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Pharmacological and antihyperalgesic properties of the novel $\alpha 2/3$ preferring GABA_A receptor ligand MP-III-024

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Abstract

γ -aminobutyric acid type A (GABA_A) receptors are located in spinal nociceptive circuits where they mediate the transmission of pain sensory signals from the periphery to higher centers. Benzodiazepine-type drugs bind GABA_A receptors containing $\alpha 1$, $\alpha 2$, $\alpha 3$, and $\alpha 5$ subunits ($\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A, and $\alpha 5$ GABA_A receptors, respectively) through which they inhibit the transmission of these signals. However, the role of these different GABA_A receptor subtypes in the antihyperalgesic properties of benzodiazepine-type drugs has not been characterized fully and is limited by currently available compounds. In the present study we describe the novel benzodiazepine site positive allosteric modulator methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024). MP-III-024 displayed preference for $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors relative to $\alpha 1$ GABA_A and $\alpha 5$ GABA_A receptors as well as an improved metabolic profile relative to subtype-selective positive modulators that are available currently. MP-III-024 produced a dose- and time-dependent reversal of mechanical sensitivity. On locomotor activity and schedule-controlled responding, MP-III-024 was ineffective across the doses tested. These data provide further evidence that $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors play an important role in the antihyperalgesic effects and may not be involved in some of the off target effects of benzodiazepine-like drugs. Further, these findings suggest that MP-III-024 is an ideal research tool for investigating the role of $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors in the behavioral properties of benzodiazepine-like drugs in mice.

1. Introduction

Neuropathic pain has been attributed, in part, to diminished GABAergic inhibition in spinal cord dorsal horn neurons (Zeilhofer, 2008). As such, drugs that facilitate spinal inhibition may be useful for the treatment of chronic pain states. Benzodiazepine-type drugs bind to an allosteric site on γ -aminobutyric acid type A (GABA_A) receptors, producing a

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conformational change in the receptor leading to an enhancement in the GABA elicited chloride currents. Previous work has shown that intrathecal administration of benzodiazepine-type drugs produces antihyperalgesic effects in animal models (Kontinen and Dickenson, 2000; Knabl et al., 2008, 2009; Witschi et al., 2011). However, the systemic use of benzodiazepine-type drugs for the treatment of various pain states is limited due to the expression of GABA_A receptors throughout the nervous system. These same receptors also mediate other characteristic effects that limit the use of benzodiazepines, such as daytime drowsiness, impairment of motor coordination and deficits in memory (for review, see Rudolph and Knoflach, 2011).

Previous molecular biological studies have revealed the existence of multiple subtypes of the GABA_A receptor (McKernan and Whiting, 1996; Rudolph et al., 2001; Olsen and Sieghart, 2008). Subsequent reports have postulated that the diverse behavioral effects of benzodiazepine-type drugs may reflect actions at different subtypes of GABA_A receptors (e.g., Knabl et al., 2008; Löw et al., 2000; McKernan et al., 2000; Rowlett et al., 2005; Rudolph et al., 1999; Tan et al., 2010). These observations suggest the possibility for a pharmacological dissociation between the clinically advantageous effects and unwanted side-effects of these compounds.

GABA_A receptors containing $\alpha 1$ subunits ($\alpha 1$ GABA_A receptors) are located ubiquitously throughout the CNS, and have been implicated in the sedative effects of benzodiazepines as well as in effects related to physical dependence and abuse (Engin et al., 2014; Fischer et al., 2013; Mirza and Nielsen, 2006; Rudolph et al., 1999; Tan et al., 2010). In contrast, GABA_A receptors containing $\alpha 2$ and $\alpha 3$ subunits ($\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors, respectively) are anatomically distributed in the cortex, limbic system and spinal cord (Rudolph and Knoflach, 2011) and have been associated with the anxiolytic effects of benzodiazepines (Fischer et al., 2010; Löw et al., 2000; McKernan et al., 2000; Rowlett et al., 2005). To date, $\alpha 2$ GABA_A receptors and possibly $\alpha 3$ GABA_A receptors have been implicated as the primary receptor subtype that mediates the antihyperalgesic effects of benzodiazepines (Knabl et al., 2008; Paul et al., 2013). Finally, GABA_A receptors containing $\alpha 5$ subunits ($\alpha 5$ GABA_A receptors) are preferentially expressed within the hippocampus and are thought to play a role in certain memory processes impacted by benzodiazepines (Collinson et al., 2002; Crestani et al., 2002; Atack, 2011; but see Perez-Sanchez 2016).

The precise roles of $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors in the pain-relieving properties of benzodiazepines has been limited due to the scarcity of available compounds that target these receptors selectively. In preclinical rodent models, antihyperalgesic effects have been demonstrated following the systemic administration of $\alpha 1$ GABA_A-sparing compounds. As an example, L-838,417, a drug described as a partial benzodiazepine site agonist at $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors but an antagonist (i.e. demonstrating no appreciable efficacy) at $\alpha 1$ GABA_A receptors' benzodiazepine site (McKernan et al., 2000) was effective in reducing mechanical sensitivity following injection of the inflammatory agent zymosin A (Knabl et al., 2008). More recently, benzodiazepine site positive allosteric modulators with preferential activity at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors were shown to exert antihyperalgesic effects under similar conditions (Di Lio et al., 2011; de Lucas et al., 2015). To date, the relationship

between antihyperalgesic effects and off-target side effects is understood poorly, and the pharmacokinetic profile of the currently available compounds limits preclinical study in rodents.

In the present study, we describe the pharmacological and behavioral profile of the novel benzodiazepine-type compound methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024). MP-III-024 displayed preference for $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors relative to $\alpha 1\text{GABA}_A$ and $\alpha 5\text{GABA}_A$ receptors in *Xenopus* oocytes. In a mouse liver microsomal assay, MP-III-024 showed a favorable metabolism profile relative to currently available compounds, making it ideal for further preclinical study. To assess further the role of $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors in the antihyperalgesic and off-target effects of benzodiazepine-type drugs, we then studied the behavioral effects of MP-III-024 on mechanical sensitivity, locomotor activity and operant behavior.

2. Materials and methods

2.1. Animals

Adult male C57BL/6 mice 10 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were group housed in standard plexiglass cages in a colony room maintained on a 12-h light–dark cycle (lights on at 7:00 AM). All mice had continuous access to food and water throughout the study and were habituated to the colony room environment for 2 weeks prior to any experimental manipulation. Mice were also exposed to the testing environment and handled for 2 days prior to initiation of an experiment. Mice were used once and were naïve to behavioral and pharmacological manipulation prior to an experiment. All testing procedures were conducted between 11:00 AM and 3:00 PM. Animals used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee of Rowan University and all testing adhered to the “Guide for the Care and Use of Laboratory Animals” (National Research Council, National Academy of Sciences, Washington, D.C., USA, 2011).

2.2. Drugs

The novel benzodiazepine analog methyl 8-ethynyl-6-(pyridin-2-yl)-4H-benzo[f]imidazo[1,5-a][1,4]diazepine-3-carboxylate (MP-III-024) was synthesized at the Department of Chemistry and Biochemistry at the University of Wisconsin-Milwaukee. MP-III-024 was suspended in 0.5% methyl cellulose and 0.9% NaCl, and administered intraperitoneally in a total volume of 10 ml/kg body weight.

2.3. Electrophysiology

cDNAs of rat GABA_A receptor subunits were used for generating the respective mRNA's that were then injected into *Xenopus laevis* oocytes (Nasco, WI) as described previously (Savic et al., 2008). For electrophysiological recordings, oocytes were placed on a nylon-grid in a bath of *Xenopus* Ringer solution (XR, containing 90 mM NaCl, 5 mM HEPES-NaOH (pH 7.4), 1 mM MgCl_2 , 1 mM KCl and 1 mM CaCl_2). For current measurements the oocytes were impaled with two microelectrodes (2–3 m Ω) 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. 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 Dagan TEV-200A two-electrode voltage clamp (Dagan Corporation, Minneapolis, MN) and a DAGAN CA-1B high performance oocyte clamp. Data were digitised, recorded and measured using a Digidata 1322A data acquisition system (Axon Instruments, Union City, CA).

2.4. Microsomal Stability Assay

4 μ L of 1 mM MP-III-024 or HZ-166 (at a final concentration of 10 μ M) in DMSO was preincubated at 37°C for 5 minutes on a digital heating shaking dry bath (Fischer scientific, Pittsburgh, PA) in a mixture containing 282 μ L of water, 80 μ L of phosphate buffer (0.5 M, pH 7.4), 20 μ L of NADPH Regenerating System Solution A (BD Bioscience, San Jose, CA) and 4 μ L of NADPH Regenerating System Solution B (BD Bioscience, San Jose, CA) in a total volume of 391.2 μ L. Following preincubation, the reaction was initiated by addition of 8.8 μ L of either human liver microsomes (BD Gentest, San Jose, CA) or mouse liver microsomes (Life technologies, Rockford, IL), at a protein concentration of 0.5 mg/mL. Aliquots of 50 μ L were taken at time intervals of 0 (without microsomes), 10, 20, 30, 40, 50 and 60 minutes. Each aliquot was added to 100 μ L of cold acetonitrile solution containing 5 μ M of verapamil as internal standard. This was followed by sonication for 10 seconds and centrifugation at 10,000 rpm for 5 minutes. 100 μ L of the supernatant was transferred into Spin-X HPLC filter tubes (Corning Incorporated, NY) and centrifuged at 13,000 rpm for 5 minutes. The filtrate was diluted 100 fold and subsequently analyzed by LC-MS/MS with Shimadzu LCMS 8040 (Shimadzu Scientific Instruments, Columbia, MD). The ratio of the peak areas of the internal standard and test compound was calculated for every time point and the natural log of the ratio were plotted against time to determine the linear slope (k). The metabolic rate ($k \cdot C_0/C$), half-life ($0.693/k$), and internal clearance ($V \cdot k$) were calculated, where k is the slope, C_0 is the initial concentration of test compound, C is the concentration of microsomes, and V is the volume of incubation in μ L per microsomal protein in mg. All experiments were repeated three times in duplicates.

2.5. Mechanical Sensitivity

Antihyperalgesic effects were studied following inflammation evoked through subcutaneous injection of 0.06 mg zymosin A suspended in 20 μ L 0.9% NaCl into the plantar surface of the right hindpaw. The non-injected left hindpaw was used as control. Mechanical sensitivity was then assessed 24 h after zymosin A injection with a Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). A Von Frey-type 0.5 mm filament applied a linear increase in force at a rate of 1 g per second and the paw withdraw thresholds for both paws was recorded. Following baseline measurements, paw withdrawal force thresholds were assessed at 10, 20, 40, 80, 160 and 320 minutes after MP-III-024 administration. For dose-effect analysis, the maximum effect irrespective of time point ($E_{\max}$) was normalized relative to the baseline measurement of the non-injected left hindpaw (BL_L) and the baseline measurement of the injected right hindpaw (BL_R) using the equation $[(E_{\max} - BL_R)/(BL_L -$

BL_R). Effects of MP-III-024 dose were evaluated by conducting *a priori* Bonferroni *t*-tests, comparing individual data points to vehicle with an alpha level set at $p = 0.05$.

2.6. Open field

Locomotor activity was assessed in an open field arena (27.3 cm x 27.3 cm x 20.3 cm) controlled by a MED-PC interface and an IBM compatible computer programmed with MED Associates software (MED Associates, St. Albans, VT). Distance traveled and vertically directed behaviors were measured by the breaking of photobeams. MP-III-024 was administered immediately prior to exposure to the chamber, following which the effects of MP-III-024 were assessed from 0–60 min post injection. For dose-effect analysis, the data were normalized to vehicle by dividing the total distance traveled across the 60 min test period following drug administration by the total distance traveled across the 60 min test period following vehicle administration. Effects MP-III-024 dose were evaluated by conducting *a priori* Bonferroni *t*-tests, comparing individual data points to baseline with an alpha level set at $p = 0.05$.

2.7. Schedule-controlled responding

Schedule-controlled responding was assessed in an experimental operant chamber (15.9 cm x 14.0 cm x 12.7 cm) equipped with a house light, ventilator fan, and two nose poke holes (1.2 cm diameter) that were located on either side of a liquid dipper. The operant chamber was controlled by a MED-PC interface and an IBM compatible computer programmed with MED Associates software (MED Associates, St. Albans, VT).

Mice were trained under a multiple-cycle procedure conducted 5 days per week (Monday through Friday). Each training cycle consisted of a pretreatment period followed by a 5-minute response period. The pretreatment times varied as to have the response periods begin at 10-, 20-, 40-, 80 and 160-minutes. During the pretreatment period, stimulus lights were not illuminated and responding had no scheduled consequences. During the response period, the right nose poke was illuminated and mice could obtain up to 5 liquid food reinforcers (8 sec access to 8 μ l Ensure®) under a fixed-ratio 3 (FR-3) schedule of food presentation. If all 5 reinforcers were earned before 5 minutes had elapsed, the light was turned off, and responding had no scheduled consequences for the remainder of the response period. The left nose poke was inactive, and responding at this hole had no scheduled consequences. Training sessions consisted of 4 consecutive cycles, and testing began once response rates were stable throughout the session.

Test sessions replaced the last training session of each week if responding was stable throughout the preceding training sessions. Test sessions were identical to training sessions except that MP-III-024 was administered i.p. at the start of the session. Data are expressed as a percentage of baseline responding using the average rate of responding from the previous day as the control value (mean of 5 cycles) for each animal. Effects of MP-III-024 dose were evaluated by conducting *a priori* Bonferroni *t*-tests, comparing the maximum effect irrespective of time point relative to vehicle with an alpha level set at $p = 0.05$.

3. Results

3.1. Electrophysiology

Figure 1 shows the chemical structure of MP-III-024 and Figure 2 the dose-response curves for stimulation of GABA-induced currents by MP-III-024 in oocytes expressing $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A or $\alpha 5$ GABA_A receptors. MP-III-024 exhibited the highest efficacy at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors. At the 100 nM concentration, MP-III-024 exhibited weak modulatory effects at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors. At higher concentrations, MP-III-024 showed moderate modulatory effects at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors while acting as a very weak modulator at $\alpha 1$ GABA_A and $\alpha 5$ GABA_A receptors. Stimulation of GABA EC3 by MP-III-024 at 100 nM or 1 μ M concentration was 109 ± 1 or 125 ± 5 , 136 ± 13 or 240 ± 42 , 134 ± 5 or 229 ± 19 and 86 ± 27 or 111 ± 31 for $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors, respectively.

3.2. Microsomal Stability Assay

Figure 3 shows the effects of MP-III-024 and HZ-166 in the mouse liver microsomal assay. Data are expressed as the peak area ratio defined as the peak area of MP-III-024 or HZ-166 divided by the peak area of the internal standard Verapamil. MP-III-024 has a good metabolic stability, with 74.8 ± 0.43 % remaining after one hour of incubation. The half-life of MP-III-024 was 141.5 ± 20 min with an intrinsic clearance of $0.491 \mu\text{L}/\text{min}/\text{mg}$ and metabolic rate of $9.815 \text{ nmol}/\text{min}/\text{mg}$. The metabolic parameters of HZ-166 were also assessed for comparison. Relative to MP-III-024, HZ-166 was less stable, with 45.1 ± 0.20 % remaining following 60 minutes and a half-life of 51.4 ± 1.88 min. HZ-166 had an intrinsic clearance of $1.35 \mu\text{L}/\text{min}/\text{mg}$ and a metabolic rate of $27.01 \text{ nmol}/\text{min}/\text{mg}$.

MP-III-024 was also assessed in a human liver microsomal assay (data not shown). MP-III-024 displayed a favorable metabolic profile in this assay which was similar to those determined in the mouse liver microsomal assay. MP-III-024 was stable in this with 76.0 ± 0.24 % remaining after one hour of incubation. The half-life of MP-III-024 was 141.8 ± 11 min with an intrinsic clearance of $0.489 \mu\text{L}/\text{min}/\text{mg}$ and metabolic rate of $9.772 \text{ nmol}/\text{min}/\text{mg}$.

3.3. Mechanical Sensitivity

The hyperalgesic effects of zymosin A administration and the antihyperalgesic properties of MP-III-024 are shown in Figure 4. Injection of zymosin A into the right hindpaw reduced mechanical sensitivity relative to the non-injected left hindpaw. MP-III-024 produced dose- and time-dependent increases in paw withdrawal thresholds in the injected paw, with a maximum effect at 40–80 minutes. Paw withdrawal thresholds of the non-injected paw were not affected. Dose response analysis of MP-III-024 revealed an ED₅₀ value of 4.05 (0.725–7.59) with significant antihyperalgesic effects observed at doses of 10 mg/kg and 32 mg/kg.

3.4. Open Field

Figure 5 shows the effects of MP-III-024 on locomotor activity. Data are expressed as the distance traveled in 5 min bins over 60 mins. Following administration of vehicle, mice engendered robust locomotor activity initially, and the distance traveled per 5 min bin

decreased slightly over the 60 min testing period. The cumulative distance traveled following vehicle administration was 6732.41 ± 630.96 cm. Administration of MP-III-024 (3.2–100 mg/kg) produced modest dose-dependent decreases in locomotor activity. However, dose response analysis revealed no significant decreases in cumulative distance traveled across the dose range tested, and an ED₅₀ value could not be determined.

3.5. Schedule-controlled responding

The effects of MP-III-024 on operant behavior are shown in Figure 6. Baseline rates of responding across all studies were 0.91 ± 0.03 responses per second. Following stable baseline response rates, doses of 3.2 mg/kg – 100 mg/kg MP-III-024 were assessed at 10, 20, 40, 80 and 160 min post-injection. There were no decreases in operant response rates observed at any time point across the doses studied. Dose response analysis revealed no significant decreases in operant responding across any dose tested, and an ED₅₀ value could not be determined.

4. Discussion

Classical benzodiazepine-type drugs bind non-selectively to $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors, and the role of these different GABA_A receptor subtypes in the pain-relieving properties of these drugs has not been characterized fully. In the present study, we describe the novel compound MP-III-024 which displayed selectivity for $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors over $\alpha 1$ GABA_A and $\alpha 5$ GABA_A receptors. MP-III-024 also demonstrated a favorable pharmacokinetic profile relative to the currently available $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptor positive modulator HZ-166 making it ideal as a preclinical research tool in rodents. MP-III-024 showed robust antihyperalgesic effects in an assay of mechanical sensitivity. In contrast, MP-III-024 was ineffective in assays of locomotor activity and schedule-controlled responding. Collectively, these data suggest that a selective $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptor positive allosteric modulator can result in robust antihyperalgesic effects while lacking sedative-like effects and effects related to behavioral toxicity.

Currently available subtype-selective GABA_A receptor modulators have been paramount to our understanding of GABA_A receptor function in preclinical studies. These compounds have been used to explore the role of $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors on various behavioral endpoints in rodents. Although many of these compounds have been shown to be stable in human liver microsomes (Namjoshi et al., 2013), poor metabolism profiles in rodents have been noted, limiting their use as preclinical tools (Scott-Stevens et al., 2005; Di Lio, 2011; Paul, 2013). Therefore, in the present study we assessed the metabolism profile of MP-III-024 relative to the positive allosteric modulator HZ-166 which was chosen based on its use in previous studies (Di Lio, 2011; Paul, 2013). The metabolism profile of MP-III-024 was markedly improved relative to HZ-166, with approximately 75% of the compound remaining following 60 minutes. The improved stability of imidazobenzodiazepine methyl esters in comparison to ethyl and especially isobutyl esters has been reported (Jahan et al., 2016) although the underlying metabolic reactions are still unknown. Although speculative, one hypothesis for the rapid metabolism

of HZ-166 in rodents may be due to ethyl esters being susceptible to beta-oxidation to form acetaldehyde; whereas MP-III-024 does not undergo this oxidation step. The stability of MP-III-024 in the presence of human liver microsomes provides further evidence that this compound is an ideal research tool for preclinical translational studies in mice.

Although benzodiazepine-type drugs are generally not considered to be analgesic when administered systemically, likely due to the sedative properties mediated through $\alpha 1\text{GABA}_A$ receptors, evidence from animal preparations suggest that non-selective benzodiazepines have pain-relieving properties with administered intrathecally (Hwang and Yaksh, 1997; Scholz et al., 2005; Tucker et al., 2004a; 2004b). This effect is likely mediated by $\alpha 2\text{GABA}_A$, $\alpha 3\text{GABA}_A$ and/or $\alpha 5\text{GABA}_A$ receptors that are located in the spinal cord (Knabl et al., 2008; Paul et al., 2013). In support of this hypothesis, $\alpha 1\text{GABA}_A$ -sparing drugs that have agonist activity at $\alpha 2\text{GABA}_A$, $\alpha 3\text{GABA}_A$ and $\alpha 5\text{GABA}_A$ receptors produce behaviors in animal models related to antinociception and reduce the associative emotional components of pain as measured by functional magnetic resonance imaging (Griebel et al., 2001; Munro et al., 2008; Knabl et al., 2008).

To date the specific role between $\alpha 2\text{GABA}_A$, $\alpha 3\text{GABA}_A$ and $\alpha 5\text{GABA}_A$ receptors in the antihyperalgesic effects of benzodiazepine-type drugs is understood poorly, and is largely dependent on the availability of drugs that target these receptor subtypes selectively. Using newly synthesized compounds that possess agonist activity at $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors, recent reports suggest that positive allosteric modulation of these receptor subtypes alone is sufficient to produce an antihyperalgesic effect (Munro et al., 2008; Di Lio, et al., 2011; Paul et al., 2013; de Lucas et al., 2015). These findings suggest that $\alpha 5\text{GABA}_A$ receptors are not necessary for a GABAergic drug to have antihyperalgesic properties. In agreement with this previous findings, we show here that MP-III-024 produces a robust reversal of hyperalgesia to a mechanical stimulus suggesting that positive allosteric modulation at $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors may be sufficient for antihyperalgesic effects to occur.

Recent studies using mutated mice lacking $\alpha 2\text{GABA}_A$ receptors in the spinal cord have implicated this receptor subtype as a primary contributor to benzodiazepine-induced antihyperalgesia (Paul et al., 2013). However, consistent with previous studies, it is interesting to note that the antihyperalgesic effects of a subtype-selective $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptor agonist were not completely lost in these mice (Knabl et al., 2008; Paul et al., 2013). These observations suggest that the antihyperalgesic effects of MP-III-024 are likely mediated by both $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors. Further, it should be noted that $\alpha 5\text{GABA}_A$ receptors are also found in spinal nociceptive circuits and may contribute to the antihyperalgesic effects of other benzodiazepine-type compounds that possess positive allosteric modulatory effects at this receptor subtype (Knabl et al., 2008; Perez-Sanchez 2016).

Pharmacological efforts to attribute the contribution of GABA_A receptor subtypes to the multiple effects of benzodiazepines have been enhanced in recent years by the increasing availability of compounds that target these receptors. Regarding $\alpha 3\text{GABA}_A$ receptors, the imidazopyridine compound TP003 has been recognized as the primary research tool used for

exploring the function of this receptor subtype (Fischer et al., 2011; Marowski et al., 2012; Shinday et al., 2013). TP003 has been described as having comparatively high agonist efficacy at $\alpha 3$ GABA_A receptors but essentially no efficacy at $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, and $\alpha 5$ GABA_A receptors (Dias et al. 2005). Because TP003 exhibits appreciable efficacy only at $\alpha 3$ GABA_A receptors, the extent to which this compound induces an effect characteristic of conventional benzodiazepines has been interpreted as evidence for a specific role of $\alpha 3$ GABA_A receptors in that particular effect. However, recent reports have called into questions the efficacy profile of TP003, and an attempted replication of the original study suggest that it may have efficacy across $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors (de Lucas, 2015). Further, to our knowledge a subtype-selective $\alpha 2$ GABA_A receptor positive allosteric modulator has not been developed. The development of subtype-selective $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptor modulators is necessary for further study into the role of these receptors in the antihyperalgesic effects of benzodiazepine-like drugs.

Previous studies suggest that the sedative properties of benzodiazepine-like drugs are mediated primarily through $\alpha 1$ GABA_A receptors (Rudolph et al., 1999; McKernan et al., 2000; DiLio et al., 2011). In the present study, MP-III-024 produced modest decreases in distance traveled in the open field procedure, however this effect did not reach statistical significance. This observation is consistent with the pharmacological profile of MP-III-024 and low efficacy $\alpha 1$ GABA_A receptors. Notably the highest dose tested in this procedure was 10-fold higher than the dose at which we saw the initial behavioral effects on antihyperalgesia. These data provide further evidence suggesting that $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors play a limited role in the sedative properties of benzodiazepine-type drugs.

On operant behavior, MP-III-024 was ineffective in reducing response rates across the dose range tested. A number of studies have demonstrated that various positive GABA_A modulators with activity at $\alpha 1$ GABA_A receptors decrease responding maintained under a variety of operant schedules suggesting an important role for $\alpha 1$ GABA_A receptors in mediating this effect (e.g., Paronis and Bergman 1999; Vanover et al., 1999; Rowlett et al., 2005; Fischer et al., 2010, Fischer et al., 2013). Further, evidence suggests that $\alpha 5$ GABA_A receptors are not involved in the rate-decreasing effects of benzodiazepine-type drugs (Fischer et al., 2013). The role of $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors is less clear, although a majority of studies suggest that drugs selective for these receptor subtypes produce either negligible or modest decreases in response rates depending on the experimental conditions (Fischer et al, 2010; Fischer et al., 2013). Together with previous studies, the present results with MP-III-024 suggest that activation of both $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors is necessary for modest behavioral disruption under certain conditions.

In the present study we describe a novel GABA_A receptor ligand which has selective efficacy at $\alpha 2$ GABA_A and $\alpha 3$ GABA_A receptors as well as a favorable metabolism profile in rodents. The behavioral data from the present set of experiments provide further evidence that the effects of benzodiazepine-like drugs are likely mediated differentially by $\alpha 1$ GABA_A, $\alpha 2$ GABA_A, $\alpha 3$ GABA_A and $\alpha 5$ GABA_A receptors. Accordingly, our studies highlight several hypotheses regarding benzodiazepine action. First, our findings suggest

that $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors play an important role in benzodiazepine-induced antihyperalgesia. Our results are also consistent with the idea that some of the sedative properties of benzodiazepines require actions additional to those at $\alpha 2\text{GABA}_A$ and $\alpha 3\text{GABA}_A$ receptors. These hypotheses should provide an important framework for studying the role of different GABA_A receptor subtypes in the behavioral effects of benzodiazepine-type drugs, which in turn should help guide the development of improved therapeutic agents for pain-related disorders.

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Figure 1.
Chemical structure of MP-III-024

Figure 2.

Concentration-effect curves for MP-III-024 on $\alpha 1\text{GABA}_A$ (upward triangles), $\alpha 2\text{GABA}_A$ (downward triangles), $\alpha 3\text{GABA}_A$ (diamonds) and $\alpha 5\text{GABA}_A$ (squares) receptors using an EC3 GABA concentration. Abscissa, dose of MP-III-024. Ordinate, percent of control current. Data represent the mean ($\pm$ SEM) from four oocytes from at least 2 batches.

Figure 3. Time course of MP-III-024 (closed symbols) and HZ-166 (open symbols) in the mouse liver microsomal assay. Abscissa, time after drug administration. Ordinate, peak area of MP-III-024 or HZ-166 divided by the peak area of internal standard. Data represent the mean ($\pm$ SEM).

Figure 4.

Effects of MP-III-024 on inflammatory pain. Time-course of 3.2 mg/kg (top left), 10 mg/kg (top right) and 32 mg/kg (bottom left) MP-III-024 following i.p. administration. Abscissa, time after MP-III-024 administration. Ordinate, paw withdrawal threshold in grams. Open symbols represent the left (non-injected) hindpaw and closed symbols represent the right (injected) hindpaw. Data points above "BL" represent predrug baseline thresholds. The panel on the bottom right represents dose response analysis of MP-III-024. Abscissa, dose of MP-III-024. Ordinate percent control of threshold relative to vehicle. Data point above "V" represent thresholds following vehicle administration. Each data point shows the mean ($\pm$ SEM) from 8 mice.

Figure 5.

Effects MP-III-024 and on locomotor activity. Left panel: Time-course of 3.2 mg/kg (upward triangles), 10 mg/kg (downward triangles), 32 mg/kg (diamonds) and 100 mg/kg (squares) MP-III-024. Abscissa, time after MP-III-024 administration. Ordinate, distance traveled in centimeters. Right panel: Dose-response analysis of MP-III-024. Abscissa, dose of MP-III-024. Ordinate percent control distance traveled relative to baseline. Points above “V” represent the distance traveled following vehicle administration. Each data point shows the mean ($\pm$ SEM) from 8 mice.

Figure 6.

Effects of MP-III-024 on operant behavior. Time-course of 10 mg/kg (left), 32 mg/kg (center) and 100 mg/kg (right) MP-III-024 following i.p. administration. Abscissa, time after MP-III-024 administration. Ordinate, percent of control response rate relative to baseline. Each data point shows the mean ($\pm$ SEM) from 8–10 mice.