Published in final edited form as:

Pharmacol Biochem Behav. 2017 June ; 157: 35–40. doi:10.1016/j.pbb.2017.04.009.

Further evaluation of the potential anxiolytic activity of imidazo[1,5-*a*][1,4]diazepin agents selective for $\alpha 2/3$ -containing GABA_A receptors

J. M. Witkin¹, R. Cerne¹, M. Wakulchik¹, J. S¹, S. D. Gleason¹, T.M. Jones¹, G. Li², L. A. Arnold², Jun-Xu Li³, J. M. Schkeryantz¹, K. R. Methuku², J. M. Cook², and M.M. Poe²

¹The Lilly Research Labs, Eli Lilly and Company, Indianapolis, IN

²Department of Chemistry & Biochemistry, University of Wisconsin Milwaukee, Milwaukee, WI

³Department of Pharmacology and Toxicology, Jacobs School of Medicine and Biomedical Sciences, University at Buffalo, Buffalo, NY

Abstract

Positive allosteric modulators of GABA_A receptors transduce a host of beneficial effects including anxiolytic actions. We have recently shown that bioavailability and anxiolytic-like activity can be improved by eliminating the ester functionality in imidazo[1,5-*a*][1,4]diazepines. In the present series of experiments, we further substantiate the value of heterocycle replacement of the ester for potential treatment of anxiety. None of the three esters was active in a Vogel conflict test in rats that detects anxiolytic drugs like diazepam. Compounds **7** and **8**, ester bioisosters, were selective for $\alpha 2$ and $\alpha 3$ over $\alpha 1$ -containing GABA_A receptors but also had modest efficacy at GABA α 5-containing receptors. Compound **7** was efficacious and potent in this anxiolytic-detecting assay without affecting non-punished responding. The efficacies of the esters and of compound **7** were predicted from their efficacies as anticonvulsants against the GABA_A antagonist pentylenetetrazole (PTZ). In contrast, the related structural analog, compound **8**, did not produce anxiolytic-like effects in rats despite anticonvulsant efficacy. These data thus support the following conclusions: 1) ancillary pharmacological actions of compound **8** might be responsible for its lack of anxiolytic-like efficacy despite its efficacy as an anticonvulsant 2) esters of imidazo[1,5-*a*][1,4]diazepines do not demonstrate anxiolytic-like effects in rats due to their low bioavailability and 3) replacement of the ester function with suitable heterocycles markedly improves bioavailability and engenders molecules with the opportunity to have potent and efficacious effects *in vivo* that correspond to human anxiolytic actions.

Author Contributions

Participated in research design: J. M. Witkin, S. D. Gleason, T. M. Jones, R. Cern, M. Wakulchik

Conducted experiments: JM Witkin, SD Gleason, M. Wakulchik

Contributed new reagents or analytic tools: K. R. Methuku², R. Cerne, T.M. Jones, M. M. Poe, G. Li, L. A. Arnold, J. M. Schkeryantz¹, and J. M. Cook²

Performed data analysis: JM Witkin, SD Gleason, R. Cern

Wrote or contributed to the writing of the manuscript: All authors

Introduction

The efficacy and value of 1,4-benzodiazepines as anxiolytic agents started prior to their identification as positive allosteric modulators of GABA (Haefley et al., 1975; Tallman et al., 1980). Their continued use as anxiolytics has been sustained for nearly 60 years. These agents are acutely active unlike the SSRI anti-anxiety drugs (Katz et al., 2004). Despite their relative safety (Woods et al., 1987; Schroeck et al., 2016) and over-the-counter sales in many countries, benzodiazepine anxiolytics have come under scrutiny in the last few decades due to reports of abuse and dependence and concerns of drug interactions (e.g., ethanol) (Kaplan and Dupont, 2005). Further, the motoric effects of ataxia and myorelaxation have raised concerns with potential impact on daily function (e.g., driving cars) (Strand et al., 2016).

Since the initial discovery of the potential for multiple subtypes of benzodiazepine receptors in 1979, a search for anxiolytics with reduced sedative and dependence liability has been ongoing (Lippa et al., 2005; Skolnick, 2012). The cloning and expression of GABA_A receptors has identified multiple subtypes based upon subunit composition (Sieghart and Ernst, 2005). A particular focus has been on the α subunits comprising the ion channel since these subunits have remarkable impact on pharmacological activity with important implication for therapeutics. Alpha 1-associated GABA_A receptors have long-been associated with sedative effects (McKernan et al., 2000) and this understanding led to the successful discovery and development of multiple α 1-selective drugs as sleep enhancers (Langer et al., 1992). Since α 2/3-containing GABA_A receptors have been associated with anxiolytic effects with reduced sedative liability (Rivas et al., 2009; Fischer et al., 2010; Attack, 2011; Ralvenius et al., 2015), this mechanism remains a viable avenue for novel drug discovery. In addition convergent data has suggested also the possibility that α 2/3-containing GABA_A receptor agonists would have reduced dependence and abuse liability (Kahot and Ator, 2008; Ator et al., 2010). However, other data has pointed out that partial agonism at these sites might also be important for these benefits (Ator et al., 2010) and that α 2 sites in the nucleus accumbens are essential for the reinforcing effects of benzodiazepines like midazolam (Engin et al., 2014).

We recently reported on several analogs of the anxiolytic ester drug HZ-166 with improved bioavailability and efficacy (Poe et al., 2016). In that study, HZ-166 (compound 2 in Poe et al., 2016) decreased marble-burying, an effect that occurs with acute dosing with anxiolytic standards including SSRIs (Li et al., 2006). Compounds 7 and 8, both ester bioisosters, tended to have greater efficacy, and in the case of compound 7, to have greater potency (Poe et al., 2016). Importantly, in a conflict test in rats that detects anxiolytic drugs, HZ-166 was not active whereas another ester bioisostere of HZ-166, KRM-II-81 (compound 9 described in Poe et al., 2016) was efficacious. Compound 9 also attenuated acid-induced pain behaviors in mice (Lewter et al., 2017). The present series of studies was conducted to characterize three additional esters and two analogs with heterocyclic replacement of the ester as in KRM-II-81 (cpd. 9). In order to deduce *in vivo* activity of these compounds, we conducted anticonvulsant studies against the GABA_A receptor antagonist pentylenetetrazole. It was predicted that the ligands with *in vivo* activity as anticonvulsants, actions known to have a large α 2 contribution (Fradley et al., 2007), would also be anxiolytic as had been suggested by another series of non-selective GABA_A receptor ligands (Lippa et al., 1979).

Methods

Potency and efficacy at GABA_A receptors

Electrophysiology methods utilized the Ion Works Barracuda (IWB) system. Unless otherwise specified, all cell culture reagents were obtained from Thermo Fisher Scientific (Waltham, MA), and all other reagents were obtained from Sigma-Aldrich (St Louis, MO). Compound serial dilutions were prepared in dimethyl sulfoxide (DMSO) and diluted into external recording solution prior to the experiment. Final DMSO concentration was 0.3%. For cell culture, HEK293 cells stably expressing hGABA_AR ($\alpha 1\beta 3\gamma 2$), hGABA_AR ($\alpha 2\beta 3\gamma 2$), hGABA_AR ($\alpha 3\beta 3\gamma 2$) and hGABA_AR ($\alpha 5\beta 3\gamma 2$) were obtained from ChanTest (Cleveland, OH). Cells were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% non-essential amino acids, G418 (200 µg/ml), hygromycin (100 µg/ml), zeocin (50 µg/ml) and puromycin (0.1 µg/ml). For IWB experiments, cells were cultured in T-150 flasks (Corning) to 80–90% confluency. Cells were rinsed twice with D-PBS without calcium and magnesium and dissociated by incubating in 3 mL of TrypLE™ (Thermo Fisher Scientific, Waltham, MA) for 5 min. Cells were then re-suspended in 10 mL of media, gently triturated, and centrifuged for 5 min at 1000 RPM. The supernatant was aspirated and the cells resuspended in 5 mL HBSS-based external solution (see below). During IWB recordings, cells were supplied and seals established in an external solution consisting of Hanks Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES with pH adjusted to 7.4 using NaOH. The internal solution used was (in mM): 90 K-Gluconate; 40 KCl, 3.2 MgCl₂; 3.2 EGTA and 5 HEPES; with pH adjusted to 7.2 using KOH. The membrane perforating agent amphotericin B was prepared as a 28 mg/mL stock solution in DMSO on the day of the experiment and added to the internal solution at a concentration of 0.1 mg/mL. Electrophysiological recordings were made as follows: The IWB instrument was primed with intracellular and extracellular solutions and 9 µL of cells (5M/mL) added to each well. Recordings were performed in population patch clamp (PPC) mode, and the whole cell recording configuration was established by 8 min incubation with amphotericin B. The holding voltage was set to –80 mV and sampling frequency was 1 kHz. Test compound was added during first compound addition. After three minute incubation with compound GABA currents were evoked with co-administration of GABA at approximately EC₁₅ concentration together with test compound. For GABA CRC experiments increasing concentrations of GABA were applied during second compound addition. Data acquisition, leak subtraction and initial analysis of peak currents during each test pulse were performed using IWB software (version 2.0.2; Molecular Devices Corporation, Union City, CA). Compounds were tested at 10 concentrations in four to six replicates. Mean and standard error of the normalized peak current amplitudes were fit to the Hill equation using GraphPad Prism 6.02 software (GraphPad Software, San Diego, CA). All error bars represent the Standard Error of the Mean (SEM).

Animal Care and Use

All studies were performed in accordance with guidelines of the National Institutes of Health and by local animal care and use committees.

Animals

Male, Sprague Dawley rats from Harlan Sprague Dawley (Indianapolis, IN) were used and weighed 90–110 g when evaluated in experiments. Rats were housed 5/cage with free access to water and food in a room with lights on at 6am and off at 6pm. Animals were maintained in the colony room for at least 3 days before testing. Animals were moved to a quiet room 1 hour prior to the start of the test.

Inverted Screen Test

Prior to dosing rats with PTZ they were tested for potential motor impairment on an inverted screen. The apparatus is made of four 13 cm × 16 cm squares of round hole, perforated stainless steel mesh (18 holes/square inch, 3/16 inch diameter, ¼ inch staggered centers, 50% open area) mounted 15 cm apart on a metal rod, 35 cm above the table top.

The rats were dosed with test compound and returned to their home cage. Twenty five min after pre-treatment, animals were tested on the inverted screen and were scored after 60 seconds as follows: (0= climbed over, 1= hanging on to screen, 2= fell off). After the inverted screen test, animals were dosed with pentylenetetrazol (PTZ) in a volume of 1 ml/kg and placed in an observation cage (40.6×20.3×15.2 cm) with a floor containing 0.25 inches of wood chip bedding material. Mean ± S.E.M. scores were analyzed by ANOVA followed by post-hoc Dunnett's test.

Pentylenetetrazole (PTZ)-Induced Seizures

Drugs were studied for their ability to prevent or dampen seizures induced in mice or rats after s.c. PTZ dosing. Animals were then observed for 30 min post PTZ for clonus (defined as clonic seizure of fore- and hindlimbs during which the mouse demonstrated loss of righting) or for tonic seizures as exemplified by loss of righting accompanied with tonic hindlimb extension. The dose of PTZ used in rat studies was 35 mg/kg, s.c., and the dose in mice was 70 mg/kg, i.p., based upon estimated ED₉₀ values for PTZ in these assays. The percentage of animals exhibiting convulsions was analyzed by Fisher's Exact probability test.

Vogel Conflict Behavior

Day 1, animals are moved from colony room into test room and put into operant chambers with water available, white noise, and houselight on (program title vogel train). A timer starts when the first lick is made. For the first 3 minutes after the first lick, data is recorded as unpunished licks. After 3 minutes, the second component becomes active for 3 minutes. All licks in the second component are recorded as punished licks. At the end of the 6 minutes, the chamber goes dark. Animals are removed and returned to home cages. After all groups of animals have been exposed to the chambers, water is made available in the home cage for 30 minutes. After 30 minutes, water is removed and animals are transported back to colony room. Data for both components is recorded as the number of licks. Sometime data for day1 and day 2 is not recorded due to operant chambers being used for other testing protocols. Data is always recorded for day 3

Day 2 conditions were identical to day one training. On day 3, animals were randomly assigned to dose groups and drugged according to route and pre-treatment times recorded below. Animals are run on program Vogel FR20, which is identical to Vogel trainxxs with the exception of during the 2nd component (punished), every 20th lick is shocked. Data for both components is recorded as the number of licks. Shock intensity= 0.5 mA, duration=100 msec. Shock is delivered through the water sipper tube. Chlordiazepoxide (CDAP) 20 mg/kg, ip, 30 minutes pre-session is used as a positive control. Injection volume is 1 ml/kg unless otherwise noted.

Compounds

Compounds were synthesized as described (Cook et al., 2009, 2010; Poe et al., 2016). Compound numbers are from Poe et al. (2016). The other compounds were obtained from commercial suppliers: valproic acid, chlordiazepoxide HCl, and pentylenetetrazole (Sigma-Aldrich, St. Louis, MO, USA). Valproate, chlordiazepoxide and pentylenetetrazole were dissolved in water. The test compounds were suspended in 1% hydroxyethylcellulose/0.05% tween 80/0.05% Dow antifoam. Compounds were administered in a volume of 1 ml/kg except at higher concentrations where dose volumes up to 3 ml/kg were used. Test compounds were given 30 min prior to testing by the i.p. route.

Results

To evaluate the potentiation of the compounds towards different subtypes of GABA_AR, GABA evoked currents were recorded on the Ion Works Barracuda (IWB) high-throughput electrophysiology platform. The negative current evoked by GABA application showed a rapid activation followed by a slow desensitization. The average maximum negative current amplitudes evoked with saturating concentration of GABA were similar for all subtypes; 0.9 nA for $\alpha 1\beta 3\gamma 2$ GABA_A receptor, 0.8 nA for $\alpha 2\beta 3\gamma 2$ GABA_A receptor, 1.0 nA for $\alpha 3\beta 3\gamma 2$ GABA_A receptor and 0.9 nA for $\alpha 5\beta 3\gamma 2$ GABA_A receptor, respectively. The potencies of GABA at individual subtypes are listed in Table 1. For that purpose of this study the concentration of GABA was individually adjusted to EC₁₅ for each GABA_A receptor subtype. From day-to-day, we saw some variability in this value, and therefore the effective concentration was determined for each run as a percent of maximum GABA (1 mM) evoked current. Only experiments with efficacies ranging from EC₁₀ to EC₂₀ were used in the analysis.

Compounds **7** and **8** potentiated GABA currents (Fig. 2, Table 1). The potency was however substantially lower at the $\alpha 1$ containing subtype than at $\alpha 2$, $\alpha 3$ or $\alpha 5$ subtypes where the EC₅₀s were approximately 10-fold lower (Table 1). In addition to lower compound potency at $\alpha 1$ containing GABA_A receptors maximum potentiation of the GABA EC₁₅-induced currents was less robust. The efficacy at $\alpha 1$ subtype was less than 25% for all three compounds (Table 1) while the efficacy at $\alpha 2$, $\alpha 3$ or $\alpha 5$ subtypes exceeded 60% for both compounds. The non-selective full agonist at benzodiazepine receptor, diazepam, produced similar potentiation (potency and efficacy) at all GABA_A receptor subtypes tested (Fig. 2, Table 1).

The effects of test compounds (evaluated at 30 mg/kg, i.p.) on motor performances and anticonvulsant efficacy are shown in Table 2. The esters (**A**, **B**, **3**) were without significant motor impact or anticonvulsant effect. In contrast, compounds **7** and **8** were effective anticonvulsants without significant motor impairing effects.

The esters (**A**, **B**, **3**) did not significantly increase punished responding nor affect non-punished responding (Fig. 3). In contrast, compound **7** increased punished drinking without significantly altering non-punished drinking (Fig. 4). Compound **8**, on the other hand, decreased both punished and non-punished drinking (Fig. 5). Given the lack of increase in punished responding with compound **8**, we did an additional study with other doses. In that study, doses up to 17 mg/kg were studied; no significant effect on either punished or non-punished drinking was observed.

Discussion

Consistent with the low bioavailability of the esters of imidazo[1,5-*a*] [1,4] diazepines (Poe et al., 2016), we found that all three esters studied were inactive in the Vogel conflict test in rats. Both oral and intraperitoneal dosing resulted in low drug exposures (Poe et al, 2016) which likely explain the lack of in vivo effects. In contrast, compound **7** was efficacious and potent in this anxiolytic-detecting assay without affecting non-punished responding. The efficacy of these molecules (**A**, **B**, **3** and **7**) was predicted from their efficacies as anticonvulsants against the GABA_A antagonist pentylenetetrazole (PTZ). The fact that the esters studied here were active in anxiolytic-detecting conflict tests in Rhesus monkeys (Fischer et al., 2010) but not in rats in the present study will require full comparative pharmacokinetic analysis to understand. Nonetheless, the higher hydrolysis of esters in rodents vs. primates (Schweri et al., 1983) is a likely reason for these differences. The mixed findings on the abilities of these esters to produce anxiolytic-like effects in rats in the elevated plus maze (Savi et al., 2008, 2010) had been suggested to be due to differences in ligand efficacies at GABA_A $\alpha 5$ receptors. Given the qualitative differences in compounds **A**, **B**, and **3** to produce anxiolytic-like effects in the present study along with the improved central exposure observed with compounds **7** and **9** (KRM-II-81) (Poe et al., 2016, compound **9**) corresponding to their effects in the same assay, we suggest that central exposure by these molecules is a key determinant of their in vivo effects. This idea is given additional support by the anticonvulsant data provided in this report.

In contrast, compound **8** did not significantly affect punished or non-punished responding in the Vogel assay. The lack of anxiolytic-like activity with compound **8** is surprising, given its ability to function as a modulator of the effects of pentylenetetrazole. We conclude that anticonvulsant efficacy against PTZ alone is not sufficient to predict anxiolytic efficacy at least for this very limited set of compounds. This conclusion is further supported by observations with HZ-166. In rats, 30 mg/kg, i.p., reduced convulsions induced by PTZ by 60%. In contrast, HZ-166 was not active in the Vogel conflict test (Poe et al., 2016) whereas the non-ester analog, KRM-II-81 (compound **9** in Poe et al., 2016) increased punished drinking in rats under the Vogel conflict test (Poe et al., 2016).

We had previously demonstrated that the plasma and brain exposures of the ester bioisosters show remarkable improvement over their ester counterparts (Poe et al., 2016). Although direct dose/exposure comparisons are not available, data document that both the anxiolytic active (compound **7**) and the anxiolytic inactive (compound **8**) compounds exhibit high exposure in both brain and plasma of rats after i.p. dosing. At 150 mg/kg, i.p., compound **8** exposed brain to 33400 ± 15900 nM and plasma to 25700 ± 10400 nM (n=3 rats). The very low efficacy of **8** at $\alpha 1$ -containing GABA_A receptors is one notable difference in the pharmacological profile of compounds **7** and **8**. However, given the association of $\alpha 1$ agonism with sedation (McKernan et al., 2000), and the ability of other $\alpha 2$ and $\alpha 3$ -selective modulators to produce anxiolytic-like effects in preclinical species (Rivas et al., 2009; Fischer et al., 2010; Atack, 2011; Ralvenius et al., 2015) and humans (Atack et al., 2006; 2011a), the lack of $\alpha 1$ efficacy of compound **8** is probably not relevant to its lack of anxiolytic activity reported here. Further studies on the differential pharmacologies of these compounds is required to properly place this negative effect in context of GABA_A $\alpha 2/3$ receptors.

To account for the differences in behavioral effects of **7** vs **8**, we hypothesize that compound **8** has ancillary actions that competed with its effects at GABA_A $\alpha 2/3$ receptors, the nature of which are not yet determined. Indeed, compound **8** showed more behavioral depressant activity than compound **7** and displayed a statistical trend to decreasing unpunished drinking (p=0.08). Compound **8** also appeared to be more cytotoxic than compound **7** in human embryonic kidney cells (HEK293T) (Poe et al., 2016).

A compelling clinical opportunity exists in the development of $\alpha 1$ sparing subtype-selective GABA_AR ligands. These ligands are expected to result in superior treatments for seizures and anxiety without causing sedation, amnesia and ataxia, or the propensity for addiction and tolerance development. NS11394 (Mirza et al., 2008) which displayed agonistic activity for the $\alpha 2$, $\alpha 3$ and $\alpha 5$ subtypes was shown to be anxiolytic; however, they were also shown to decrease memory, likely due to the activation of the $\alpha 5$ subtype (Mirza et al., 2008). In fact, NS11394 had greater efficacy at GABA_A $\alpha 5$ receptors than GABA_A receptors constituted by other alpha subunits (Mirza et al., 2008). In contrast, the ligands reported here (cpds **7** and **8**) also had affinity and efficacy as agonists of GABA_A $\alpha 5$ receptors though were generally less than that at the other alpha subtypes examined as also exemplified from GABA currents recorded from *Xenopus* oocytes (Lewter et al., 2017). NS11821, another molecule that positively modulates $\alpha 2$, $\alpha 3$, and $\alpha 5$ -comprised GABA_A receptors, was less sedating and produced less body sway and subjectively-reported sedation than lorazepam in humans (Zuiker et al., 2016). Additionally, continued research has seen a number of promising candidates fail due to pharmacokinetic complications or other adverse effects. One ligand, the $\alpha 2/\alpha 3$ -positive allosteric modulator TPA023 (Atack et al., 2006) was shown to be a relatively non-sedating anxiolytic in humans (Atack, 2009) but ultimately led to cataracts (Mohler, 2011). In contrast, other attempts to create reduced motor impacting anxiolytic drugs have not been as successful. For example, MRK-409 (MK-0343) was sedating in humans in contrast to preclinical predictions (Atack et al., 2011b). These results indicate that a ligand which activates only the $\alpha 2/\alpha 3$ -GABA_AR subtypes may be useful for

the treatment of anxiety but the proof of this has yet to be achieved in pill form (Skolnick, 2012).

The data presented here support the following conclusions: 1) esters of imidazo[1,5-*a*][1,4]diazepines have low bioavailability, 2), replacement of the ester function with suitable heterocycles markedly improves bioavailability (Poe et al., 2016) and imbues molecules with potent and efficacious effects *in vivo* that can correspond to human anxiolytic actions. In conclusion, the present series of experiments has provided further substantiation of the viability of imidazo[1,5-*a*][1,4]diazepines as novel allosteric modulators of GABA_A receptors which may have clinical utility as anxiolytics. Moreover, an additional promise of such sub-type selective drugs is in the treatment of pain (Ralvenius et al., 2015). Given the co-morbidity of pain and anxiety (Bandelow, 2015) and the recent findings that molecules selective for $\alpha 2/\alpha 3$ or $\alpha 2/\alpha 3/\alpha 5$ -containing GABA_A receptors (de Lucas et al., 2015) including compound **9** (Lewter et al., 2017) were efficacious in rodent pain models as amply predicted for these alpha protein subunit targets (Ralvenius et al., 2015), such potentiators of $\alpha 2/\alpha 3$ -constituted GABA_A receptors are predicted to be valuable additions to the therapeutic arsenal for patients in the future.

Acknowledgments

We thank the following granting agencies for support: MH-096463 and NS-076517. We also acknowledge UW-Milwaukee's Shimadzu Laboratory for Advanced and Applied Analytical Chemistry and support from the Milwaukee Institute of Drug Discovery and the University of Wisconsin Research Foundation.

References

- Atack JR. GABAA receptor subtype-selective modulators. I. $\alpha 2/\alpha 3$ -selective agonists as non-sedating anxiolytics. *Curr Top Med Chem*. 2011; 11:1176–1202. [PubMed: 21050172]
- Atack, JR. Subtype-Selective GABA_A Receptor Modulation Yields a Novel Pharmacological Profile: The Design and Development of TPA023. In: Enna, SJ., Williams, M., editors. *Advances in Pharmacology Contemporary Aspects of Biomedical Research Drug Discovery*. Vol. 57. Academic Press; 2009. p. 137-185.
- Atack JR, Hallett DJ, Tye S, Wafford KA, Ryan C, Sanabria-Bohórquez SM, Eng WS, 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*. 2011a; 25:329–344. [PubMed: 20156926]
- Atack JR, Wafford KA, Street LJ, Dawson GR, Tye S, Van Laere K, Bormans G, Sanabria-Bohórquez 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*. 2011b; 25:314–328. [PubMed: 20147571]
- Atack JR, Wafford KA, Tye SJ, Cook SM, Sohal B, Pike A, Sur C, Melillo D, Bristow L, Bromidge F, Ragan I, Kerby J, Street L, Carling R, Castro JL, Whiting P, Dawson GR, McKernan RM. TPA023 [7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-ylmethoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazine], an agonist selective for $\alpha 2$ - and $\alpha 3$ -containing GABA_A receptors, is a nonsedating anxiolytic in rodents and primates. *J Pharmacol Exp Ther*. 2006; 316:410–422. [PubMed: 16183706]
- Ator NA, Atack JR, Hargreaves RJ, Burns HD, Dawson GR. Reducing abuse liability of GABAA/benzodiazepine ligands via selective partial agonist efficacy at $\alpha 1$ and $\alpha 2/3$ subtypes. *J Pharmacol Exp Ther*. 2010; 332:4–16. [PubMed: 19789360]

- Bandelow B. Generalized Anxiety Disorder and Pain. *Mod Trends Pharmacopsychiatri*. 2015; 30:153–65.
- 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]
- Cook, JM., Zhou, H., Huang, S., Srirama Sarma, PVV., Zhang, C. Stereospecific anxiolytic and anticonvulsant agents with reduced muscle-relaxant, sedative-hypnotic and ataxic effects. US Patent. 7,618,958 B2. 2009. Issued November 17, 2009
- Cook JM, Edwankar R, Edwankar C, Huang S, Jain H, Rivas F, Yang J, Zhou H. Selective anticonvulsant agents and their uses, T08025US Utility, Serial # 12/725,763, Filed March 17, 2010. 2010
- Engin E, Bakhurin KI, Smith KS, Hines RM, Reynolds LM, Tang W, Sprengel R, Moss SJ, Rudolph U. Neural basis of benzodiazepine reward: requirement for α 2 containing GABA_A receptors in the nucleus accumbens. *Neuropsychopharmacology*. 2014; 39(8):1805–1815. [PubMed: 24553732]
- Fischer BD, Licata SC, Edwankar RV, Wang ZJ, Huang S, He X, Yu J, Zhou H, Johnson EM Jr, Cook JM, Furtmüller 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]
- Fradley RL, Guscott MR, Bull S, Hallett DJ, Goodacre SC, Wafford KA, Garrett EM, Newman RJ, O'Meara GF, Whiting PJ, Rosahl TW, Dawson GR, Reynolds DS, Atack JR. Differential contribution of GABA(A) receptor subtypes to the anticonvulsant efficacy of benzodiazepine site ligands. *J Psychopharmacol*. 2007; 21:384–391. [PubMed: 17092983]
- Haefely W, Kulcsár A, Möhler H, Pieri L, Polc P, Schaffner R. Possible involvement of GABA in the central actions of benzodiazepines. *Adv Biochem Psychopharmacol*. 1975; 14:131–151.
- Kaplan EM, DuPont RL. Benzodiazepines and anxiety disorders: a review for the practicing physician. *Curr Med Res Opin*. 2005; 21:941–950. [PubMed: 15969894]
- Kohut SJ, Ator NA. Novel discriminative stimulus effects of TPA023B, subtype-selective gamma-aminobutyric-acid(A)/benzodiazepine modulator: comparisons with zolpidem, lorazepam, and TPA023. *Pharmacol Biochem Behav*. 2008; 90:65–73. [PubMed: 18395780]
- Langer SZ, Faure-Halley C, Seeburg PH, Graham D, Arbilla S. The selectivity of zolpidem and alpidem for the α 1-subunit of the GABAA receptor. *Eur J Pharmacol*. 1992; 2:232–234.
- Lewter LA, Janet L, Fisher2, Justin N, Siemian1, Methuku Kashi Reddy, Po Michael M, Cook James M, Li Jun-Xu. Antinociceptive effects of a novel α 2/ α 3-subtype selective GABA_A receptor positive allosteric modulator. *ACS Chemical Neuroscience*. in press.
- Lippa A, Czobor P, Stark J, Beer B, Kostakis E, Gravielle M, Bandyopadhyay S, Russek SJ, Gibbs TT, Farb DH, Skolnick P. Selective anxiolysis produced by ocinaplon, a GABA(A) receptor modulator. *Proc Natl Acad Sci U S A*. 2005; 102:7380–7385. [PubMed: 15870187]
- Lippa, AS., Nash, PA., Greenblatt, EN. Preclinical neuropsychopharmacological testing procedures for anxiolytic drugs. In: Fielding, S.Lal, H.Lippa, AS.Nash, PA., Greenblatt, EN., editors. *Anxiolytics*. Mount Kisco, N.Y: Futura Pub. Co; 1979. p. 41-81.
- 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. *Nat Neurosci*. 2000; 3:587–592. [PubMed: 10816315]
- Mirza NR, Atack J, Wafford K. GABA(A) Receptor Subtypes: Novel Targets for Novel Medicines. *Adv Pharmacol Sci*. 2012; 2012:529861. [PubMed: 22570647]
- 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]
- Möhler H. The rise of a new GABA pharmacology. *Neuropharmacology*. 2011; 60:1042–1049. [PubMed: 21035473]

- Poe MM, Kashi R, Methuku, Li Guanguan, Teske Kelly A, Stafford Douglas C, Arnold Leggy A, Cramer Jeffrey W, Jones Timothy M, McDermott Jeff S, Witkin Jeffrey M, Cook James M, Schkeryantz Jeffrey M. Synthesis and characterization of a novel GABA_A receptor ligand, 5-(8-Ethynyl-6-(pyridin-2-yl)-4H-benzof[imidazo[1,5-a][1,4]diazepin-3-yl)oxazole (KRM-II-81), that combines exceptional drug stability, pharmacokinetics, and anxiolytic efficacy. *J Med Chem*. 2016 in press.
- Ralvenius WT, Benke D, Acuña MA, Rudolph U, Zeilhofer HU. Analgesia and unwanted benzodiazepine effects in point-mutated mice expressing only one benzodiazepine-sensitive GABAA receptor subtype. *Nat Commun*. 2015 Apr 13.:6.
- Rivas FM, Stables JP, Murphree L, Edwankar RV, Edwankar CR, Huang S, Jain HD, Zhou H, Majumder S, Sankar S, Roth BL, Ramerstorfer J, Furtmüller R, Sieghart W, Cook JM. Antiseizure activity of novel gamma-aminobutyric acid (A) receptor subtype-selective benzodiazepine analogues in mice and rat models. *J Med Chem*. 2009; 52(7):1795–8. [PubMed: 19275170]
- Savi MM, Majumder S, Huang S, Edwankar RV, Furtmüller R, Joksimovi S, Clayton T Sr, Ramerstorfer J, Milinkovi MM, Roth BL, Sieghart W, Cook JM. Novel positive allosteric modulators of GABAA receptors: do subtle differences in activity at alpha1 plus alpha5 versus alpha2 plus alpha3 subunits account for dissimilarities in behavioral effects in rats? *Prog Neuropsychopharmacol Biol Psychiatry*. 2010 Mar 17; 34(2):376–86. [PubMed: 20074611]
- Savi MM, Huang S, Furtmüller R, Clayton T, Huck S, Obradovi DI, et al. Are GABAA receptors containing alpha5 subunits contributing to the sedative properties of benzodiazepine site agonists? *Neuropsychopharmacology*. 2008; 33:332–339. [PubMed: 17392731]
- Schroeck JL, Ford J, Conway EL, Kurtzhals KE, Gee ME, Vollmer KA, Mergenhagen KA. Review of Safety and Efficacy of Sleep Medicines in Older Adults. *Clin Ther*. 2016 Nov; 38(11):2340–2372. [PubMed: 27751669]
- Schweri MM, Martin JV, Mendelson WB, Barrett JE, Paul SM, Skolnick P. Pharmacokinetic and pharmacodynamic factors contributing to the convulsant action of beta-carboline-3-carboxylic acid esters. *Life Sci*. 1983 Oct 10; 33(15):1505–10. [PubMed: 6621253]
- 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.
- Skolnick P. Anxiolytic drugs: on a quest for the Holy Grail. *Trends Pharmacol Sci*. 2012; 33(11):611–620. [PubMed: 22981367]
- Strand MC, Gjerde H, Mørland J. Receptors for the age of anxiety: pharmacology of the benzodiazepines. Driving under the influence of non-alcohol drugs--An update. Part II: Experimental studies. *Forensic Sci Rev*. 2016 Jul; 28(2):79–101. [PubMed: 27257716]
- Tallman JF, Paul SM, Skolnick P, Gallager DW. Receptors for the age of anxiety: pharmacology of the benzodiazepines. *Science*. 1980; 207:274–281. [PubMed: 6101294]
- 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]

Figure 1.

Structures of the compounds studied. Compounds **7**, **8**, and **9** correspond to compounds reported in Poe et al. (2016). Compounds **A**, **B**, and **3** were originally reported in Cook et al. (2009, 2010) and Fisher et al. (2010).

Figure 2.

Potentiation of human recombinant GABA_A receptor currents measured by IW-Barracuda automated patch-clamp. Potentiation of GABA EC₁₅ evoked currents in HEK293 cells expressing human recombinant $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$ GABA_AR or $\alpha 5\beta 3\gamma 2$ GABA_AR. The concentration response curves for Cpd **7** (A) and, Cpd **8** (B) are compared to the concentration response curve of clinically approved benzodiazepines (diazepam, D). Currents are measured in PPC mode of measured by IW-Barracuda automated patch-clamp. Potentiation is expressed as % increase in the peak GABA EC₁₅-evoked current. All values shown are mean $\pm$ SEM for 4–6 replicates.

Figure 3.

Effects of the esters **A**, **B**, and **3** on punished (unfilled circles) and non-punished drinking (filled circles) of rats. Each point represents the mean $\pm$ S.E.M. of 8 rats/dose condition. Chlordiazepoxide (20 mg/kg, i.p.) was studied as a comparator. * $p < 0.05$ by post-hoc Dunnett's test.

Figure 4.

Effects of compound **7** on punished (unfilled circles) and non-punished (filled circles) drinking of rats. Each point represents the mean $\pm$ S.E.M. of 8 rats/dose condition. Chlordiazepoxide (20 mg/kg, i.p.) was studied as a comparator. Data were analyzed by ANOVA followed by post-hoc Dunnett's test. *: $p < 0.05$). Non-punished responding: $F_{3,28}=2.4$, $p=0.1.1$. Punished responding: $F_{3,28}=8.7$, $p < 0.001$. * $p < 0.05$ by post-hoc Dunnett's test.

Figure 5.

Effects of compound **8** on punished (unfilled circles) and non-punished drinking (filled circles) of rats. Each point represents the mean $\pm$ S.E.M. of 8 rats/dose condition.

Chlordiazepoxide (20 mg/kg, i.p.) was studied as a comparator. Data were analyzed by ANOVA followed by post-hoc Dunnett's test. *: $p < 0.05$). Non-punished responding: $F_{3,28} = 2.5$, $p = 0.08$. Punished responding: $F_{3,28} = 1.45$, $p = 0.25$. * $p < 0.05$ by post-hoc Dunnett's test.

Data summary for test compounds **7** and **8** and a clinically approved benzodiazepine anxiolytic (diazepam). Electrophysiological measurements of HEK293 cells expressing human recombinant $\alpha 1\beta 3\gamma 2$, $\alpha 2\beta 3\gamma 2$, $\alpha 3\beta 3\gamma 2$, or $\alpha 5\beta 3\gamma 2$ GABA_AR in the presence of compounds were measured by IW-Barracuda automated patch-clamp. Efficacy is expressed as % increase in the peak GABA EC₁₅-evoked current. All values shown are mean $\pm$ SEM for a minimum of 4–6 replicates. Average EC₅₀s of GABA (n=2) are listed for each channel subtype.

Table 1

Compound	GABAA- $\alpha 1$			GABAA- $\alpha 2$			GABAA- $\alpha 3$			GABAA- $\alpha 5$		
	EC ₅₀ (nM)	Max (%GABA)	Relative Effic. (%)	EC ₅₀ (nM)	Max (%GABA)	Relative Effic. (%)	EC ₅₀ (nM)	Max (%GABA)	Relative Effic. (%)	EC ₅₀ (nM)	Max (%GABA)	Relative Effic. (%)
7	2413	115.0	20.8	102.1	178.0	75.5	102	208.6	65.6	61.3	173.6	61.0
	816.7	121.3	29.6	25.87	205.2	101.7	66.7	274.3	105.3	12.9	191.6	75.9
Diazepam	7.428	171.9	100.0	16.5	203.4	100.0	55.23	265.6	100.0	11.1	220.7	100.0
GABA EC ₅₀	12.27 μ M			11.58 μ M			33.38 μ M			8.52 μ M		

Table 2

Effects on motor function and anticonvulsant efficacy.

Assay	Veh	Valpro	A	B	3	7	8
Motor Effect	0.2 (0.2)	2.0 (0) [*]	1.2 (0.49)	1.2 (0.49)	0.8 (0.49)	0.94 (0.58)	0.8 (0.49)
Anticonvulsant Effect	5	100 [*]	0	0	0	100 [*]	80 [*]

Compounds were tested at 30 mg/kg, i.p., 30 min prior to testing. Motor impairment was assessed on an inverted screen with scores of 0 being no impairment and 2 being full impairment (see Methods). F₆, 43 = 2.91, p<0.05. Anticonvulsant efficacy was assessed against 35 mg/kg pentylentetrazole (s.c). Data are expressed as the percent of animals protected (no clonic convulsions observed). Data are means ± S.E.M. of 5 or 20 rats (veh).

^{*} p<0.05 by post-hoc Dunnett's test (motor) or by Fisher's Exact probability test (anticonvulsant).

Veh: Compound vehicle; Valpro: valproic acid (300 mg/kg, i.p.). Compounds **A**, **B**, **3**, **7**, and **8** are shown in Figure 1.