 0.338 mmol) in THF (20 mL) was added. The mixture, which resulted, was heated at reflux for 8 h. The reaction mixture was then cooled to rt, after which acetic acid (40.6 mg, 0.676 mmol) was added. After the solution was stirred for 10 min, the mixture was filtered through celite. The filtrate was diluted with CH_2Cl_2 (50 mL) and washed sequentially with water, brine, and then dried (K_2CO_3). Evaporation of the solvent under reduced pressure afforded a pale yellow solid, which was purified by flash column chromatography (silica gel, EtOAc/hexane, 2:3) to furnish **10** as a white solid (52 mg, 40%): mp 221–222°C; IR (KBr)ν 3297, 3105, 1603, 1570, 1495, 1310, 938 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 8.07 (s, 1H), 7.80 (dd, 1H, J_1 = 8.4 Hz, J_2 = 1.8 Hz), 7.64-7.6 (m, 2H), 7.53-7.37 (m, 5H), 6.12 (d, 1H, *J* = 12.9 Hz), 4.21 (d, 1H, *J* = 12.9 Hz), 3.20 (s, 1H), 2.88 and 2.83 (Abq, 2H, *J* = 7.6 Hz), 1.41 (t, 3H, *J* = 7.6 Hz); ^{13}C NMR (300 MHz, $CDCl_3$) δ 171.8, 170.6, 168.8, 139.1, 136.6, 135.8, 135.4 (2C), 135.1, 130.7, 129.3 (2C), 128.3 (2C), 128.1, 124.7, 122.7, 121.6, 81.2, 80, 44.7, 19.7, 11.5. MS(EI) m/e (relative intensity) 379 (M^+, 100).

7.1.6. 5-(8-Ethynyl-6-phenyl-4H-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-isopropyl-1,2,4-oxadiazole (11)[36]—Isopropyl amido oxime (95 mg, 0.931 mmol) was added to a stirred suspension of powdered 4 Å molecular sieves (100 mg) in anhydrous THF (30 mL) under argon. After the mixture was allowed to stir at rt for 10 min, NaH (60 % dispersion in mineral oil; 0.931 mmol) was added to the mixture. After the mixture was stirred for a further 30 min, a solution of the forgoing ester **1** (165 mg, 0.465 mmol) in THF (30 mL) was added. The mixture which resulted was heated to reflux for 8 h. The reaction mixture was then cooled to rt, after which acetic acid (56 mg, 0.931 mmol) was added. After the solution was stirred for 10 min, the mixture was filtered through celite. The filtrate was diluted with CH_2Cl_2 (75 mL) and washed with water, brine and dried (K_2CO_3). Evaporation of the solvent under reduced pressure afforded a pale yellow solid, which was purified by flash column chromatography (silica gel, EtOAc/hexane, 2:3) to furnish **11** as a white solid (82 mg, 0.209 mmol, 45%): mp 190°C; IR (KBr)ν 3291,3057, 2972, 1613, 1574, 1494, 1466, 1303, 1264, 939, 832, 781, 734, 699, 666 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ8.07(s, 1H), 7.81-7.79(dd, 1H), 7.64-7.61(m, 2H), 7.53-7.37(m, 5H), 6.14(d, 1H, *J* = 13.1 Hz), 4.19(d, 1H, *J* = 12.8 Hz), 3.20(s, 1H), 3.24-3.15(m, 1H), 1.44-1.41(d, 6H, *J* = 6.93 Hz); MS(EI) m/e (relative intensity) 393(M^+, 100). HRMS (TOF) Calcd for $C_{24}H_{19}N_5ONa(M+Na)^+$ 416.1487, found: 416.1501. CHN Analysis: Anal. Calcd. for $C_{24}H_{19}N_5O$•0.37 CH_2Cl_2: C, 68.89; H, 4.68; N, 16.48. Found: C, 68.94; H, 4.59; N, 16.32. (CHN sample was transferred to a vial for drying with CH_2Cl_2 which may explain the contaminant.)

7.1.7. 5-(8-Ethynyl-6-(pyridin-2-yl)-4H-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-yl)-3-isopropyl-1,2,4-oxadiazole (12)—Oxadiazole **12** was prepared in 40% yield from **2** using the same procedure employed for the synthesis of **11. 12**: mp 200

°C; ^{1}H NMR ($CDCl_3$) δ 1.42–1.44 (d, 6H, *J* = 6.93 Hz), 3.13–3.27 (m, 2H), 4.27–4.31 (d, 1H, *J* = 10.8 Hz), 6.14–6.18 (d, 1H, *J* = 10.8 Hz), 7.36–7.40 (m, 1H), 7.59–7.62 (m, 2H), 7.77–7.86 (m, 2H), 8.04–8.09 (m, 2H), 8.58–8.60 (m, 1H); ^{13}C NMR (300 MHz, $CDCl_3$) δ 20.5, 26.7, 44.8, 79.5, 81.5, 121.3, 122.7, 123.9, 124.8, 127, 135.2, 135.3, 135.7, 136, 136.2, 136.8, 148.7, 156.3, 167.8, 170.6, 175.2, 190.2; HRMS (ESI) Calcd for $C_{23}H_{19}N_6O$ $(M+H)^+$ 395.1620, found: 395.1635.

7.1.8. 5-(8-Ethynyl-6-(2-fluorophenyl)-4H-benzo-[*f*]-imidazo-[1,5-*a*]-[1,4]-diazepine-3-yl)-3-isopropyl-1,2,4-oxadiazole (13)—Oxadiazole **13** was prepared in 45% yield from **3** using the procedure for the synthesis of **11. 13**: mp 160–165°C; IR (neat) ν3194, 2961,2924, 2854, 1631, 1610, 1495, 1450, 1414, 1394, 1367, 1342, 1312, 1259, 1221, 1071, 1011, 940, 903, 862, 793, 767, 754, 697, 671cm$^{-1;}$ ^{1}H NMR ($CDCl_3$) δ8.09(s, 1H), 7.80(dd, 1H, *J* = 1.78, 1.78 Hz), 7.69(m, 3H), 7.51(m, 2H), 7.07(m, 1H), 6.26(brs, 1H), 4.40(brs, 1H), 3.24 (m, 2H), 1.43(d, 6H, *J* = 6.93 Hz); MS (EI) m/e (relative intensity) 411(43), 383(M^+, 98), 325(100), 299(74), 178(74), 57(57); HRMS(ESI) Calcd for $C_{24}H_{18}FN_5O$ $(M+H)^+$ 412.1644, found: 412.1628.

7.2. Electrophysiological experiments

Xenopus oocytes were injected with rat cDNA's of $GABA_A$ receptor β3 and γ2 subunits as well as α1, α2, α3, or α5 subunits[21] 36 hours after injection, the enveloping follicle cell layers of the oocytes were removed and 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). For current measurements the oocytes were impaled with two microelectrodes (2–3 mΩ), which were filled with 2 mM KCl. 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 two applications, oocytes were washed in XR for up to 15 min to ensure full recovery from desensitization. All recordings were performed at room temperature at a holding potential of −60 mV using a Warner OC-725C two-electrode voltage clamp. Data were digitised, recorded and measured using a Digidata 1322A data acquisition system. Results of concentration response experiments were fitted using GraphPad Prism 3.00 (GraphPad Software, San Diego, CA).

The equation used for fitting concentration response curves was Y=Bottom + (Top −Bottom)/(1+10$^{((LogEC50-X)*HillSlope))}$; X represents the logarithm of concentration, Y represents the response; Y starts at Bottom and goes to Top with a sigmoid shape. This is identical to the "four parameter logistic equation".

7.3. Metabolic Stability for $GABA_A$ receptor ligands using human liver microsomes (SRI study No. 400-10)

The test articles were incubated at two concentrations (1 and 10 μM) in 96-well plate format with active or heat-inactivated human liver microsomes and cofactors. Aliquots were removed at 0, 15, 30 and 60 minutes and mixed with acetonitrile containing internal standard for analysis. Samples were extracted and assayed using a liquid chromatography/tandem mass spectrometry (LC-MS/MS) analytical method. The test articles were prepared as stock solutions in DMSO and stored in aliquots at −20 °C. On the day of the experiment, the test articles were diluted in the 100 mM phosphate buffer (pH 7.4) to achieve appropriate final concentrations.

Pooled human liver microsomes (Lot # 38289, pool of 150 different male and female donor livers) were obtained from BD Biosciences Corporation (Woburn, MA). Microsomes were stored at ~ −135°C until use.

The test articles (1 and 10 μM) were incubated with human liver microsomes (0.5 mg protein/ml) and appropriate cofactors (2.5 mM NADPH and 3.3 mM magnesium chloride) in 100 mM phosphate buffer, pH 7.4 (0.1% final DMSO), in a 37°C water bath. Incubations with all compounds were initiated with the addition of microsomes. At selected time points (0, 15, 30 and 60 min), a single 100 μl aliquot was removed from each sample and mixed with 200 μl of chilled acetonitrile containing internal standard. Following brief vortexing and centrifugation, the samples were further diluted into a 96-well plate for subsequent LC-MS/MS analysis. All samples were assayed in duplicate.

Experimental controls consisted of: a) incubation of all components except test article for 0 and 60 min, b) incubation of midazolam (positive control) at 10 μM for 0, 15, 30 and 60 min, and c) incubation of 1 and 10 μM test article and 10 μM midazolam with heat-inactivated microsomes (0.5 mg protein/ml) for 0 and 60 min. All controls were assayed in duplicate.

Samples were analyzed by LC-MS/MS in multiple reaction monitoring mode using positive-ion electrospray ionization. The details of the LC-MS/MS method can be provided upon request.

Data from the metabolic stability assays were transferred to and processed in a Microsoft Excel spreadsheet. To determine metabolic stability, the percent remaining at each time point was calculated by dividing the peak area ratio of test article/internal standard at each time point by the peak area ratio at 0 min multiplied by 100.

7.4. The Light/Dark Cycle Test and Locomotor Activity Test on oxadiazole 11

Rats used for behavior were housed in a reverse light/dark cycle (lights on from 1900 to 0700 h) for at least 10 days before the start of behavioral experiments. Rats were administered the diazepam (3 mg/kg, i.p.), test compound (10 mg/kg, i.p.), or SAL (2 ml/kg) 20 min before being placed in an open-field arena (Coulbourn Instruments, Allentown, PA) in which spontaneous locomotor activity in the x–y plane was determined for 30 min by beam breaks and recorded with TruScan software (Coulbourn Instruments). Rats were then injected with D-amphetamine sulfate (0.5 mg/kg, i.p.) and locomotor activity recorded for an additional 90 min.

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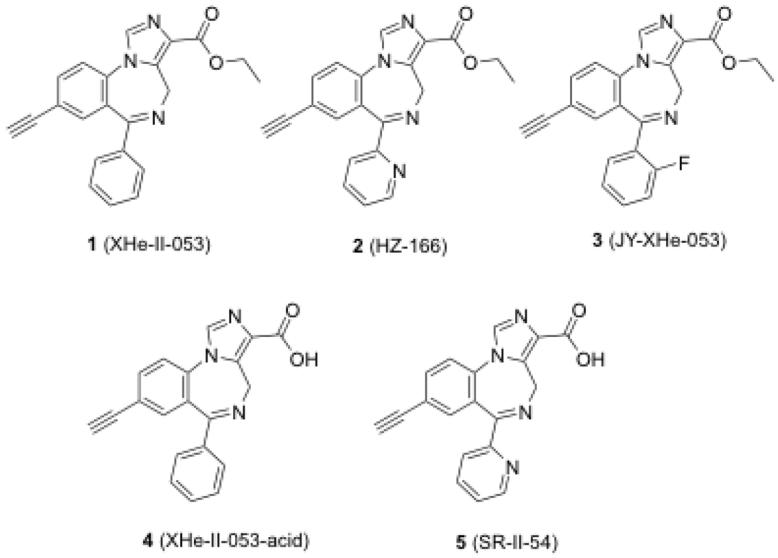
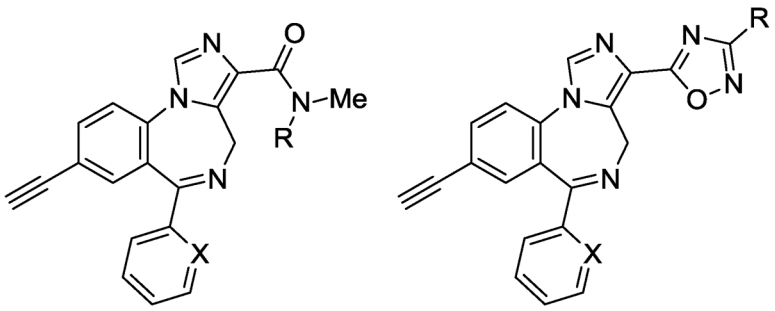


Figure 1.
Structures of imidazobenzodiazepine-related ligands **1-5**



6-9

6: X = CH; R = H
7: X = N; R = H
8: X = CF; R = H
9: X = N; R = Me

10-13

10: X = CH; R = Et
11: X = CH; R = *i*-Pr
12: X = N; R = *i*-Pr
13: X = CF; R = *i*-Pr

Figure 2.
Pharmacokinetically-based bioisosteric analogs of the lead compounds **1**-**3** to increase stability.

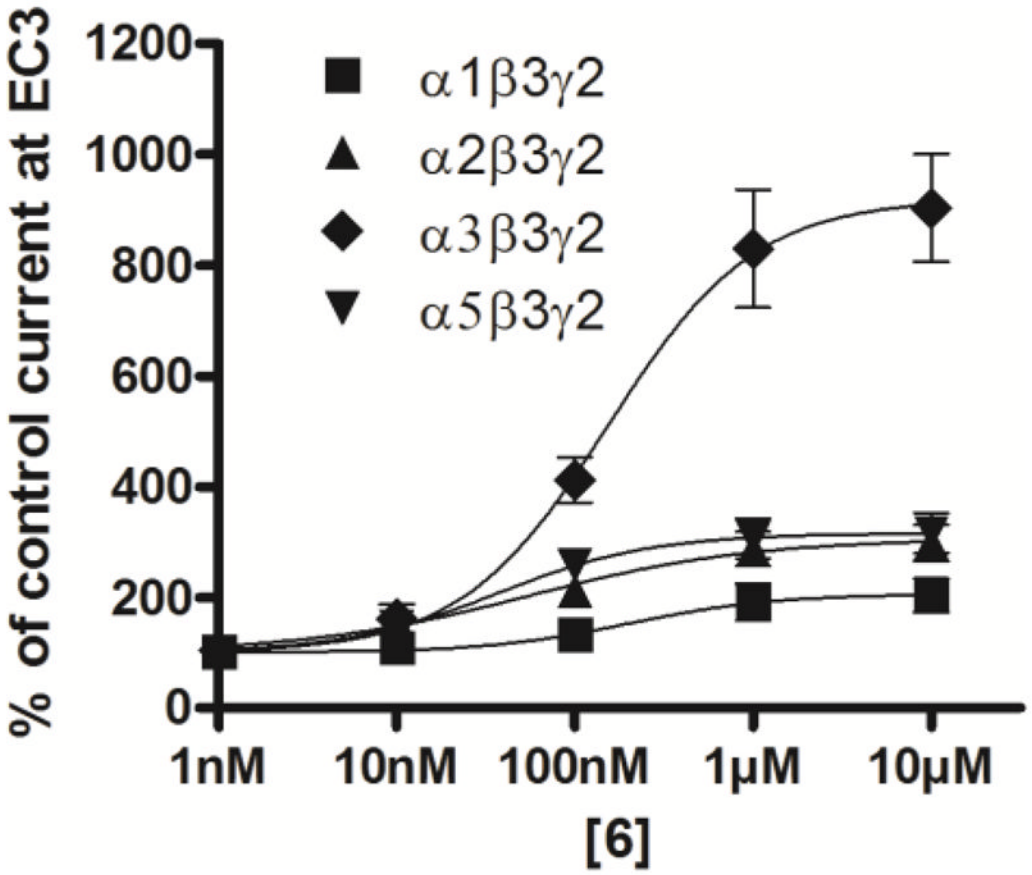


Figure 3.

Concentration–effect curves for **6** (YT-III-31) on α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (◆), and α5β3γ2 (▼) GABAA receptors, using an EC3 GABA concentration. Data points represent the mean ± SEM from four oocytes (for each receptor subtype) from 2 batches. Stimulation of GABA EC3 by compound 6 at 100 nM or 1 μM concentration was 132±11 or 192±30, 211±23 or 287±31, 412±41 or 831±106, and 257±28 or 307±36, for α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors, respectively.

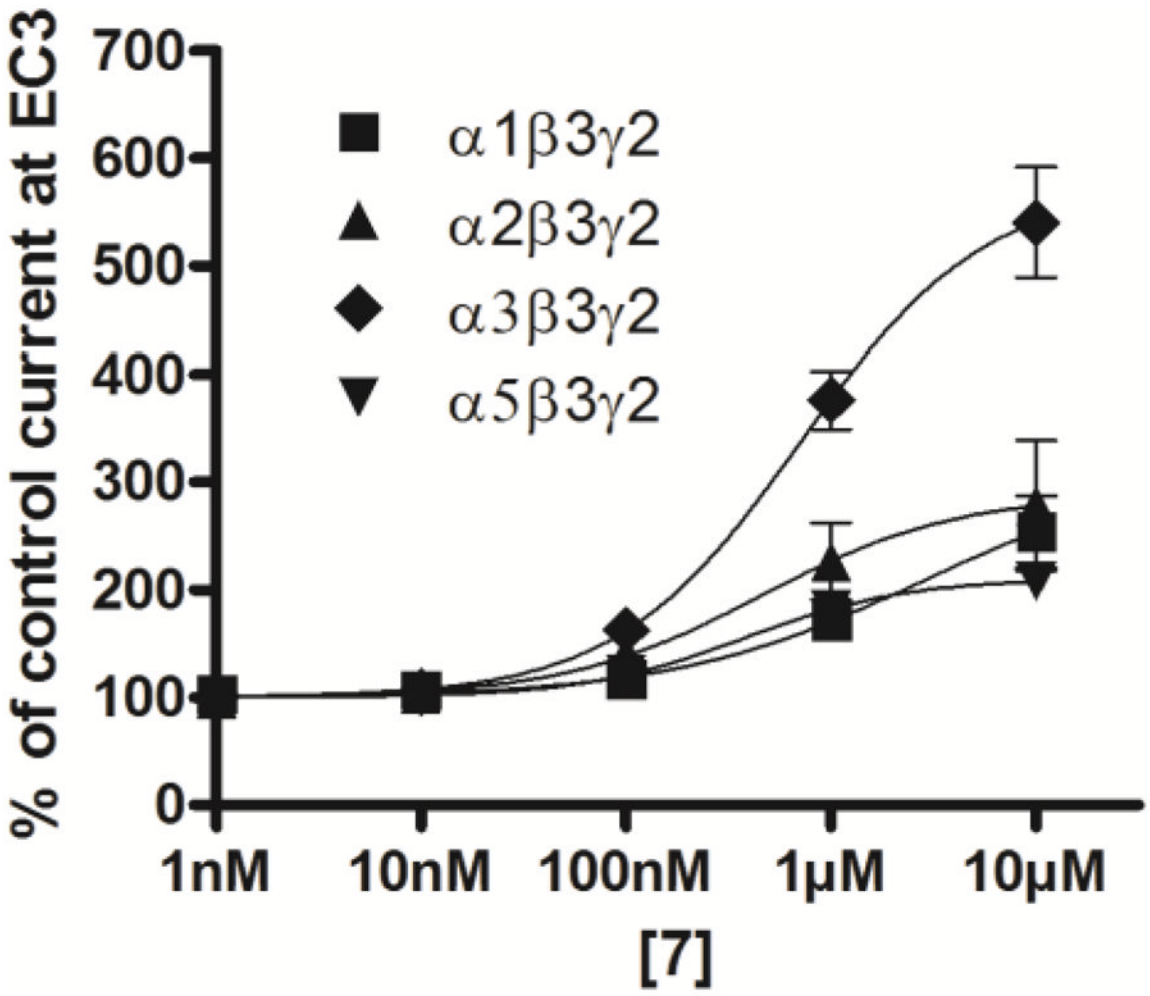


Figure 4.

Concentration–effect curves for **7** (HJ-I-40) on α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (▼), andα5β3γ2 (◆) GABAA receptors, using an EC3 GABA concentration. Data points represent the mean±SEM from at least three oocytes (for each receptor subtype) from ≥2 batches. Stimulation of GABA EC3 by compound 7 at 100 nM or 1 μM concentration was 117±3 or 171±12, 140±15 or 226±35, 163±5 or 376±27, and 122±5 or 182±9, for α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors, respectively.

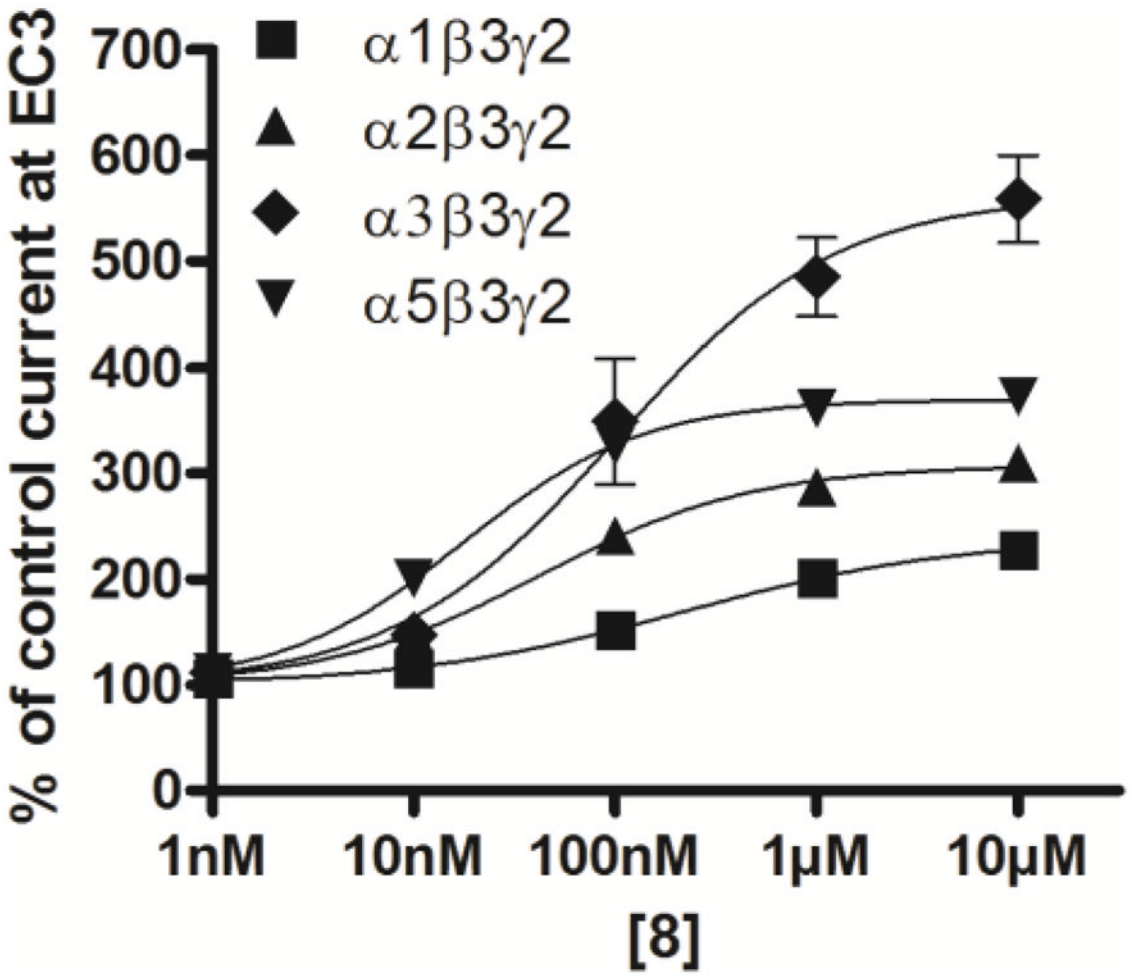


Figure 5.

Concentration–effect curves for **8** (HJ-I-37) on α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (◆), and α5β3γ2 (▼) GABAA receptors, using an EC3 GABA concentration. Data points represent the mean±SEM from at least three oocytes (for each receptor subtype) from 2 batches. Stimulation of GABA EC3 by compound 8 at 100 nM or 1 μM concentration was 152±6 or 201±15, 241±12 or 287±16, 349±60 or 485±37, and 326±9 or 361±4, for α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors, respectively.

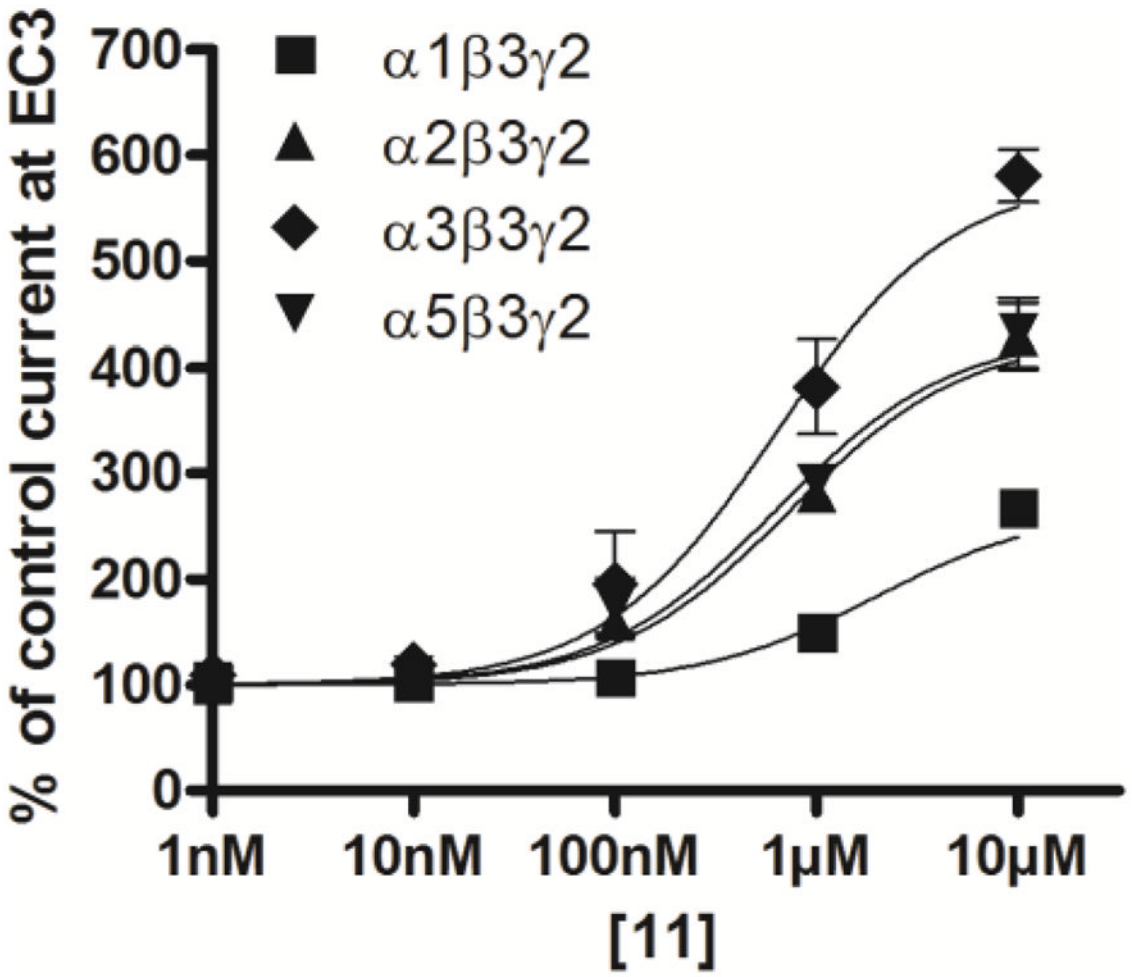


Figure 6.

Concentration–effect curves for **11** (EMJ-I-026) on α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (◆), and α5β3γ2 (▼) GABAA receptors, using an EC3 GABA concentration. Data points represent the mean ± SEM from at least three oocytes (for each receptor subtype) from 2 batches. Stimulation of GABA EC3 by compound 11 at 100 nM or 1 μM concentration was 107±4 or 150±10, 160±3 or 281±13, 195±5 or 381±45, and 175±66 or 291±13, for α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors, respectively.

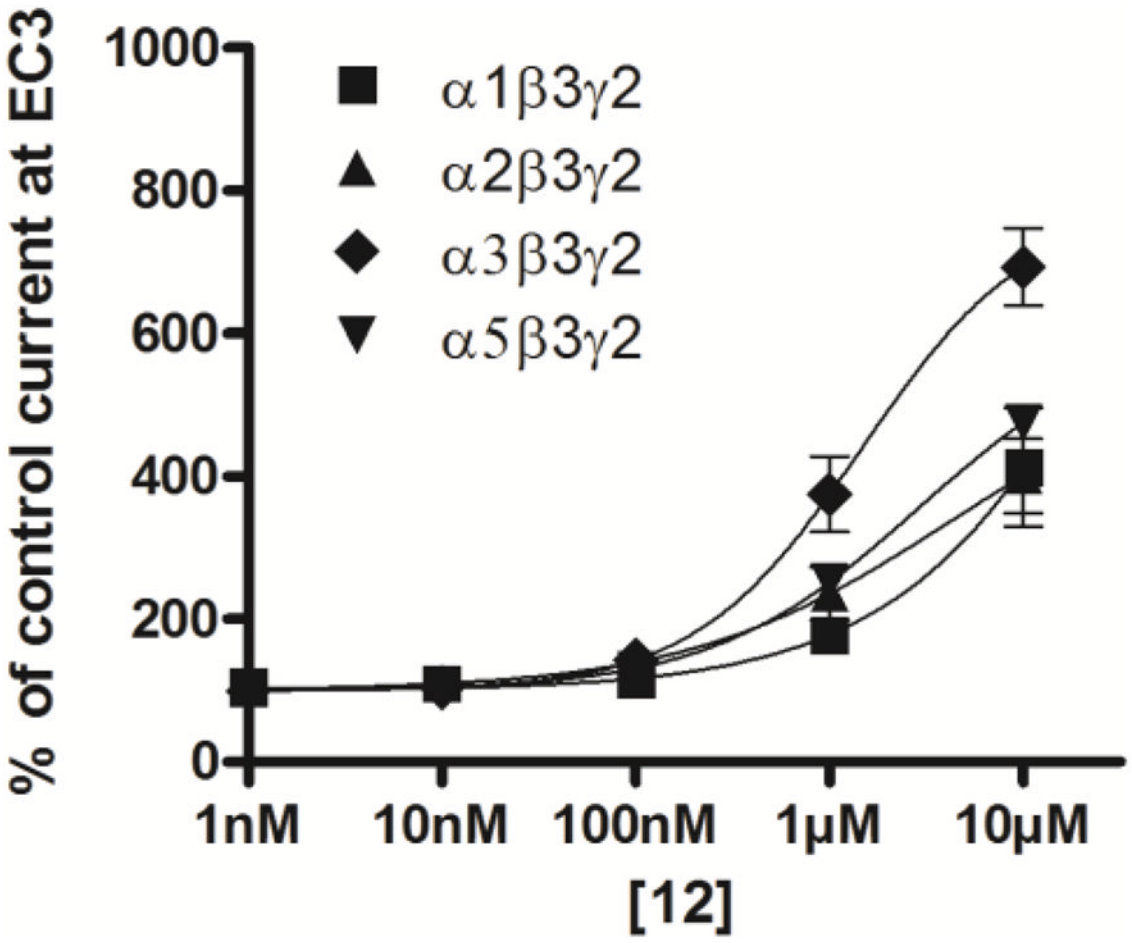


Figure 7.

Concentration–effect curves for **12** (ZJW-II-40) on α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (▼), and α5β3γ2 (◆) GABAA receptors, using an EC3 GABA concentration. Data points represent the mean ± SEM from four oocytes (for each receptor subtype) from ≥2 batches. Stimulation of GABA EC3 by compound 12 at 100 nM or 1 μM concentration was 117±2 or 178±19, 139±6 or 236±37, 143±5 or 375±52, and 130±1 or 252±11, for α1β3γ2, α2β3γ2, α3β3γ2, and α5β3γ2 receptors, respectively.

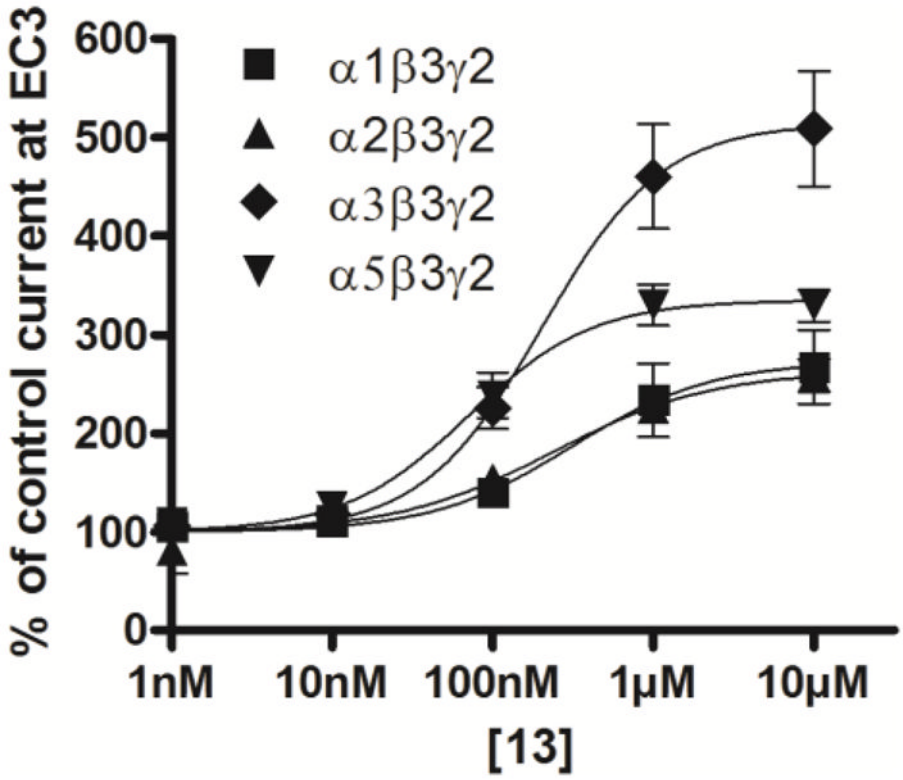


Figure 8.

Concentration–effect curves for **13** (YT-III-40) on α1β3γ2 (■), α2β3γ2 (▲), α3β3γ2 (◆), and α5β3γ2 (▼) GABAA receptors, using an EC3 GABA concentration. Data points represent the mean ± SEM from four oocytes (for each receptor subtype) from ≥2 batches. Stimulation of GABA EC3 by compound 13 at 100 nM or 1 μM concentration was 140±8 or 234±37, 154±2 or 225±9, 226±53 or 460±53, and 239±23 or 331±21, for α1β3γ2, α2β3γ2,, α3β3γ2, and α5β3γ2 receptors, respectively.

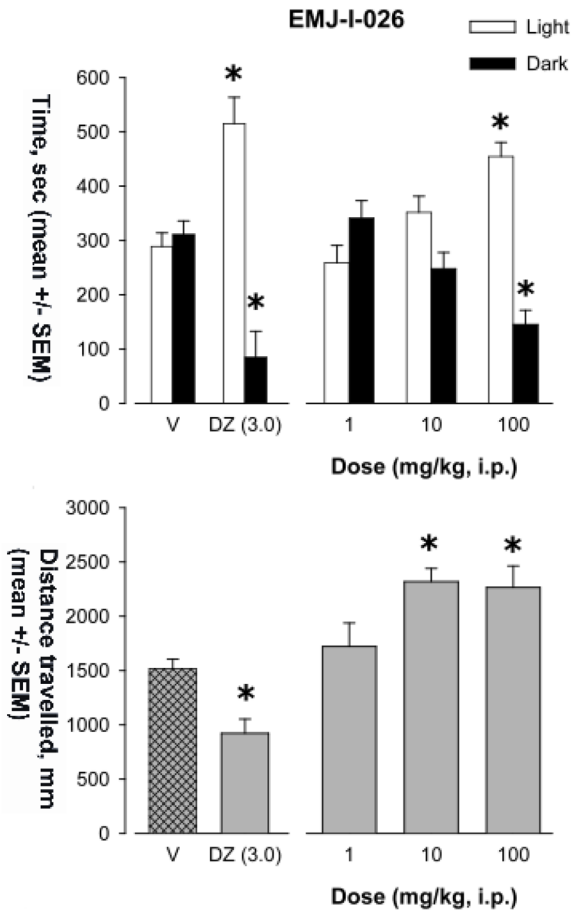
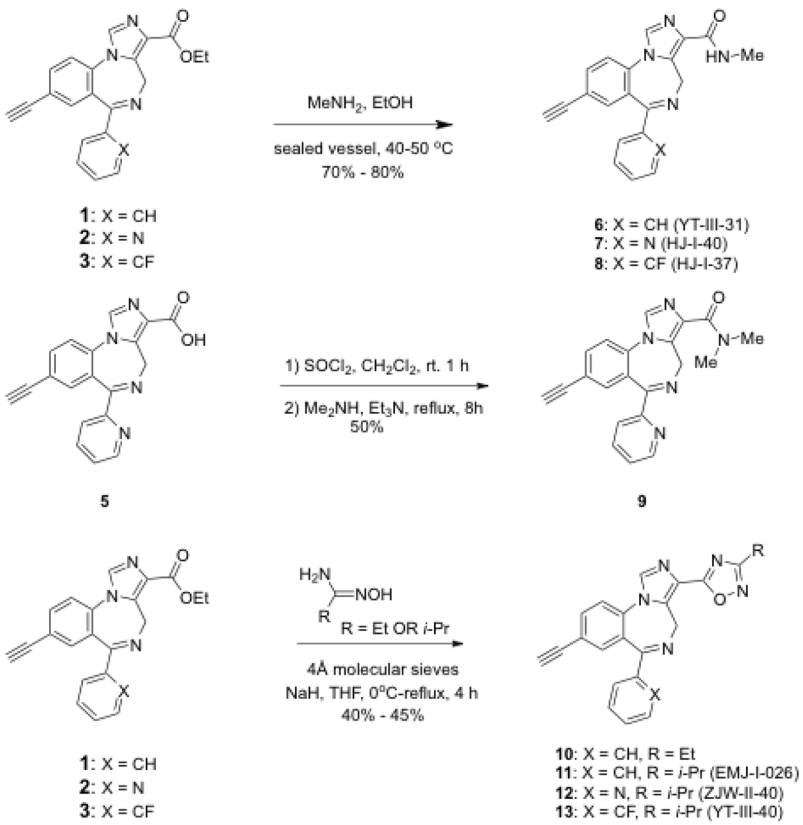


Figure 9.
The light/dark cycle test and locomotor activity test on oxadiazole agonist **11** (EMJ-I-026)



Scheme 1.
Synthesis of **6-13**

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Table 1

In vitro metabolic stability of **1**-**5** using human liver microsomes by SRI International.

Test Article	Time, min	Mean % Remaining vs T = 0 min[a]	
		1 μM[b]	10 μM[b]
1	15	41.4	47.6
	30	11.2	13.9
	60	1.5	1.7
1 with HI[c] microsomes	60	107.4	101.8
2	15	106.0	103.4
	30	95.6	90.4
	60	80.2	76.1
2 with HI[c] microsomes	60	93.8	95.4
3	15	13.5	20.0
	30	2.2	3.6
	60	0.3	0.6
3 with HI[c] microsomes	60	101.9	110.5
4	15	107.6	95.3
	30	110.4	94.8
	60	110.7	94.8
4 with HI[c] microsomes	60	105.8	97.2
5	15	101.9	91.4
	30	104.6	86.1
	60	109.6	85.9
5 with HI[c] microsomes	60	97.0	88.3

[a] % remaining at T =0 is 100%

[b] Samples were assayed in duplicates

[c] HI = Heat Inactivated, for control purposes

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Table 2

In vitro metabolic stability of **6-12** on human liver microsomes

Test Article	Time, min	Mean % Remaining vs T = 0 min[a]	
		1 μM[b]	10 μM[b]
6	15	87.3	107.4
	30	37.5	104.3
	60	5.2	82.8
6 with HI[c] microsomes	60	114.2	113.6
7	15	108.7	104.4
	30	126.0	106.3
	60	136.4	110.0
7 with HI[c] microsomes	60	109.6	109.5
8	15	97.3	96.8
	30	95.9	102.8
	60	92.3	103.3
8 with HI[c] microsomes	60	116.2	108.5
11	15	100.0	109.2
	30	109.7	108.0
	60	90.8	96.6
11 with HI[c] microsomes	60	137.5	118.9
12	15	97.8	105.6
	30	98.7	99.3
	60	89.5	95.1
12 with HI[c] microsomes	60	112.7	91.7

[a] % remaining at T =0 is 100%

[b] Samples were assayed in duplicates

[c] HI = Heat Inactivated, for control purposes