Published in final edited form as:

J Med Chem. 2016 December 08; 59(23): 10800–10806. doi:10.1021/acs.jmedchem.6b01332.

Synthesis and characterization of a novel gamma-aminobutyric acid type A (GABA_A) receptor ligand that combines outstanding metabolic stability, pharmacokinetics, and anxiolytic efficacy

Michael M. Poe^{1,‡}, Kashi Reddy Methuku^{1,‡}, Guanguan Li¹, Ashwini R. Verma¹, Kelly A. Teske^{1,#}, Douglas C. Stafford¹, Leggy A. Arnold¹, Jeffrey W. Cramer², Timothy M. Jones², Rok Cerne², Michael J. Krambis², Jeffrey M. Witkin², Enrique Jambrina³, Sabah Rehman⁴, Margot Ernst⁴, James M. Cook¹, and Jeffrey M. Schkeryantz^{2,*}

¹Department of Chemistry and Biochemistry and the Milwaukee Institute for Drug Discovery, University of Wisconsin-Milwaukee, Milwaukee, WI, 53201

²Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 42685

³Lilly Research Laboratories, Eli Lilly and Company, Alcobendas, Spain

⁴Department for Molecular Neurosciences, Medical University of Vienna, Vienna, Austria

Abstract

1,4-Benzodiazepines are used in the treatment of anxiety disorders but have limited long term use due to adverse effects. HZ-166 (**2**) has been shown to have anxiolytic-like effects with reduced sedative/ataxic liabilities. A 1,3-oxazole KRM-II-81 (**9**) was discovered from a series of six bioisosteres with significantly improved pharmacokinetic and pharmacodynamic properties as compared to **2**. Oxazole **9** was further characterized and exhibited improved anxiolytic-like effects in a mouse marble burying assay and a rat Vogel conflict test.

Graphical Abstract

*Corresponding Author: schkeryantz_jeffrey@lilly.com. Phone: (317)-433-5175.

[‡]Author Contributions

M.M.P. and K.R.M. contributed equally.

[#]Present Addresses: K.A.T. is now at the School of Pharmacy, University of Connecticut, Storrs, CT, 06269.

ASSOCIATED CONTENT

Supporting Information. The electrophysiology using the Ion-Works Barracuda high-throughput platform and the unbound concentrations time-response curve can be found in the Supporting Information. Additional tables, figures, methods and experimental are also included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

The γ -aminobutyric acid type A receptor (GABA_AR), a heteropentameric chloride ion channel,^{1, 2} is the principle target for the major inhibitory neurotransmitter, γ -aminobutyric acid (GABA), within the central nervous system (CNS). Benzodiazepines (BZDs) are a general class of drugs known to bind to the GABA_AR and are primarily used to treat anxiolytic and obsessive-compulsive disorders.³ The pharmacological action exerted by a BZD is dependent on the discrete subunits of the receptor complex. Classical BZDs, such as diazepam, bind non-selectively to $\alpha 1$ - $\beta 2$ GABA_AR at the well-documented BZD binding site located at the $\alpha^+ \gamma^-$ interface.^{4, 5}

A compelling clinical opportunity exists in the development of $\alpha 2/\alpha 3$ selective GABA_AR ligands.^{6, 7} Based on the literature, these ligands are expected to result in superior treatments for seizures,^{8, 9} antihyperalgesia^{10, 11} and anxiety^{12–14} without causing sedation, amnesia and ataxia, or the propensity for addiction/dependence^{15, 16} or tolerance.¹⁷ The clinical value of classical BDZs is evidenced by the fact that some have been marketed for over 50 years without a suitable replacement. Continued research has seen a number of promising candidates^{8, 18–21} fail due to pharmacokinetic complications²² or a variety of adverse effects.^{19, 23, 24}

Over the past decade a series of $\alpha 2/\alpha 3$ subtype selective imidazobenzodiazepines, using a privileged scaffold, have been designed, synthesized and studied. Some of the first compounds, ligands **1** – **3** (XHe-II-053, HZ-166 and JY-XHe-053, respectively; Chart 1) have been characterized previously for their selectivity at GABA_AR containing $\alpha 2/\alpha 3$ subunits over $\alpha 1$.²⁵ Previous investigations indicated that ligand **2** can engender anxiolytic-like effects in primates without sedative-like liabilities of non-selective positive allosteric modulators (e.g. diazepam).²⁶ However, ligand **2**, as well as ligands **1** and **3**, suffered from poor exposure and high clearance in rodents due to hydrolysis of the ester to the carboxylic acid **4**, which exhibited low penetration across the blood-brain-barrier. Therefore, the goal of the present study was to discover a lead-like molecule that would retain the anxiolytic-like properties of BZDs **1** – **3** with lower systemic clearance in rats and thus increase plasma and brain exposure. Our design strategies to prevent formation of the carboxylic acid metabolite included blocking a site of oxidation on the ester by replacing the $-\text{CH}_2$ with a $-\text{CD}_2$ (ligand **5**, see Scheme S1) and replacing the ester altogether with an ester bioisostere.

The newly synthesized ligands were assessed *in vitro* and *in vivo* to determine their metabolic stability, general anxiolytic-like effects, and possible adverse motor effects. As a result, a novel lead compound, KRM-II-81 (**9**), was identified with exceptional drug stability, pharmacokinetics, anxiolytic-like efficacy, and relatively benign motor-impairing characteristics.

CHEMISTRY

The ester bioisosteres **6** – **10** were prepared from compounds **1** – **3**, as depicted in Scheme 1. The methyl and ethyl oxadiazoles **6** and **7** were synthesized by treating **2** with an amidoxime in the presence of sodium hydride. The alkyl group was limited to methyl and ethyl to

resemble the ethyl ester functionality of **2**. The 1,3-oxazoles **8** – **10** were synthesized via the aldehyde derived from **1** – **3** through a two-step reduction-oxidation protocol. The reduction of the ester in the presence of LiAlH_4 afforded the alcohol which was subsequently oxidized with activated manganese dioxide. The oxazoles **8** (KRM-II-82), **9** and **10** (KRM-II-18B) were synthesized individually from the corresponding aldehydes using toluenesulfonylmethyl isocyanide (TosMIC) in the presence of potassium carbonate.²⁷

RESULTS and DISCUSSION

In Vitro Characterization

The GABA_AR ligands depicted in Chart 1, Schemes 1 and 2 were triaged through a combination of *in vitro* potency, microsomal stability, and *in vivo* pharmacodynamics (PD) and motor side-effect liabilities to identify optimized compounds for further testing in electrophysiological and *in vivo* assays (Tables 1 and 2, see Supporting Information for methods).

Preferred potency for the $\alpha 3\beta 3\gamma 2$ subtype over the $\alpha 1\beta 3\gamma 2$ (all ligands $\text{EC}_{50} > 20 \mu\text{M}$) GABA_AR in the FLIPR assay was displayed by all compounds; however, the oxadiazoles **6** ($\text{EC}_{50} = 5.15 \mu\text{M}$) and **7** ($\text{EC}_{50} = 3.02 \mu\text{M}$) were consistently less potent than their oxazole counterpart **9** ($\text{EC}_{50} = 0.94 \mu\text{M}$) at $\alpha 3\beta 3\gamma 2$ GABA_AR.

Ligands containing a 2'-F in the pendant phenyl ring, **3** and **10** were especially potent at $\alpha 3\beta 3\gamma 2$ with EC_{50} values of 29 nM and 11 nM, respectively. The deuterated ethyl ester **5** had a similar activity in comparison to non-deuterated **2**, while the acid **4** displayed an EC_{50} of 0.47 μM for the $\alpha 3\beta 3\gamma 2$ GABA_AR. The lack of interaction with the $\alpha 1\beta 3\gamma 2$ GABA_AR predicted low risk of motor impairment and sedative effects with these ligands.^{8, 9} In addition, these compounds are expected to be devoid of tolerance and dependence.^{15–17} Based on physicochemical properties, we anticipated that most molecules would have adequate blood-brain-barrier penetration, with the exception of the hydrophilic carboxylic acid **4**. As expected, appreciable quantities of carboxylic acid **4** were not detected in the brain either by dosing **4** itself or monitoring for **4** as the metabolite of esters **2** and **5** (Table 2).

To assess metabolic stability all of the compounds were incubated for 30 minutes with human, mouse, or rat liver microsomes (Table 1). Consistent with our hypothesis of blocking a possible oxidation site in the ester, replacing the $-\text{CH}_2$ of **2** with a $-\text{CD}_2$ in **5** resulted in increased metabolic stability across all species. In addition, replacement of the C(3)-ester in **1** – **3** with a heterocycle, **6** – **10**, resulted in improved metabolic stability for all related 2'-X ligands in liver microsomes. Cytotoxicity was evaluated by exposing human embryonic kidney cells (HEK293T) to the compounds for 18 hours. Ligand **2** and bioisosteres **6**, **7**, and **9** were non-toxic at a dose of 100 μM (Table 1). However, oxazoles **8** and **10** displayed LD_{50} 's of 66.8 μM and 61.5 μM , respectively, while all other ligands had LD_{50} values higher than 100 μM .

In Vivo Exposure Characterization

Selected compounds were dosed *in vivo* in rats to determine their plasma and brain exposure profiles (Table 2). Both esters **2** and **5** had poor plasma and exposure when dosed either i.v. (1 mg/kg, see Table S1) or p.o. (Table 2). Unfortunately, the increased metabolic stability of **5** as compared to **2** in rat liver microsomes did not translate into increased *in vivo* exposure. However, we observed appreciable plasma levels 1 hour after dosing (10 mg/kg p.o.) of oxadiazole **7** and excellent levels of oxazole **9** (1720 nM and 3910 nM, respectively). Significant concentrations in the brain were observed for both compounds 60 minutes after administration.

Pharmacodynamic Assessment

Marble burying is a useful model of anxiety and has been used to evaluate potential anxiolytic compounds.²⁸ Herein, we evaluated the anxiolytic-like effects of compounds **2**, **6** – **10** in mice using a marble burying assay (Figure 1).^{28, 29}

At 10 mg/kg, only oxadiazole **7** produced a significant reduction in marble burying as compared to vehicle. At 30 mg/kg all compounds significantly decreased marble-burying. Mice treated with 30 mg/kg of **7**, however, showed weak signs of sedation. To evaluate the potential for anti-anxiety versus motor side effects of these compounds, a motorsensory study using mice on a rotating rod (rotarod) was carried out (Figure 2) with the same mice as studied for marble-burying.

At a concentration of 10 mg/kg little change in motorsensory behavior was observed for all the compounds tested with the exception of compounds **6** and **10**, which showed very minor impairment. At 30 mg/kg, the effective anxiolytic dose for most compounds, we observed a very weak increase in ataxia for all compounds except **9**, which importantly did not significantly alter rotarod performance compared to vehicle and displayed zero failures.

From an *in vivo* perspective, the mouse marble burying assay allowed us to screen bioisosteres **6** – **10** for PD as well as potential anxiolytic-like effects while the rotarod assay permitted detection of motor side-effect liabilities. By triaging the molecules through a combination of *in vitro* potency, microsomal stability, and *in vivo* PD and motor side-effect liabilities we selected oxazole **9** for further characterization in electrophysiology, pharmacokinetic and *in vivo* rodent anxiolytic activity.

Electrophysiology

Oxazole **9** and for comparison ester **2** were assessed for electrophysiological efficacy profiles in recombinant $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$ and $\alpha 5\beta 3\gamma 2$ GABA_A receptors expressed in *Xenopus laevis* oocytes as described previously.³⁰ To minimize cell-to-cell variability, oxazole **9** was compared directly with ethyl ester **2** in the same cells at 100 nM, 1 μ M, and 10 μ M (Table 3).

While oxazole **9** is slightly more efficacious than ethyl ester **2** in modulating $\alpha 1\beta 3\gamma 2$ receptors at 10 μ M, it is of comparable efficacy at $\alpha 2\beta 3\gamma 2$ and $\alpha 3\beta 3\gamma 2$ receptors at all concentrations. Most importantly and indicated by the electrophysiological results above,

oxazole **9** is less active at $\alpha 1\beta 3\gamma 2$ receptors than at $\alpha 2\beta 3\gamma 2$ and $\alpha 3\beta 3\gamma 2$ receptors at all concentrations rendering it an $\alpha 1$ sparing GABA_AR ligand. Interestingly, it is significantly more efficacious than **2** at $\alpha 5\beta 3\gamma 2$ receptors (but only at the 10 μ M dose), which very recently has been mechanistically-connected to possible anxiolytic activity.³¹

The oxazole **9**, along with **2** and **5**, were also assessed with the high-throughput Ion Works Barracuda (IWB) electrophysiology platform and compared to zolpidem and diazepam in HEK293 cells expressing human $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, and $\alpha 3\beta 3\gamma 2$ GABA_ARs (see Supporting Information, Figure S1). The results of these concentration-dependent studies indicate that oxazole **9** modulates $\alpha 2$ (EC_{50} = 118 nM) and $\alpha 3$ (EC_{50} = 205 nM) containing GABA_ARs to a greater extent than $\alpha 1$ -containing receptors (EC_{50} = 489 nM).

Characterization of Oxazole **9**

Following a 1 mg/kg i.v. dose in rats, oxazole **9** exhibited a clearance of 21.7 ml/min/kg with a low V_{dss} of 1.4 L/kg and a good $T_{1/2}$ of 1.4 hours. Following a 10 mg/kg i.p. dose in rats, the AUC was 16500 nM*hrs with a C_{max} of 3090 nM occurring at 2.0 hours. Oxazole **9** had an i.p. $T_{1/2}$ of 3.1 hours and very good bioavailability of 69%.

Understanding the relationship between *in vivo* CNS effects and the unbound concentration of ligand in the brain can be important when assessing dose-response relationships. Thus in a second experiment, the total plasma and brain concentrations were determined 4 hrs following a 10 mg/kg i.p. dose (Table 4). The fraction unbound in plasma and brain was then measured as 25.9% and 17.7%, respectively using *in vitro* equilibrium dialysis. Subsequently, the unbound concentration in each compartment was calculated by multiplying free fraction by total concentration to provide a $C_{u,plasma}$ = 552 nM and a $C_{u,brain}$ = 363 nM at 4 hours, which leads to a $K_{p,uu}$ = 0.66 since $K_{p,uu} = C_{u,brain}/C_{u,plasma}$. A full unbound concentration-time profile in plasma and brain of unbound **9** is illustrated in the supporting information (Figure S2).

Next, oxazole **9** was evaluated further in the “Vogel conflict test” to detect anxiolytic-like behavioral effects. In this assay, water deprived rats were allowed to drink water during an unpunished and punished period in which licking was suppressed by a mild shock during punished periods. Anxiolytics cause significantly increased drinking during the punished period.³² Included in this assay was ester **2** for comparison and chlordiazepoxide (CDAP) as positive control³² (Figure 4).

Ligand **2** was tested at 30 mg/kg, where it had been active in the marble burying assay (Figure 1). However, no discernable difference was observed from the vehicle in the Vogel assay. In contrast, CDAP induced a large and significant increase in drinking behavior during the punished period without significantly affecting drinking during the non-punished period. The 1,3-oxazole **9** produced no increase in response in comparison to the control when given a 3 mg/kg i.p. dose with a 30 min pretreatment. However, when dosed at 10 mg/kg, **9** increased the rate of response by 240% over the control ($P < 0.05$). Doses at 30 mg/kg and 60 mg/kg showed similar increases of about 220% and 200% over the control, respectively. In comparison, the marketed anxiolytic, CDAP, produced a 330% response over the control ($P < 0.05$) when dosed at 20 mg/kg. Neither **9** (up to 60 mg/kg) nor CDAP (20

mg/kg) affected the response rate in the unpunished session. Thus oxazole **9** possesses a significant anxiolytic signature at 10 mg/kg i.p. in the punished session without decreasing the response rate in the unpunished session. The lack of anxiolytic-like effects of **2** in rats using the Vogel conflict test is likely due to the instability of the ester function in rodents and further underscores the unique and improved drug-like properties of **9**.

CONCLUSION

It has long been known that targeting specific GABA_AR α subunits can result in desired pharmacological effects; however, due to the similar topology of the BzR binding site of the various α subunits,^{5, 33} designing selective ligands devoid of adverse effects, such as sedation, ataxia, amnesia, tolerance, and dependence, has been difficult. Results presented here indicate that oxazole **9** has potential as an anxiolytic devoid of these adverse effects. Consequently, oxazole **9** represents a unique and promising lead compound with attributes that include a desirable $\alpha 2/\alpha 3$ -GABA_AR selective profile, low molecular weight (MW = 351), superb lipophilicity (clogP = 2.3; calculated used the ChemAxon program), good pH=7 solubility (by light scattering), excellent total exposure in plasma and brain and favorable measured fraction unbound in plasma and brain (26% and 18% respectively), leading to *in vivo* activity in pre-clinical rodent models of anxiety. Preliminary studies have begun in models of epilepsy and neuropathic pain (data to be published) to further characterize **9**.

EXPERIMENTAL SECTION

5-(8-Ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole (**9**)

Step 1—8-Ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepine-3-carbaldehyde. Ethyl ester **2** (3 g, 8.4 mmol) was placed in an oven dried two neck round bottom flask and dry THF (300 mL) was added. The reaction mixture was cooled to 0 °C and the mixture allowed to stir after which LiAlH₄ (320 mg, 8.42 mmol) was added to the reaction mixture at 0 °C. After 10 min the reaction mixture was stirred at rt with continued stirring for 45 min under an argon atmosphere. After 45 min at rt, analysis of the mixture by TLC (silica gel 1:9 MeOH/EtOAc) indicated the absence of starting ester **2**. The reaction mixture was slowly quenched with a cold saturated aq solution of Na₂SO₄ (20 mL) at 0 °C and then the reaction mixture diluted with EtOAc (50 mL). After this, the mixture was filtered through a small pad of celite, followed by an EtOAc wash (30 mL) of the celite, and the filtrate mixture washed and extracted with EtOAc (3 × 30 mL). The combined organic layers were washed with water, brine, and the solvent removed under reduced pressure to furnish the mixture of alcohols (imine alcohol 40% and reduced imine alcohol 60%, via analysis by ¹H NMR spectroscopy) as a yellow solid. This mixture of alcohols was used directly in the next step. The mixture of 2'-pyridylalcohols was dissolved in dry DCM (300 mL) under an argon atmosphere, and activated MnO₂ (10.9 g, 126 mmol) and Na₂CO₃ (2.7 g, 25.3 mmol) were added to the reaction mixture at 0 °C. The mixture was allowed to stir at rt overnight. After complete conversion of alcohol to aldehyde in the reaction mixture as indicated by TLC (silica gel), the reaction mixture was diluted with DCM (50 mL) and filtered through a small pad of celite. This pad of celite was washed with DCM (50 mL).

The solvent was removed under reduced pressure and the residue that resulted purified by flash column chromatography (silica gel, EtOAc/DCM 2:1 with 1% each Et₃N and CH₃OH) to afford the pure aldehyde as a white solid (1.03 g, 39% over two steps). **¹H NMR** (300 MHz, CDCl₃) δ 10.05 (s, 1H), 8.56 (d, *J* = 5.0 Hz, 1H), 8.08 (d, *J* = 7.5 Hz, 1H), 7.97 (s, 1H), 7.78 (ddd, *J* = 1.5, 6.0 Hz, 1H), 7.77 (dd, *J* = 1.5, 7.0 Hz, 1H), 7.55–7.57 (m, 2H), 7.38 (ddd, *J* = 1.5, 5.0 Hz, 1H), 6.00 (br s, 1H), 4.17 (br s, 1H), 3.16 (s, 1H); **¹³C NMR** (75 MHz, CDCl₃) δ 186.9, 167.7, 156.2, 148.6, 137.7, 137.1, 136.7, 136.3, 135.4, 135.3, 135.0, 127.1, 124.9, 124.0, 122.8, 121.5, 81.5, 79.7, 44.4; **HRMS** (LCMS-IT-TOF) Calc. for C₁₉H₁₃N₄O (M + H)⁺ 313.1080, found 313.1084.

Step 2—5-(8-Ethynyl-6-(pyridin-2-yl)-4*H*-benzo[*f*]imidazo[1,5-*a*][1,4]diazepin-3-yl)oxazole (KRM-II-81, **9**). The toluenesulfonylmethyl isocyanide (TosMIC, 750 mg, 3.84 mmol) was placed in a dry two neck round bottom flask and dry MeOH (100 mL) added under an argon atmosphere. At rt, solid K₂CO₃ (1.33 g, 9.6 mmol) and the aldehyde from the preceding step (1.0 g, 3.2 mmol) were added to the reaction mixture and the mixture heated to reflux for 3 to 4 h. After completion of the reaction on analysis by TLC (silica gel, 1:10 MeOH and EtOAc), which was indicated by the absence of aldehyde starting material and complete conversion to the 1,3-oxazole (lower R_f), the reaction mixture was quenched with cold water. After the mixture was quenched, 1/3 of the solvent was removed under reduced pressure and the product extracted with ethyl acetate (3 × 30 mL). The combined organic layers were washed with water, brine and dried (Na₂SO₄). The solvent was then removed under reduced pressure and the residue purified by flash chromatography (silica gel) to give the pure oxazole **9** as white solid (821 mg, 73%). **¹H NMR** (300 MHz, CDCl₃) δ 8.62 (d, *J* = 4.2 Hz, 1H), 8.12 (s, 1H), 8.06 (d, *J* = 7.8 Hz, 1H), 7.96 (s, 1H), 7.85 (ddd, *J* = 1.8, 6.0 Hz, 1H), 7.79 (dd, *J* = 1.8, 6.6 Hz, 1H), 7.62 (d, *J* = 8.4 Hz, 1H), 7.55 (d, *J* = 1.5 Hz, 1H), 7.53 (s, 1H), 7.41 (ddd, *J* = 1.5, 4.8 Hz, 1H), 5.78 (d, *J* = 12.9 Hz, 1H), 4.31 (d, *J* = 12.9 Hz, 1H), 3.71 (s, 1H). **¹³C NMR** (75 MHz, CDCl₃) δ 167.9, 156.7, 149.9, 149.0, 146.6, 137.0, 136.4, 135.8, 135.5, 135.3, 129.8, 127.5, 127.0, 124.9, 124.0, 122.8, 122.7, 121.0, 81.8, 79.5, 45.3; **HRMS** (LCMS-IT-TOF) Calc. for C₂₁H₁₄N₅O (M + H)⁺ 352.1188, found 352.1193.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

We would like to thank the NIH (MH096463, NS076517) for generous financial support (JMC), and the Austrian Science Fund for supporting ME with project FWF P 27746.

We are grateful for the expert experimental work of Xia Li, Denise Morrow, and Scott Gleason in contributing behavioral pharmacology data for this manuscript. Maria Cuadrado and Camino de los Llanos Martinez are gratefully acknowledged for their help in generating the FLIPR data. We would also like to acknowledge the Lilly Open Innovation Drug Discovery Program (OIDD) for its role in the initiation of this collaboration. In addition, the authors acknowledge support from the Milwaukee Institute for Drug Discovery and University of Wisconsin-Milwaukee's Shimadzu Laboratory for Advanced and Applied Analytical Chemistry.

ABBREVIATIONS

BQL	below quantification limit
BZD(s)	benzodiazepine(s)
CDAP	chlordiazepoxide
CNS	central nervous system
EC₅₀	half maximal effective concentration
Et₃N	trimethylamine
FLIPR	Fluorescence Imaging Plate Reader
GABA	gamma-aminobutyric acid
GABA_AR	gamma-aminobutyric acid type A receptor
HRMS	high-resolution mass spectrometry
PD	pharmacodynamics
TosMIC	toluenesulfonylmethyl isocyanide

References

1. Cromer BA, Morton CJ, Parker MW. Anxiety over GABA_A receptor structure relieved by AChBP. *Trends Biochem Sci.* 2002; 27:280–287. [PubMed: 12069787]
2. Sieghart W, Sperk G. Subunit composition, distribution and function of GABA_A receptor subtypes. *Curr Top Med Chem.* 2002; 2:795–816. [PubMed: 12171572]
3. Baldwin DS, Polkinghorn C. Evidence-based pharmacotherapy of generalized anxiety disorder. *Int J Neuropsychopharmacol.* 2005; 8:293–302. [PubMed: 15576000]
4. Sieghart W, Ernst M. Heterogeneity of GABA_A receptors: revived interest in the development of subtype-selective drugs. *Curr Med Chem: Cent Nerv Syst Agents.* 2005; 5:217–242.
5. Clayton T, Chen JL, Ernst M, Richter L, Cromer BA, Morton CJ, Ng H, Kaczorowski CC, Helmstetter FJ, Furtmuller R, Ecker G, Parker MW, Sieghart W, Cook JM. An updated unified pharmacophore model of the benzodiazepine binding site on γ -aminobutyric acid_A receptors: Correlation with comparative models. *Curr Med Chem.* 2007; 14:2755–2775. [PubMed: 18045122]
6. Atack JR. GABAA receptor subtype-selective modulators. I. α 2/ α 3-selective agonists as non-sedating anxiolytics. *Curr Top Med Chem.* 2011; 11:1176–1202. [PubMed: 21050172]
7. Da Settimo F, Taliani S, Trincavelli ML, Montali M, Martini C. GABAA/Bz receptor subtypes as targets for selective drugs. *Curr Med Chem.* 2007; 14:2680–2701. [PubMed: 17979718]
8. McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, Whiting PJ. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA_A receptor α ₁ subtype. *Nat Neurosci.* 2000; 3:587–592. [PubMed: 10816315]
9. Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H. Benzodiazepine actions mediated by specific γ -aminobutyric acid_A receptor subtypes. *Nature.* 1999; 401:796–800. [PubMed: 10548105]
10. Knabl J, Witschi R, Hosl K, Reinold H, Zeilhofer UB, Ahmadi S, Brockhaus J, Sergejeva M, Hess A, Brune K, Fritschy JM, Rudolph U, Mohler H, Zeilhofer HU. Reversal of pathological pain

through specific spinal GABA_A receptor subtypes. *Nature*. 2008; 451:330–334. [PubMed: 18202657]

11. Di Lio A, Benke D, Besson M, Desmeules J, Daali Y, Wang Z-j, Edwankar R, Cook JM, Zeilhofer HU. HZ166, a novel GABA_A receptor subtype-selective benzodiazepine site ligand, is antihyperalgesic in mouse models of inflammatory and neuropathic pain. *Neuropharmacology*. 2011; 60:626–632. [PubMed: 21145329]
12. Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U. Molecular and neuronal substrate for the selective attenuation of anxiety. *Science*. 2000; 290:131–134. [PubMed: 11021797]
13. Morris HV, Dawson GR, Reynolds DS, Attack JR, Stephens DN. Both $\alpha 2$ and $\alpha 3$ GABA_A receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. *Eur J Neurosci*. 2006; 23:2498–2504.
14. Dias R, Sheppard WFA, Fradley RL, Garrett EM, Stanley JL, Tye SJ, Goodacre S, Lincoln RJ, Cook SM, Conley R, Hallett D, Humphries AC, Thompson SA, Wafford KA, Street LJ, Castro JL, Whiting PJ, Rosahl TW, Attack JR, McKernan RM, Dawson GR, Reynolds DS. Evidence for a significant role of $\alpha 3$ -containing GABA_A receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci*. 2005; 25:10682–10688. [PubMed: 16291941]
15. Tan KR, Brown M, Labouebe G, Yvon C, Creton C, Fritschy JM, Rudolph U, Luscher C. Neural bases for additive properties of benzodiazepines. *Nature*. 2010; 463:769–775. [PubMed: 20148031]
16. Licata S, Platt D, Cook J, Van Linn M, Rowlett J. Contribution of alpha1 subunit-containing gamma-aminobutyric acid A (GABAA) receptors to motor-impairing effects of benzodiazepines in squirrel monkeys. *Psychopharmacology (Berl)*. 2009; 203:539–546. [PubMed: 19031072]
17. van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM, Crestani F. Requirement of $\alpha 5$ -GABA_A receptors for the development of tolerance to the sedative action of diazepam in mice. *J Neurosci*. 2004; 24:6785–6790. [PubMed: 15282283]
18. Attack JR, Hallett DJ, Tye S, Wafford KA, Ryan C, Sanabria-Bohorquez SM, Eng W-s, Gibson RE, Burns HD, Dawson GR, Carling RW, Street LJ, Pike A, De Lepeleire I, Van Laere K, Bormans G, De Hoon JN, Van Hecken A, McKernan RM, Murphy MG, Hargreaves RJ. Preclinical and clinical pharmacology of TPA023B, a GABA_A receptor $\alpha 2/\alpha 3$ subtype-selective partial agonist. *J Psychopharmacol*. 2011; 25:329–344. [PubMed: 20156926]
19. Attack JR, Wafford KA, Street LJ, Dawson GR, Tye S, Van Laere K, Bormans G, Sanabria-Bohorquez SM, De Lepeleire I, de Hoon JN, Van Hecken A, Burns HD, McKernan RM, Murphy MG, Hargreaves RJ. MRK-409 (MK-0343), a GABA_A receptor subtype-selective partial agonist, is a non-sedating anxiolytic in preclinical species but causes sedation in humans. *J Psychopharmacol*. 2011; 25:314–328. [PubMed: 20147571]
20. Mirza NR, Larsen JS, Mathiasen C, Jacobsen TA, Munro G, Erichsen HK, Nielsen AN, Troelsen KB, Nielsen EO, Ahring PK. NS11394 [3'-[5-(1-Hydroxy-1-methyl-ethyl)-benzoimidazol-1-yl]-biphenyl-2-carbonitrile], a unique subtype-selective GABA_A receptor positive allosteric modulator: In vitro actions, pharmacokinetic properties and in vivo anxiolytic Efficacy. *J Pharmacol Exp Ther*. 2008; 327:954–968. [PubMed: 18791063]
21. Zuiker RGJA, Chen X, Osterberg O, Mirza NR, Muglia P, de Kam M, Klaassen ES, van Gerven JMA. NS11821, a partial subtype-selective GABA_A agonist, elicits selective effects on the central nervous system in randomized controlled trial with healthy subjects. *J Psychopharmacol*. 2016; 30:253–262. [PubMed: 26655084]
22. Scott-Stevens P, Attack JR, Sohal B, Worboys P. Rodent pharmacokinetics and receptor occupancy of the GABA_A receptor subtype selective benzodiazepine site ligand L-838417. *Biopharm Drug Dispos*. 2005; 26:13–20. [PubMed: 15578749]
23. Mohler H. The rise of a new GABA pharmacology. *Neuropharmacology*. 2011; 60:1042–1049. [PubMed: 21035473]
24. Hofmann M, Kordas KS, Gravius A, Bolcskei K, Parsons CG, Dekundy A, Danysz W, Dezsi L, Wittko-Schneider IM, Saghy K, Gyertyan I, Horvath C. Assessment of the effects of NS11394 and L-838417, $\alpha 2/3$ subunit-selective GABA_A receptor-positive allosteric modulators, in tests of pain, anxiety, memory and motor function. *Behav Pharmacol*. 2012; 23:790–801. [PubMed: 23075708]

25. Cook, JM.; Zhou, H.; Huang, S.; Sarma, PVVS.; Zhang, C. Stereospecific anxiolytic and anticonvulsant agents with reduced muscle-relaxant, sedative-hypnotic and ataxic effects. US. 7,618,958 B2. Nov 17. 2009
26. Fischer BD, Licata SC, Edwankar RV, Wang Z-j, Huang S, He X, Yu J, Zhou H Jr, EMJ, Cook JM, Furtmuller R, Ramerstorfer J, Sieghart W, Roth BL, Majumder S, Rowlett JK. Anxiolytic-like effects of 8-acetylene imidazobenzodiazepines in a rhesus monkey conflict procedure. *Neuropharmacology*. 2010; 59:612–618. [PubMed: 20727364]
27. Webb MR, Donald C, Taylor RJK. A general route to the *Streptomyces*-derived inthomycin family: the first synthesis of (+)-inthomycin B. *Tetrahedron Lett*. 2006; 47:549–552.
28. Shimazaki T, Iijima M, Chaki S. Anxiolytic-like activity of MGS0039, a potent group II metabotropic glutamate receptor antagonist, in a marble-burying behavior test. *Eur J Pharmacol*. 2004; 501:121–125. [PubMed: 15464070]
29. Li X, Morrow D, Witkin JM. Decreases in nestlet shredding of mice by serotonin uptake inhibitors: Comparison with marble burying. *Life Sci*. 2006; 78:1933–1939. [PubMed: 16182315]
30. Ramerstorfer J, Furtmuller R, Vogel E, Huck S, Sieghart W. The point mutation gamma 2F77I changes the potency and efficacy of benzodiazepine site ligands in different GABA_A receptor subtypes. *Eur J Pharmacol*. 2010; 636:18–27. [PubMed: 20303942]
31. Behlke LM, Foster RA, Liu J, Benke D, Benham RS, Nathanson A, Yee BK, Zeilhofer HU, Engin E, Rudolph U. A pharmacogenetic ‘restriction-of-function’ approach reveals evidence for anxiolytic-like actions mediated by α 5-containing GABA_A receptors in mice. *Neuropsychopharmacology*. 2016; 41:2492–2501. [PubMed: 27067130]
32. Millan MJ, Brocco M. The Vogel conflict test: procedural aspects, gamma-aminobutyric acid, glutamate and monoamines. *Eur J Pharmacol*. 2003; 463:67–96. [PubMed: 12600703]
33. Clayton T, Poe MM, Rallapalli S, Biawat P, Savic MM, Rowlett JK, Gallos G, Emala CW, Kaczorowski CC, Stafford DC, Arnold LA, Cook JM. A review of the updated pharmacophore for the alpha 5 GABA(A) benzodiazepine receptor model. *Intl J Med Chem*. 2015; Article ID 430248. doi: 10.1155/2015/430248

Figure 1.

Assessment of anxiolytic-like activity of **2** and **6** – **10** in the marble burying assay. Male, NIH Swiss Webster mice ($n = 10$) were injected i.p. with either vehicle or a test compound (10 or 30 mg/kg) 30 minutes prior to testing. Data were analyzed using ANOVA (Dunnett's test: * $P < 0.05$ vs. vehicle). ^a Mild sedation-like behavior observed at 30 mg/kg.

Figure 2.

Assessment of ataxic effects of **2** and **6 – 10** in the rotarod assay. Mice, as treated in the marble burying assay, were placed on a rotarod set at 4 r.p.m. and testing time was 2 min. Mice not falling off during the test were given a “Success” designation, while mice that fell once were assigned a “Partial” designation. Mice falling twice during the 2 minute time period failed the test

Figure 3.

Total plasma concentration-time profile of **9**. Male Sprague-Dawley rats ($n = 3$ per time point) were dosed i.v. (1 mg/kg) or i.p. (10 mg/kg) and plasma concentrations measured at time points noted.

Figure 4. Vogel Conflict assessment of anxiolytic-like activity with male, Sprague-Dawley rats ($n = 6-8$). **A.** Evaluation of **2** and the anxiolytic CDAP given i.p. at 30 mg/kg and 20 mg/kg respectively; **B.** Dose response effects of **9** in comparison to CDAP. All compounds were administered 30 min prior to testing. Results were analyzed using ANOVA (Dunnett's test: * $P < 0.05$; ** $P < 0.01$).

Scheme 1.
 Synthesis of heterocyclic bioisosteres **6** – **10**.

X = CH; XHe-II-053 (1)
N; HZ-166 (2)
CF; JY-XHe-053 (3)

R = H; SR-II-54 (4)
CD₂CH₃; MP-III-068 (5)

Chart 1.
Imidazobenzodiazepines **1 – 5**.

Table 1

In vitro data of compounds 1 – 10.

	α 3 FLIPR ^a	Liver microsomal stability ^b (% Remaining, 0.5h, 37 0176C, 4 μ M)			Cytotoxicity ^c (18 h)	
	EC50 (μ M)	Human	Mouse	Rat	LDS0 (μ M)	
1	0.146	35	6.3	36	n.d.	
2	0.844	86	57	58	> 100	
3	0.029	6.1	6.1	0.5	n.d.	
4	0.47	96	93	99	n.d.	
5	1.17	95	63	77	n.d.	
6	5.15	81	85	93	> 100	
7	3.02	91	92	97	> 100	
8	0.321	74	73	72	66.8 $\pm$ 9	
9	0.937	91	90	90	>100	
10	0.011	78	78	68	61.5 $\pm$ 10	

^aMembrane potential (fluorescence) changes were measured by FLIPR assay using a FLIPR Tetra instrument from Molecular Devices and stably transfected HEK293T cells expressing either α 1 β 3 γ 2 (n = 2; all compounds EC50 > 20 μ M) or α 3 β 3 γ 2 (n = 1) GABAARs; details are listed in methods section.

^bCompounds (n = 1) were incubated with liver microsomes for 30 minutes at 37 °C at a final concentration of 4 μ M. The percentage of compound remaining after incubation was determined by LC-MS/MS.

^cCompounds (n = 4) were incubated with HEK293T cells for 18 hours followed by the quantification of viable cells using CellTiter-Glo (Promega); n.d., not determined

Table 2

Summary of exposure data

Ligand	Total Plasma Concentrations (nM) ^a				Brain concentrations (T in min) (nM) ^b	
	15	30	60	120	Conc. of dosed ligand	Conc. of 4 (as a metabolite)
p.o. (10 mg/kg)						
2	BQL ^c	BQL ^c	BQL ^c	BQL ^c	(120) BQL ^c	(120) 10 ^d
5	BQL ^c	BQL ^c	BQL ^c	BQL ^c	(120) BQL ^c	(120) BQL ^c
4	248	293	161	37.5	(120) BQL ^c	(120) BQL ^c
7	n.d.	n.d.	1720	n.d.	(60) 3090	n.d.
9	n.d.	n.d.	3910	n.d.	(60) 3640	n.d.

Sprague-Dawley rats (n = 3 per time point) were dosed p.o. (10 mg/kg).

^aBlood samples were collected at 15, 30, 60 and 120 minutes, extracted, and quantified by LC-MS/MS.

^bBrains were harvested after 60 or 120 minutes followed by extraction and quantification by LC-MS/MS;

^cQuantifiable limit < 12.2 nM;

^dThe three samples averaged 10 nM; n.d. not determined.

Table 3

Efficacy of 9 and 2 at α x β 3 γ 2 GABA_AR subtypes as % of control current.

Change of GABA EC _{3,6} current (100%), n=3-4						
9			2			
	100 nM	1 μ M	10 μ M	100 nM	1 μ M	10 μ M
α 1	n.d.	155.2 $\pm$ 9.9	231.7 $\pm$ 13.75	n.d.	147.4 $\pm$ 11.43	178.4 $\pm$ 19.55
α 2	100 $\pm$ 11.85	200.6 $\pm$ 15.67	258.3 $\pm$ 18.07	111 $\pm$ 6.48	207.4 $\pm$ 19.72	244 $\pm$ 18
α 3	109.3 $\pm$ 6.33	187.5 $\pm$ 25.25	332 $\pm$ 48.14	119.1 $\pm$ 8.62	195.2 $\pm$ 32.82	305 $\pm$ 31
α 5	n.d.	157.5 $\pm$ 11	246.4 $\pm$ 6.15	n.d.	134 $\pm$ 9.4	158.5 $\pm$ 2.6

Dose-dependent modulation of GABA (EC₃₋₆ concentration) by ligands 9 and 2 on *Xenopus laevis* oocytes expressing GABA_AR subtypes α 1- α 5, β 3, γ 2 was determined by two-electrode voltage clamp current recordings. Data represents mean $\pm$ SEM.

Table 4

Total and unbound concentrations at 4 hours of 9 following a 10 mg/kg i.p. dose (n = 3).

	Total Concentration (nM)	Unbound Concentration (C _u) (nM) ^a	Free Fraction ^b F _u
Plasma	2130	552	0.259
Brain	2050	363 (K _{p,uu} = 0.66)	0.177

^aCalculated from total concentration (nM) and fraction coefficient (F_u);

^bDetermined with rat plasma protein and rat brain *in vitro* using equilibrium dialysis.