JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.
JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250

Quantitative analyses of antagonism: combinations of midazolam and either flunitrazepam or pregnanolone in rhesus monkeys discriminating midazolam

Lisa R. Gerak and Charles P. France

Departments of Pharmacology (LRG and CPF) and Psychiatry (CPF), The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX, USA 78229-3900

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Copyright 2011 by the American Society for Pharmacology and Experimental Therapeutics.

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Running head: Combinations of positive $GABA_A$ modulators

Corresponding author: Lisa R. Gerak

Department of Pharmacology

University of Texas Health Science Center

7703 Floyd Curl Dr, mail code 7764

San Antonio, TX 78229

Phone: 210 567 0106

Fax: 210 567 4303

Email: gerak@uthscsa.edu

Text pages: 19

Number of tables: 1

Number of figures: 6

Number of references: 28

Number of words in Abstract: 239

Number of words in Introduction: 747

Number of words in Discussion: 1499

Recommended section assignment: Behavioral Pharmacology

Non-standard abbreviations: none

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Adverse effects of benzodiazepines limit their clinical use; these effects might be reduced without altering therapeutic effects by administering other positive $GABA_A$ modulators (i.e., neuroactive steroids) with benzodiazepines. One concern with this strategy involves reversing these combined effects in case of overdose. The current study examined whether flumazenil can attenuate the combined effects of two benzodiazepines, midazolam and flunitrazepam, and the combined effects of midazolam and the neuroactive steroid pregnanolone, in four monkeys discriminating midazolam. Each positive modulator produced ≥80% midazolam-lever responding. Interactions between midazolam and either flunitrazepam or pregnanolone were additive. Flumazenil antagonized the benzodiazepines when they were administered alone or in combination. Schild analyses yielded slopes that did not deviate from unity, regardless of whether benzodiazepines were administered alone or together; the pA_2 value for flumazenil was 7.58. In contrast, flumazenil enhanced the effects of pregnanolone with 0.32 mg/kg flumazenil shifting the pregnanolone dose-effect curve 2-fold leftward. Flumazenil attenuated the combined effects of midazolam and pregnanolone, although antagonism was not dose dependent. Thus, the interaction between two benzodiazepines was similar to that of a benzodiazepine and a neuroactive steroid; however, flumazenil more efficiently attenuated a combination of two benzodiazepines, as compared with a combination of a benzodiazepine and a neuroactive steroid. While the magnitude of antagonism of a benzodiazepine combined with a neuroactive steroid was reduced, these results support continued exploration of the use of combinations of positive modulators to enhance therapeutic effects while reducing adverse effects.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Benzodiazepines have been widely used to treat anxiety, insomnia, convulsions and ethanol withdrawal. While they are safe and effective, long-term use has revealed adverse effects, particularly tolerance and dependence. One strategy that might retain the therapeutic effects of benzodiazepines while reducing adverse effects is to develop other positive modulators of γ-aminobutyric acid$_A$ ($GABA_A$) receptors, such as neuroactive steroids. Although benzodiazepines and neuroactive steroids act at distinct sites on $GABA_A$ receptors, they both facilitate the actions of GABA, thereby increasing Cl^- flux and producing similar acute behavioral effects. Like benzodiazepines, neuroactive steroids have anxiolytic (Wieland et al., 1997), sedative (Lancel, 1999; Vanover et al., 1999) and anticonvulsant effects (Gasior et al., 2000; Kokate et al., 1994; Reddy and Rogawski, 2001) and can reverse ethanol withdrawal (Finn et al., 2000). Despite these similarities, the effects of neuroactive steroids and benzodiazepines are not identical with differences emerging during chronic treatment; for example, tolerance and dependence are less likely to develop during chronic treatment with neuroactive steroids, as compared with benzodiazepines (Eppolito and Gerak, 2010; Kokate et al., 1998; Reddy and Rogawski, 2000). Although the ramifications of long-term therapeutic use of neuroactive steroids are not known, lack of tolerance could provide a clinical advantage for neuroactive steroids over benzodiazepines. In contrast, there are other factors that might make the therapeutic use of benzodiazepines more appealing than that of neuroactive steroids, such as the availability of a pharmacological antagonist (e.g., flumazenil), which can reverse effects of benzodiazepines in the event of overdose; no such compound is available to reverse the effects of neuroactive steroids. Thus, clinical benefits are different among positive $GABA_A$ modulators,

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

and if benzodiazepines and neuroactive steroids could be combined in one therapeutic, that drug might retain the clinical effectiveness of benzodiazepines with fewer adverse effects and could be at least partially attenuated by flumazenil.

One way to combine the benefits of benzodiazepines and neuroactive steroids might be to administer them concurrently. Drug combinations have been used successfully to treat other conditions. For example, when drugs (e.g., opioids and nonsteroidal anti-inflammatory drugs) are given together to relieve pain, smaller doses of each drug are needed to produce the desired effect; those smaller doses along with actions through different mechanisms reduce adverse effects. A similar approach might be used with positive modulators acting at different sites on $GABA_A$ receptors to retain positive aspects of each drug while reducing less desirable features.

Studies in monkeys suggest that combinations of benzodiazepines and neuroactive steroids might provide clinical advantages by retaining therapeutic effects while reducing adverse effects. For example, combinations of the benzodiazepine triazolam and the neuroactive steroid pregnanolone produced supra-additive effects in a conflict procedure, which provides a measure of anxiolytic effects (Fischer and Rowlett, 2011). When discriminative stimulus effects or rates of lever pressing were measured, the interaction was additive (McMahon and France, 2005; Fischer and Rowlett, 2011), and in monkeys self administering a combination of triazolam and pregnanolone, the interaction was either additive or infra-additive, depending on the ratio of doses used (Fischer and Rowlett, 2011). Thus, the combined effects of positive $GABA_A$ modulators can vary, enhancing some effects more than others.

Although benzodiazepines are generally safe, severe respiratory depression can occur when benzodiazepines are given with other drugs (e.g., ethanol), and toxicity can be reduced with flumazenil, which reverses the benzodiazepine component of the mixture. Flumazenil

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

might also be expected to attenuate the combined effects of a benzodiazepine and a neuroactive steroid. Flumazenil antagonizes benzodiazepines in monkeys discriminating midazolam, shifting dose-effect curves rightward (Lelas et al., 1999; 2000; McMahon et al., 2002); however, it enhances the effects of neuroactive steroids, shifting dose-effect curves leftward (McMahon and France, 2005; Gerak and France, 2011), which reflects the positive efficacy of flumazenil (Dantzer and Pério, 1982). Thus, flumazenil could enhance or attenuate the combined effects of benzodiazepines and neuroactive steroids depending on the proportion of each drug in the mixture.

This study examined the ability of flumazenil to alter the effects of pregnanolone, midazolam and another benzodiazepine flunitrazepam, given alone or together. Drug combinations were administered using a fixed-ratio design such that the proportion remained constant based on the potency of each drug to produce midazolam-lever responding. According to receptor theory, flumazenil should attenuate the effects of all benzodiazepines, regardless of whether they are administered alone or together. These studies tested that hypothesis by comparing the combined effects of two benzodiazepines to the combined effects of a benzodiazepine and a neuroactive steroid and determining the extent to which flumazenil attenuates each drug combination.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Subjects. Five adult female rhesus monkeys were housed individually on a 14 hr light and 10 hr dark cycle. Their weights (4.0-9.0 kg) did not markedly change throughout the experiments and were maintained with primate chow (Harlan Teklad, High Protein Monkey Diet, Madison, WI), fresh fruit and peanuts provided in the home cage. Monkeys had free access to water while in their home cages. They were trained to discriminate midazolam at least one year before the start of these studies (Gerak and France, 2011). Three monkeys (subjects SA, LI, and NI) contributed to all data presented. A fourth monkey (subject RO) participated in studies in which 1) midazolam was given alone and with flumazenil, 2) flunitrazepam was given alone, and 3) a mixture of midazolam and flunitrazepam was administered with and without flumazenil. That monkey was replaced by a fifth monkey (subject HE) who participated in studies in which 1) midazolam was studied alone, 2) pregnanolone was given alone and with flumazenil, and 3) a mixture of midazolam and pregnanolone was administered with and without flumazenil. Monkeys used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio, San Antonio, TX, and with the 1996 *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources on Life Science, National Research Council, National Academy of Sciences).

Apparatus. During experimental sessions, monkeys were seated in chairs (Primate Products, Miami, FL) with their feet placed in shoes mounted on the front of the chair. Shoes contained brass electrodes to which brief (250 msec, 3mA) electric shock could be delivered. During sessions, chairs were placed in ventilated, sound-attenuating chambers that were equipped with two stimulus lights and two response levers. A commercially available interface

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

(Med Associates Inc., East Fairfield, VT, USA) connected experimental chambers to computers which controlled experiments and recorded data using MED-PC IV software (MED Associates, Inc., St. Albans, VT). White noise was present in each chamber.

Procedure. At the beginning of these studies, three monkeys discriminated 0.178 mg/kg of midazolam and two other monkeys discriminated 0.32 mg/kg of midazolam from vehicle while responding under a fixed-ratio 10 schedule of stimulus-shock termination (Gerak and France, 2011). For the two monkeys that began the experiment discriminating the larger dose of midazolam (subjects RO and LI), the training dose was decreased to 0.178 mg/kg during these studies because the larger dose decreased rate of lever pressing; dose-effect curves for midazolam and flunitrazepam (subject RO) and for midazolam and pregnanolone (subject LI) determined before and after the change in training dose were similar. Daily experimental sessions were divided into 15-minute cycles with up to eight cycles in each session. Cycles began with 10-minute timeout periods during which chambers were dark and responding had no programmed consequence. Response periods occurred during the last five minute of each cycle and were signaled by illumination of red stimulus lights. Under the schedule of stimulus-shock termination, monkeys could extinguish stimulus lights and postpone the shock schedule for 30 sec by responding 10 consecutive times (fixed-ratio 10) on the lever designated correct by an injection administered during the first minute of the cycle. The lever designated correct after administration of midazolam was counterbalanced across monkeys. Incorrect responses reset the response requirement on the correct lever. If monkeys did not satisfy the response requirement within 15 sec of illumination of red lights, brief electric shock was delivered every 15 sec until the response requirement was satisfied or the cycle ended, which occurred either when four shocks were delivered or five minute elapsed since the beginning of the response period.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Injections were given during the first minute of each cycle. For some training sessions, the training dose of midazolam was administered at the beginning of one cycle; during that cycle as well as during one subsequent cycle, which began with a sham injection, responding on the drug-appropriate lever extinguished lights and postponed the shock schedule. The session ended after the sham cycle. The cycle during which midazolam was administered was preceded by zero to six cycles during which saline or sham injections were administered. For other training sessions, monkeys received saline or sham injections prior to all cycles, with the number of cycles varying between two and eight. Stimulus control was considered adequate for testing when the following criteria were satisfied: ≥80% responding on the injection-appropriate lever during each cycle and fewer than 10 responses on the incorrect lever prior to completion of the first fixed ratio on the correct lever. Before their first test session, monkeys satisfied these criteria for five consecutive or six of seven training sessions; thereafter, test sessions were conducted when these criteria were satisfied for two consecutive training sessions. When monkeys did not satisfy the testing criteria, training continued until the criteria were satisfied during two consecutive sessions.

Test sessions were identical to training sessions except that 10 consecutive responses on either lever postponed shock and various doses of test compounds were administered during the first minute of each cycle. Dose-effect curves for midazolam, flunitrazepam and pregnanolone were generated by administering vehicle on the first cycle and test compound during subsequent cycles with the cumulative dose increasing by ¼ log unit per cycle. Dosing continued until monkeys responded ≥80% on the midazolam lever. From these dose-effect curves, the dose needed to produce 50% responding on the drug lever (ED_{50}) was estimated for individual monkeys using linear regression. The discriminative stimulus effects of combinations of positive

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

modulators were studied by administering increasing doses during each cycle while maintaining a constant proportion of the two drugs; the proportion was based on the group mean ED_{50} values (e.g., McMahon and France, 2005). During the first minute of the cycle, two separate injections of vehicle were administered; one injection was saline and the second injection was the vehicle appropriate for either flunitrazepam or pregnanolone, depending on which drug was studied in combination with midazolam during subsequent cycles. Thereafter, two drugs were given in separate injections on each cycle. When midazolam was studied in combination with flunitrazepam, doses that were 1/8 of the group mean ED_{50} value for each drug were administered during the second cycle; when midazolam was studied in combination with pregnanolone, doses that were ¼ of the group mean ED_{50} value for each drug were administered during the second cycle. The dose of each drug doubled on each subsequent cycle up to the dose combination that produced ≥80% responding on the midazolam lever. To examine whether flumazenil alters the discriminative stimulus effects of positive modulators administered alone or in combination, a single dose of flumazenil (0.01-1 mg/kg) was given on the first cycle followed by cumulative doses of the test compound(s) up to doses that produced ≥80% responding on the midazolam lever. When one positive modulator was administered after flumazenil, doses increased in ¼ log unit increments, whereas when two positive modulators were administered concurrently, doses of both drugs doubled on each subsequent cycle. Dose-effect curves for a single positive modulator or a combination of two positive modulators were determined twice in the absence of flumazenil: once before and once after they were studied in the presence of three doses of flumazenil.

Drugs. Midazolam hydrochloride (Bedford Laboratories, Bedford, OH) was purchased as a commercially prepared solution and diluted with sterile water. Flunitrazepam (Sigma-

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Aldrich Co., St. Louis, MO) was dissolved in a vehicle containing 50% emulphor and 50% ethanol. Pregnanolone (5β-pregnan-3α-ol-20-one; Bujno Synthesis, Warsaw, Poland) was dissolved in 45% (w/v) hydroxypropyl-γ-cyclodextrin. Flumazenil was dissolved in a vehicle comprising 40% propylene glycol, 50% sterile water, and 10% ethanol. Doses are expressed in terms of the forms listed above in mg/kg body weight. Drugs were administered s.c. in the back.

Data Analyses. Discrimination data are expressed as the average percentage of total responses emitted on the midazolam lever ± 1 S.E.M. and plotted as a function of dose. Control response rates were determined when monkeys received only saline or sham injections and satisfied the testing criteria during each cycle of the session. A mean response rate for each training session was calculated by averaging response rates for each cycle; control response rates represent the average of the means for 5 training sessions. Once an individual monkey responded at least 80% on the midazolam lever during drug test sessions, cumulative dosing stopped and a percentage of 100 was used in the analyses for larger doses. Effects of positive modulators administered in the absence of flumazenil were doubly determined and averaged for individual monkeys before the mean of the group was obtained.

ED_{50} values were estimated by fitting straight lines to individual dose-effect curves for each monkey; each line was estimated by linear regression using one dose that produced not more than 25% responding on the drug lever, one dose that produced at least 75% responding on the drug lever, and all doses in between. Potency ratios were calculated for each animal by dividing ED_{50} values obtained after administration of flumazenil by ED_{50} values obtained after administration of vehicle. A significant change in the potency of a positive modulator or combination of positive modulators was detected when the 95% confidence intervals of the potency ratios averaged among monkeys did not include 1. When flumazenil was studied with

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

either 1 or 2 benzodiazepines, dose ratios were also used to construct Schild plots. Using the method of Arunlakshana and Schild (1959), log (dose ratio-1) was determined for individual monkeys, averaged, and plotted as a function of flumazenil dose (–log [mol/kg]). Straight lines were fitted to each Schild plot using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) and the following equation: log (dose ratio-1) = -log(molar dose of flumazenil)*slope + intercept. Schild plots generated using midazolam and flunitrazepam either alone or in combination were compared using an F-ratio test (Kenakin, 1993; Koek et al., 2000). The slope of the Schild plot was constrained to unity in the simpler model and allowed to vary in the more complex model. If the calculated F value was not significant, then the data were best fit by the simpler model (i.e., slope constrained to -1). When slopes of Schild plots were constrained to unity, apparent pA_2 values were compared using an F-ratio test. When slopes were not constrained, apparent pA_2 values (95% CI) were determined using linear regression of Schild plots. Significance was set at $P<0.05$.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Among the four monkeys that received combinations of midazolam and flunitrazepam (subjects RO, SA, LI and NI), the group mean response rate (mean ± 1 S.E.M.) obtained in the absence of drug was 2.53 ± 0.32 responses/s, and among the four monkeys that received combinations of midazolam and pregnanolone (subjects HE, SA, LI and NI), the group mean response rate was 2.62 ± 0.24 responses/s. Mean response rates were lower following administration of the training dose of midazolam: 1.47 ± 0.15 responses/s in the first group and 1.44 ± 0.51 responses/s in the second. When monkeys received saline, they responded on the saline-appropriate lever and response rates were similar to control rates (data not shown). Midazolam, flunitrazepam and pregnanolone dose-dependently increased responding on the midazolam-appropriate lever (left panel, Figure 1). When midazolam and flunitrazepam were administered alone (subjects RO, SA, LI, and NI), ED_{50} values (mean ± 1 S.E.M.) were 0.097 ± 0.028 mg/kg and 0.12 ± 0.03 mg/kg, respectively; when midazolam and pregnanolone were administered alone (subjects HE, SA, LI, and NI), ED_{50} values were 0.051 ± 0.011 mg/kg and 3.45 ± 0.49 mg/kg, respectively. To study the combined effects of drugs, multiples of these ED_{50} values were administered. The smallest dose combination of midazolam and flunitrazepam (0.013 mg/kg and 0.015 mg/kg, respectively, which were 1/8 of their ED_{50} values) produced 1.4% responding on the midazolam lever; as the dose doubled on each subsequent cycle, responding on the midazolam lever increased and was ≥80% when the ED_{50} values were administered concurrently (upright triangles, right panel, Figure 1). The dose combination that was estimated to produce 50% midazolam-lever responding was 0.042 ± 0.010 mg/kg of midazolam together with 0.051 ± 0.012 mg/kg of flunitrazepam. Similarly, the smallest dose combination of midazolam and pregnanolone studied (0.013 mg/kg and 0.86 mg/kg,

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

respectively) was ¼ of their ED_{50} values and produced 4.4% responding on the midazolam lever; as the dose doubled on each subsequent cycle, responding on the midazolam lever increased, with concurrent administration of the ED_{50} values resulting in ≥80% drug-lever responding (inverted triangles, right panel, Figure 1). The dose combination that was estimated to produce 50% midazolam-lever responding was 0.023 ± 0.003 mg/kg of midazolam together with 1.54 ± 0.23 mg/kg of pregnanolone. The effects of positive $GABA_A$ modulators, administered alone or in combination, on rate of lever pressing were similar to those obtained with the training dose of midazolam; doses producing ≥80% drug-lever responding decreased response rates to <60% of control rates (data not shown).

Flumazenil dose dependently antagonized the discriminative stimulus effects of midazolam and flunitrazepam, whether the benzodiazepines were administered alone or together. Doses of 0.01, 0.032 and 0.1 mg/kg of flumazenil shifted the midazolam dose-effect curve 2.3-, 6.5- and 16.5-fold to the right, respectively (data not shown) and shifted the flunitrazepam dose-effect curve 1.9-, 6.4- and 22.8-fold, respectively (Figure 2). When midazolam and flunitrazepam were given concurrently, flumazenil also produced orderly, rightward shifts in the dose-effect curve (2.3-, 4.8-, 18.5-fold, respectively; Figure 3). Schild plots were constructed from data obtained when flumazenil was studied with midazolam alone, flunitrazepam alone, or a combination of midazolam and flunitrazepam (Figure 4); straight lines fitted to the data indicated that slopes [$F(2,27)=0.806$, n.s.] and intercepts [$F(2,29)=0.082$, n.s.] were not significantly different among the three Schild plots. Moreover, the common slope was not different from unity [$F(1,29)=1.134$, n.s.]. Thus, the simplest model that could be used to fit the data obtained when flumazenil was studied with midazolam, flunitrazepam or a combination of midazolam and flunitrazepam was one with a common slope and a common intercept. With the

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

common slope constrained to unity, the apparent pA_2 value was 7.58. When the three Schild plots were analyzed independently, the unconstrained slope of each line was not significantly different from unity yielding apparent pA_2 values that were also not significantly different (Table 1).

Although flumazenil antagonized the discriminative stimulus effects of benzodiazepines, it enhanced the discriminative stimulus effects of pregnanolone, shifting the pregnanolone dose-effect curve to the left (Figure 5). Larger doses of flumazenil were needed to shift the pregnanolone dose-effect curve leftward, as compared with doses that shifted the benzodiazepine dose-effect curves rightward (Figures 2 and 3). Mean potency ratios (95% CI) obtained following administration of 0.032, 0.1 and 0.32 mg/kg of flumazenil were 0.85 (0.42, 1.29), 0.66 (0.07, 1.24), 0.48 (0.27, 0.69); the largest dose of flumazenil produced a significant (2-fold) increase in the potency of pregnanolone.

A dose of 0.32 mg/kg of flumazenil, which significantly enhanced the effects of pregnanolone when it was administered alone, attenuated the combined effects of midazolam and pregnanolone (Figure 6). Mean dose ratios (95% CI) obtained following administration of 0.1, 0.32 and 1 mg/kg of flumazenil were 1.28 (0.51, 2.05), 1.97 (1.44, 2.49), and 1.31 (0.59, 2.02). Although a dose of 0.32 mg/kg of flumazenil significantly decreased the potency of the combination of midazolam and pregnanolone, this effect was not dose dependent.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

Benzodiazepines have high positive efficacy and act at the benzodiazepine binding site on $GABA_A$ receptors. When two benzodiazepines are given together, their discriminative stimulus effects are additive in monkeys (current study; McMahon and France, 2005). A different type of interaction occurs when a benzodiazepine is given with flumazenil, which has low positive efficacy and acts at the benzodiazepine binding site. Flumazenil antagonizes the discriminative stimulus effects of benzodiazepines, producing orderly, rightward shifts in dose-effect curves. Schild analyses of these antagonism data yield slopes that are not different from unity and are consistent with a simple, competitive and reversible interaction. The pA_2 values, which indicate the potency of flumazenil to antagonize benzodiazepines, has been determined for flumazenil with several different benzodiazepines in monkeys discriminating midazolam; pA_2 values range from 7.41 to 7.69 (Lelas et al., 1999; 2000; McMahon et al., 2002), suggesting that the same population of receptors mediates the discriminative stimulus effects of all benzodiazepines studied to date. When flumazenil is examined with midazolam and with flunitrazepam, results are similar to previous data with slopes not different from unity and pA_2 values for flumazenil within the range of values obtained previously (pA_2=7.59 with flunitrazepam; pA_2=7.60 with midazolam). Thus, the potency of flumazenil in antagonizing various benzodiazepines is remarkably consistent across a wide range of conditions (Lelas et al., 1999; 2000; McMahon et al., 2002).

Given the similarities in the potency of flumazenil to antagonize benzodiazepines across many conditions, it might be expected that its potency would be the same when two benzodiazepines are given together. Consistent with that hypothesis, flumazenil antagonizes the discriminative stimulus effects of a combination of midazolam and flunitrazepam, yielding pA_2

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

values that are not different from those obtained when flumazenil is studied with a single benzodiazepine. These results further demonstrate the similarity in the potency of flumazenil across a range of drugs and conditions.

There are multiple modulatory sites on $GABA_A$ receptors, and positive modulators acting at several of these sites can produce midazolam-lever responding in monkeys. Moreover, concurrent administration of high-efficacy positive modulators acting at different sites results in additive effects between drugs (current study; McMahon and France, 2005). Thus, when high-efficacy drugs are administered together, their combined effects are qualitatively the same, regardless of which modulatory site mediates those effects. One way that actions at different sites can be detected is to study high-efficacy drugs with a low-efficacy drug that acts at one of the two sites (e.g., flumazenil). Under these conditions, flumazenil is expected to attenuate the effects of drugs acting at the same (e.g., benzodiazepine) site and should not alter the effects of drugs acting at different sites. Flumazenil enhances the discriminative stimulus effects of pregnanolone, shifting its dose-effect curve leftward and confirming that flumazenil has some positive efficacy (Dantzer and Pério, 1982).

Given that interactions between flumazenil and positive modulators vary according to the type of positive modulator being studied, antagonism by flumazenil should be less for a combination of midazolam and pregnanolone, as compared with antagonism of midazolam alone or of a combination of midazolam and flunitrazepam. For example, 0.1 mg/kg of flumazenil shifts a benzodiazepine dose-effect curve 17- to 23-fold rightward and does not alter the pregnanolone dose-effect curve. This dose of flumazenil might be expected to attenuate the combination of midazolam and pregnanolone; however, it does not change their combined discriminative stimulus effects (Figure 6). Although flumazenil is less potent in antagonizing a

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

combination of midazolam and pregnanolone, as compared to its potency in antagonizing benzodiazepines, it can attenuate the combined effects of midazolam and pregnanolone with 0.32 mg/kg producing a small, rightward shift. Moreover, flumazenil fully antagonizes the effects of midazolam in the mixture, as evidenced by similar ED_{50} values for pregnanolone when it is administered alone (3.45 ± 0.49 mg/kg), as compared to the ED_{50} value for pregnanolone when it is given in a mixture with midazolam and that mixture is maximally shifted by flumazenil (3.08 ± 0.71 mg/kg). Thus, even under conditions where flumazenil enhances the effects of one component of the mixture, a combination of a benzodiazepine and a neuroactive steroid can be attenuated by flumazenil.

One possible strategy for retaining the therapeutic effects of benzodiazepines while reducing their adverse effects is to administer benzodiazepines and neuroactive steroids together. This strategy would allow smaller doses of each drug to be used, which might decrease adverse effects, such as the development of tolerance. The consequences of repeated daily treatment are different for benzodiazepines, as compared with neuroactive steroids. During chronic benzodiazepine treatment, tolerance develops readily to many effects of benzodiazepines, including decreases in lever pressing (McMahon and France, 2002b) and anticonvulsant effects (Gonsalves and Gallagher, 1987; Löscher et al., 1996), although cross tolerance does not develop to decreases in lever pressing produced by neuroactive steroids (Gerak, 2009). During chronic treatment with neuroactive steroids, tolerance can develop to some (e.g., decreases in locomotor activity, Marshall et al.,1997; impairment in Morris Water Maze, Türkmen et al., 2006) but not all (e.g., decreases in lever pressing, McMahon and France, 2002a; anticonvulsant effects, Kokate et al., 1998; Reddy and Rogawski, 2000) effects of neuroactive steroids. Thus, giving smaller doses of benzodiazepines and neuroactive steroids together, as compared with doses of

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

either drug that would be needed when administered alone, might decrease the likelihood of tolerance and cross tolerance developing. In addition, the proportion of each drug in the mixture might be titrated to optimize the therapeutic effects while minimizing the possibility that tolerance develops.

Unlike the development of tolerance and dependence, some undesired effects are common among positive modulators, regardless of the binding site that mediates their effects, and would be expected to occur when they are administered in combination. For example, positive modulators decrease ventilation and they can produce severe respiratory depression when administered together (e.g., benzodiazepines and ethanol). While these effects will only occur when large doses are administered concurrently, it is important to know whether the effects could be reversed in case of overdose. In the current study, flumazenil antagonizes the effects of a benzodiazepine given alone and with another benzodiazepine. Although flumazenil enhances the effects of pregnanolone administered alone, it does not enhance the effects of a combination of midazolam and pregnanolone and modestly attenuates their combined effects at a dose of 0.32 mg/kg. It is not clear whether a combination of benzodiazepines and neuroactive steroids would produce life-threatening adverse effects; however, flumazenil might be useful in reducing toxicity caused by the combination. Moreover, because one component of the mixture, the neuroactive steroid, will be resistant to antagonism by flumazenil, the proportion of each component in the mixture could be adjusted to obtain the most ideal combination of a benzodiazepine and a neuroactive steroid; this proportion would decrease the likelihood that tolerance and dependence develop to the mixture while retaining the ability to attenuate the effects in case of overdose.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

The generality of these findings to other dependent variables needs to be determined empirically; however, drug discrimination seems to be predictive of effects across a wide range of conditions. For example, many, if not all, effects of benzodiazepines are mediated by benzodiazepine sites on $GABA_A$ receptors, including their discriminative stimulus, therapeutic and adverse effects. While absolute potency of drugs acting at benzodiazepine sites can vary across procedures, relative potencies do not change (Lelas et al., 1999; Rowlett et al., 2006). Drug discrimination has been used extensively to examine interactions between $GABA_A$ modulators that vary in efficacy and site of action on $GABA_A$ receptors (current study; Lelas et al., 2000; McMahon and France, 2005; Gerak and France, 2011), providing an empirical and theoretical framework against which results could be compared and demonstrating that drug discrimination studies are highly reliable and predictable. Finally, under the conditions used in the current study, additive interactions have been observed between positive modulators acting at different sites, and flumazenil has been shown to enhance the effects of pregnanolone, providing a baseline against which the relative enhancement or attenuation of the combined effects of positive modulators can be compared. Consequently, there appears to be little evidence to suggest that results would be different with other dependent variables and a number of reasons to believe that the discriminative stimulus effects of positive $GABA_A$ modulators will be predictive of other effects.

In summary, the ability of flumazenil to antagonize benzodiazepines administered concurrently could not be distinguished from its ability to antagonize a single benzodiazepine. Moreover, despite qualitative differences in the interaction between flumazenil and either benzodiazepines or neuroactive steroids, flumazenil attenuated the discriminative stimulus effects of a combination of midazolam and pregnanolone, although the magnitude of antagonism

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

was less for the combination, as compared with antagonism of midazolam alone. While different interactions with flumazenil might have been observed had different proportions of midazolam and pregnanolone been studied (Fischer and Rowlett, 2011), the data obtained to date support the view that combinations of benzodiazepines and neuroactive steroids could be useful in retaining the therapeutic effects of benzodiazepines and reducing their individual adverse effects while at least partially retaining the ability to reverse accidental overdose.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors wish to thank B. Harrington, A. Hernandez, M. Hernandez, D. Logan, B. Taylor, and J. Wallis for their excellent technical assistance.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Author Contributions

Participated in research design: Gerak and France

Conducted experiments:

Contributed new reagents or analytic tools:

Performed data analyses: Gerak

Wrote or contributed to the writing of the manuscript: Gerak and France

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

References

Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. *Br J Pharmacol* **14**: 48-58.

Dantzer R and Pério A (1982) Behavioural evidence for partial agonist properties of RO 15-1788, a benzodiazepine receptor antagonist. *Eur J Pharmacol* **81**: 655-658.

Eppolito AK and Gerak LR (2010) Tolerance to the rate-increasing and not rate-decreasing effects of pregnanolone in rats. *Behav Pharmacol* **21**: 736-744.

Finn DA, Gallaher EJ, and Crabbe JC (2000) Differential change in neuroactive steroid sensitivity during ethanol withdrawal. *J Pharmacol Exp Ther* **292**: 394-405.

Fischer BD and Rowlett JK (2011) Anticonflict and reinforcing effects of triazolam + pregnanolone combinations in rhesus monkeys *J Pharmacol Exp Ther* **337**: 805-811.

Gasior M, Ungard JT, Beekman M, Carter RB, and Witkin JM (2000) Acute and chronic effects of the synthetic neuroactive steroid, ganaxolone, against the convulsive and lethal effects of pentylenetetrazol in seizure-kindled mice: comparison with diazepam and valproate. *Neuropharmacology* **39**: 1184-1196.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Gerak LR (2009) Selective changes in sensitivity to benzodiazepines, and not other positive $GABA_A$ modulators, in rats receiving flunitrazepam chronically. *Psychopharmacology* **204**: 667-677.

Gerak LR and France CP (2011) Chronic benzodiazepine treatment does not alter interactions between positive $GABA_A$ modulators and flumazenil or pentylenetetrazole in monkeys. *Behav Pharmacol* **22**: 49-57.

Gonsalves SF and Gallagher DW (1987) Time course for development of anticonvulsant tolerance and GABAergic subsensitivity after chronic diazepam. *Brain Res* **405**: 94-99.

Kenakin T (1993) *Pharmacologic analysis of drug-receptor interaction*, 2nd ed. Raven Press, New York

Koek W, Assié MB, Zernig G, and France CP (2000) In vivo estimates of efficacy at $5\text{-}HT_{1A}$ receptors: effects of EEDQ on the ability of agonists to produce lower-lip retraction in rats. *Psychopharmacology* **149**: 377-387.

Kokate TG, Svensson BE, and Rogawski MA (1994) Anticonvulsant activity of neurosteroids: correlation with gamma-aminobutyric acid-evoked chloride current potentiation. *J Pharmacol Exp Ther* **270**: 1223-1229.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Kokate TG, Yamaguchi S, Pannell LK, Rajamani U, Carroll DM, Grossman AB, and Rogawski MA (1998) Lack of anticonvulsant tolerance to the neuroactive steroid pregnanolone in mice. J *Pharmacol Exp Ther* **287**: 553-558.

Lancel M (1999) Role of $GABA_A$ receptors in the regulation of sleep: initial sleep responses to peripherally administered modulators and agonists. *Sleep* **22**: 33-42.

Lelas S, Gerak LR, and France CP (1999) Discriminative-stimulus effects of triazolam and midazolam in rhesus monkeys. *Behav Pharmacol* **10**: 39-50.

Lelas S, Gerak LR, and France CP (2000). Antagonism of the discriminative stimulus effects of positive γ-aminobutyric $acid_A$ modulators in rhesus monkeys discriminating midazolam. *J Pharmacol Exp Ther* **294**: 902-908.

Löscher W, Rundfeldt C, Hönack D, and Ebert U (1996) Long-term studies on anticonvulsant tolerance and withdrawal characteristics of benzodiazepine receptor ligands in different seizure models in mice. I. Comparison of diazepam, clonazepam, clobazam and abecarnil. *J Pharmacol Exp Ther* **279**: 561-572.

Marshall FH, Stratton SC, Mullings J, Ford E, Worton SP, Oakley NR and Hagan RM (1997) Development of tolerance in mice to the sedative effects of the neuroactive steroid minaxolone following chronic exposure. *Pharmacol Biochem Behav* **58**: 1-8.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

McMahon LR and France CP (2002a) Acute and chronic effects of the neuroactive steroid pregnanolone on schedule-controlled responding in rhesus monkeys. *Behav Pharmacol* **13**: 545-555.

McMahon LR and France CP (2002b) Daily treatment with diazepam differentially modifies sensitivity to the effects of γ-aminobutyric $acid_A$ modulators on schedule-controlled responding in rhesus monkeys. *J Pharmacol Exp Ther* **300**: 1017-1025.

McMahon LR, Gerak LR, Carter L, Ma C, Cook JM, and France CP (2002) Discriminative stimulus effects of benzodiazepine (BZ1) receptor-selective ligands in rhesus monkeys. *J Pharmacol Exp Ther* **300**: 505-512.

McMahon LR and France CP (2005) Combined discriminative stimulus effects of midazolam with other positive $GABA_A$ modulators and $GABA_A$ receptor agonists in rhesus monkeys. *Psychopharmacology* **178**: 400-409.

Reddy DS and Rogawski MA (2000) Chronic treatment with the neuroactive steroid ganaxolone in the rat induces anticonvulsant tolerance to diazepam but not to itself. *J Pharmacol Exp Ther* **295**: 1241-1248.

Reddy DS and Rogawski MA (2001) Enhanced anticonvulsant activity of neuroactive steroids in a rat model of catamenial epilepsy. *Epilepsia* **42**: 337-344.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Rowlett JK, Lelas S, Tornatzky W, Licata SC (2006) Anti-conflict effects of benzodiazepines in rhesus monkeys: relationship with therapeutic doses in humans and role of $GABA_A$ receptors. *Psychopharmacology* **184**: 201-211.

Türkmen S, Löfgren M, Birzniece V, Bäckström T, and Johansson IM (2006) Tolerance development to Morris water maze test impairments induced by acute allopregnanolone. *Neuroscience* **139**: 651-659.

Vanover KE, Suruki M, Robledo S, Huber M, Wieland S, Lan NC, Gee KW, Wood PL, and Carter RB (1999). Positive allosteric modulators of the $GABA_A$ receptor: differential interaction of benzodiazepines and neuroactive steroids with ethanol. *Psychopharmacology* **141**: 77-82.

Wieland S, Belluzzi J, Hawkinson JE, Hogenkamp D, Upasani R, Stein L, Wood PL, Gee KW, and Lan NC (1997). Anxiolytic and anticonvulsant activity of a synthetic neuroactive steroid Co 3-0593. *Psychopharmacology* **134**: 46-54.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

The project described was supported by the National Institutes of Health National Institute on Drug Abuse [Grant DA009157] and a Senior Scientist Award [Grant K05 DA17918 (CPF)]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute on Drug Abuse or the National Institutes of Health.

Reprint requests: Lisa R. Gerak

Department of Pharmacology

University of Texas Health Science Center

7703 Floyd Curl Dr, mail code 7764

San Antonio, TX 78229

Email: gerak@uthscsa.edu

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Figure Legends

Figure 1. Discriminative stimulus effects midazolam, flunitrazepam and pregnanolone administered alone (left panel) and midazolam administered in combination with flunitrazepam or pregnanolone (right panel). Each dose-effect curve represents data from 4 monkeys; dose-effect curves shown in this figure for midazolam, flunitrazepam and a combination of midazolam and flunitrazepam were determined in subjects RO, SA, LI and NI and dose-effect curves for pregnanolone and a combination of midazolam and pregnanolone were determined in subjects HE, SA, LI and NI. Abscissa, left panel: dose in milligrams per kilogram body weight; right panel: dose expressed as multiples of ED_{50} values which are denoted by x. In monkeys that received combinations of midazolam and flunitrazepam, the ED_{50} value for midazolam when it was administered alone was 0.097 mg/kg and the ED_{50} for flunitrazepam alone was 0.12 mg/kg. In monkeys that received combinations of midazolam and pregnanolone, the ED_{50} value for midazolam when it was studied alone was 0.051 mg/kg and the ED_{50} for flunitrazepam alone was 3.45 mg/kg. Data above V represent the effects of vehicle. Ordinates: mean (± 1 SEM) percentage of total responding that occurred on the midazolam-appropriate lever.

Figure 2. Discriminative stimulus effects of flunitrazepam administered alone (circles) or following administration of various doses of flumazenil in 3 monkeys (subjects SA, LI, and NI). Abscissa: dose in milligrams per kilogram body weight; data above V represent the effects of vehicle. Ordinate: mean (± 1 SEM) percentage of total responding that occurred on the midazolam-appropriate lever.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3. Discriminative stimulus effects of a combination of midazolam and flunitrazepam following administration of vehicle (circles) or various doses of flumazenil in 4 monkeys (subjects RO, SA, LI and NI). Abscissa: dose in milligrams per kilogram body weight with the top label on the abscissa indicating the dose of midazolam and the bottom label indicating the dose of flunitrazepam; data above V represent the effects of vehicle. Doses of midazolam and flunitrazepam were multiples of ED_{50} values obtained when that drug was studied alone; those values were 0.097 mg/kg for midazolam and 0.12 mg/kg for flunitrazepam. Ordinate: mean (± 1 SEM) percentage of total responding that occurred on the midazolam-appropriate lever.

Figure 4. Schild plots for flumazenil studied with midazolam, flunitrazepam or a combination of the two benzodiazepines. Raw data are shown in Figure 2 (flumazenil with flunitrazepam) and Figure 3 (flumazenil with the combination of midazolam and flunitrazepam) or not shown (flumazenil with midazolam). Abscissa: negative log of the molar dose of flumazenil. Ordinate: log (dose-ratio – 1).

Figure 5. Discriminative stimulus effects of pregnanolone administered alone (circles) or following administration of various doses of flumazenil in 4 monkeys (subjects HE, SA, LI and NI). Abscissa: dose in milligrams per kilogram body weight; data above V represent the effects of vehicle. Ordinate: mean (± 1 SEM) percentage of total responding that occurred on the midazolam-appropriate lever.

Figure 6. Discriminative stimulus effects of a combination of midazolam and pregnanolone following administration of vehicle (circles) or various doses of flumazenil in 4 monkeys

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

(subjects HE, SA, LI and NI). Abscissa: dose in milligrams per kilogram body weight with the top label on the abscissa indicating the dose of midazolam and the bottom label indicating the dose of pregnanolone; data above V represent the effects of vehicle. Doses of midazolam and pregnanolone were multiples of ED_{50} values obtained when that drug was studied alone; those values were 0.051 mg/kg for midazolam and 3.45 mg/kg for pregnanolone. Ordinate: mean (± 1 SEM) percentage of total responding that occurred on the midazolam-appropriate lever.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

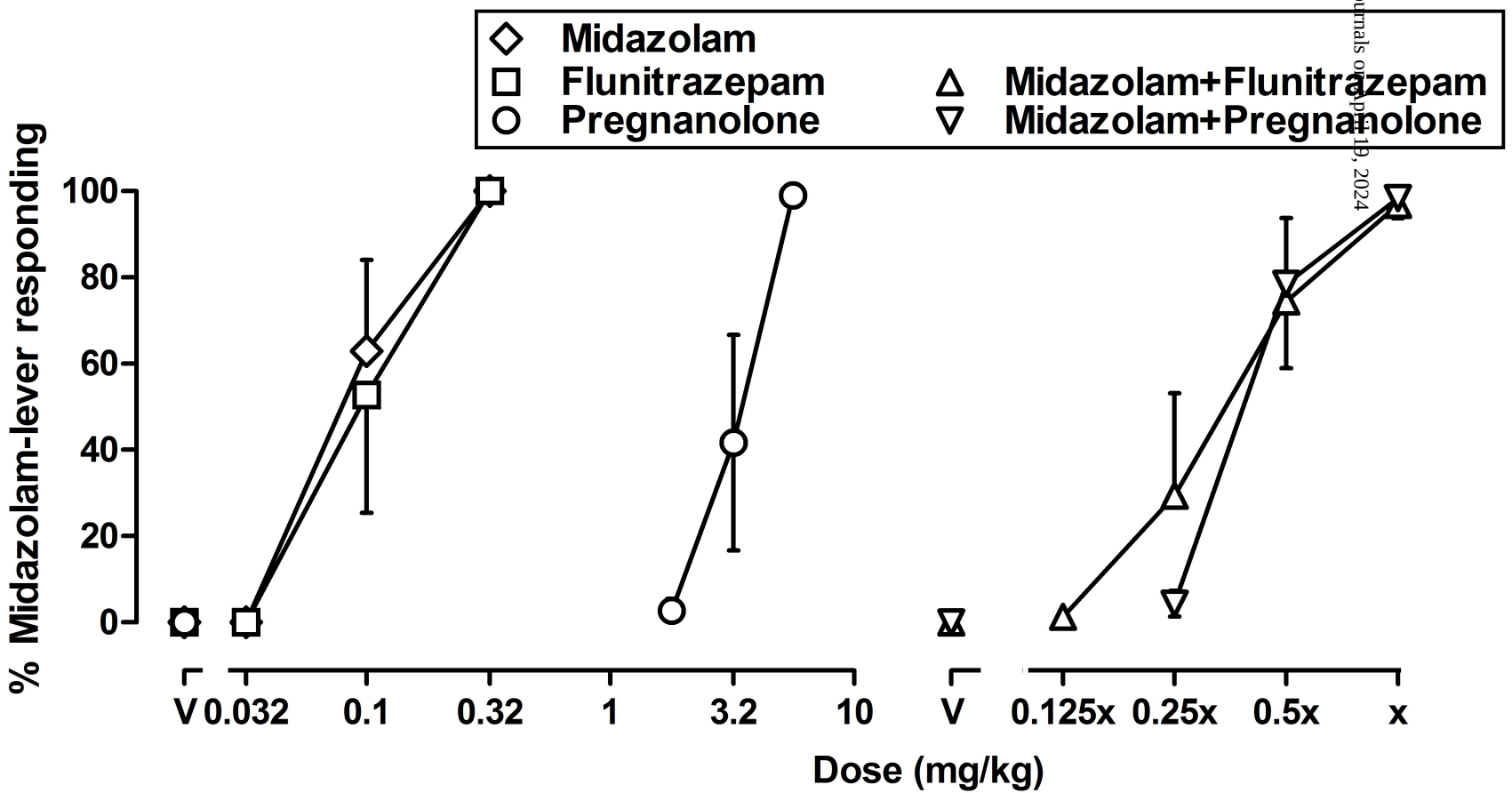
JPET Fast Forward. Published on December 15, 2011 as DOI: 10.1124/jpet.111.188250
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Slopes of Schild plots and pA_2 values (95% confidence limits) of flumazenil determined with constrained and unconstrained slopes.

	Slope (unconstrained)	pA_2 (unconstrained)	pA_2 (constrained)
Midazolam	-1.02 (-1.43, -0.61)	7.59 (7.36, 8.05)	7.60 (7.44, 7.76)
Flunitrazepam	-1.36 (-1.88, -0.84)	7.43 (7.24, 7.77)	7.59 (7.36, 7.82)
Midazolam+Flunitrazepam	-1.04 (-1.48, -0.60)	7.54 (7.31, 7.99)	7.56 (7.39, 7.73)

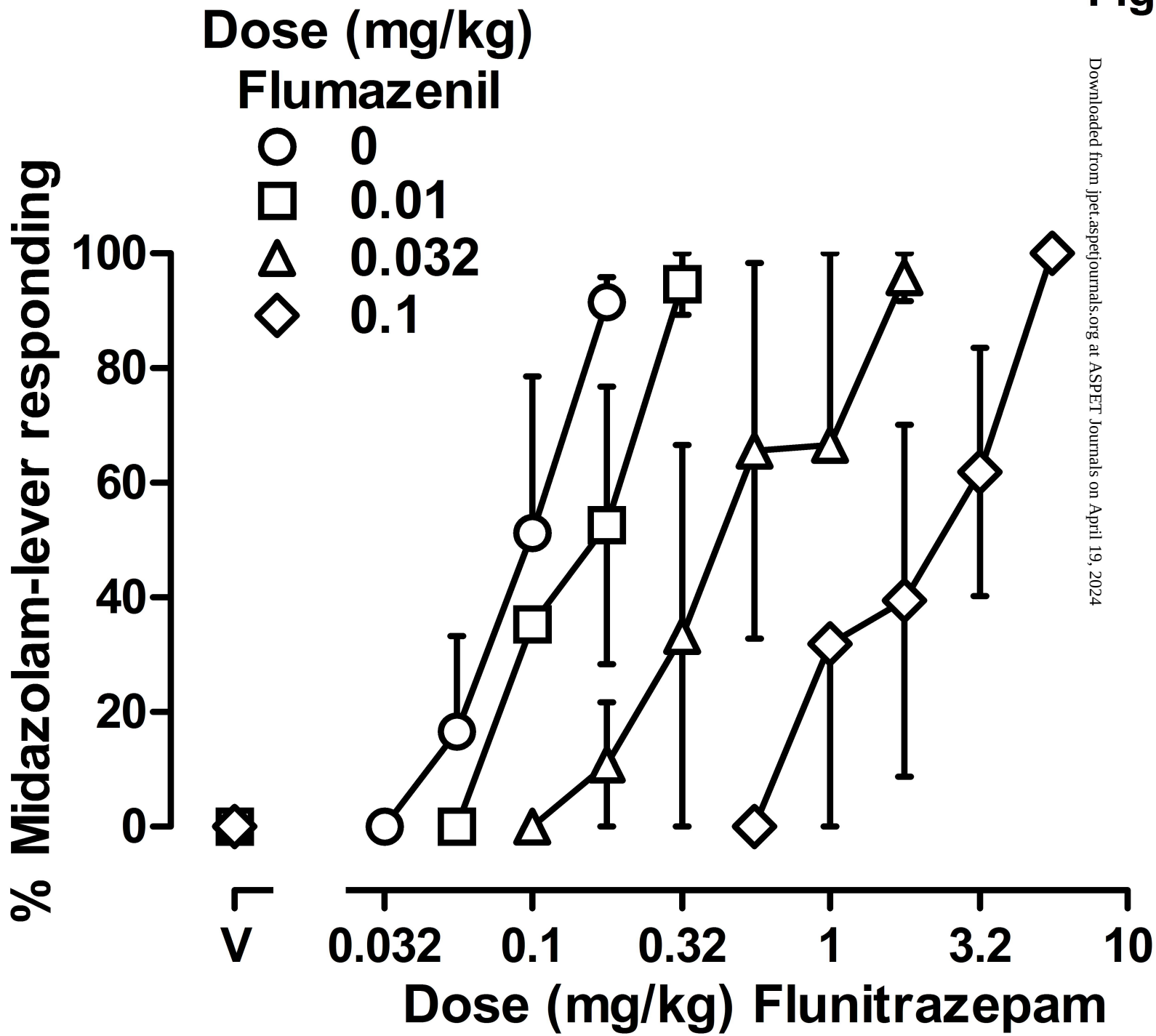
Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 1



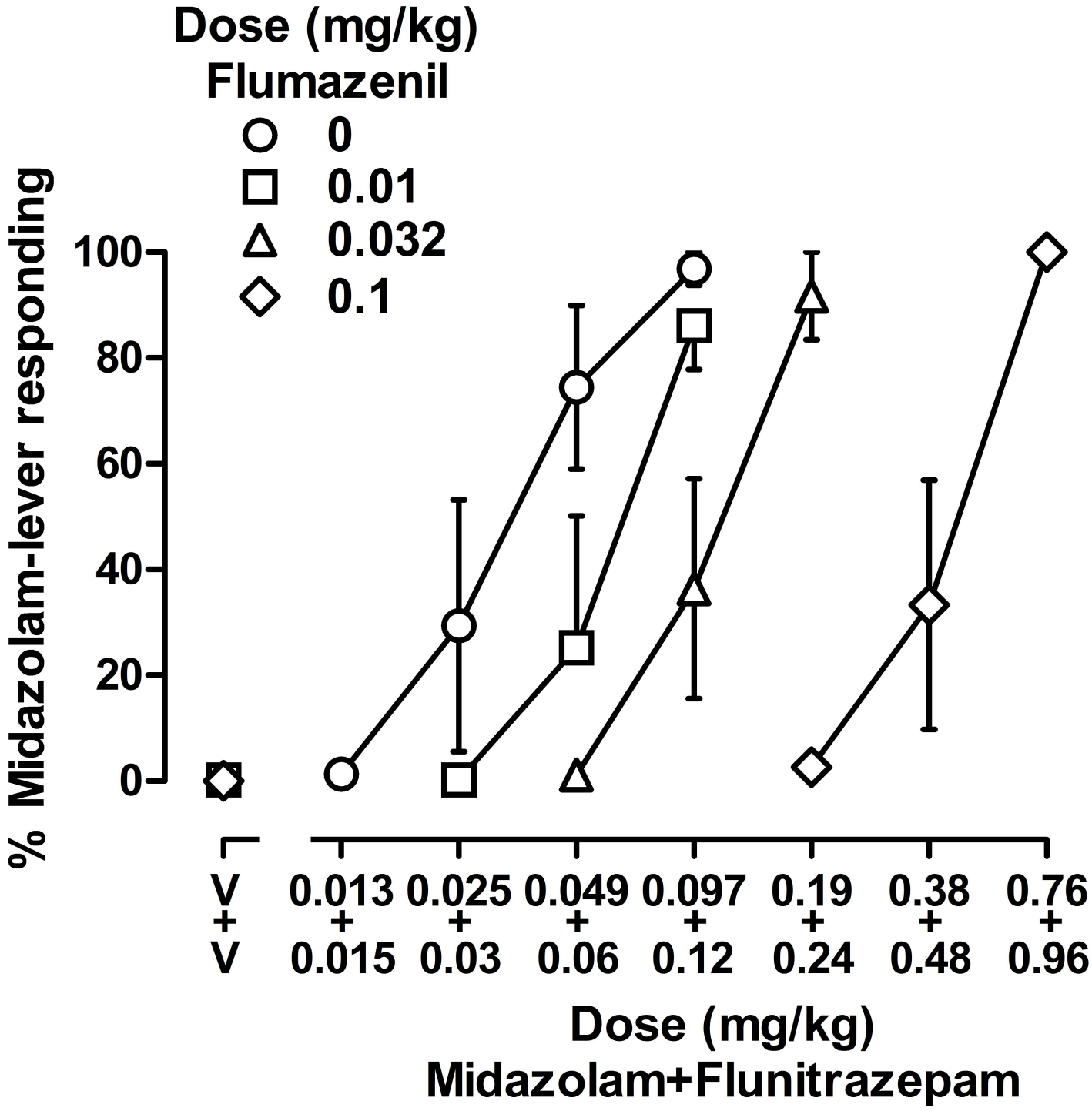
jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 2



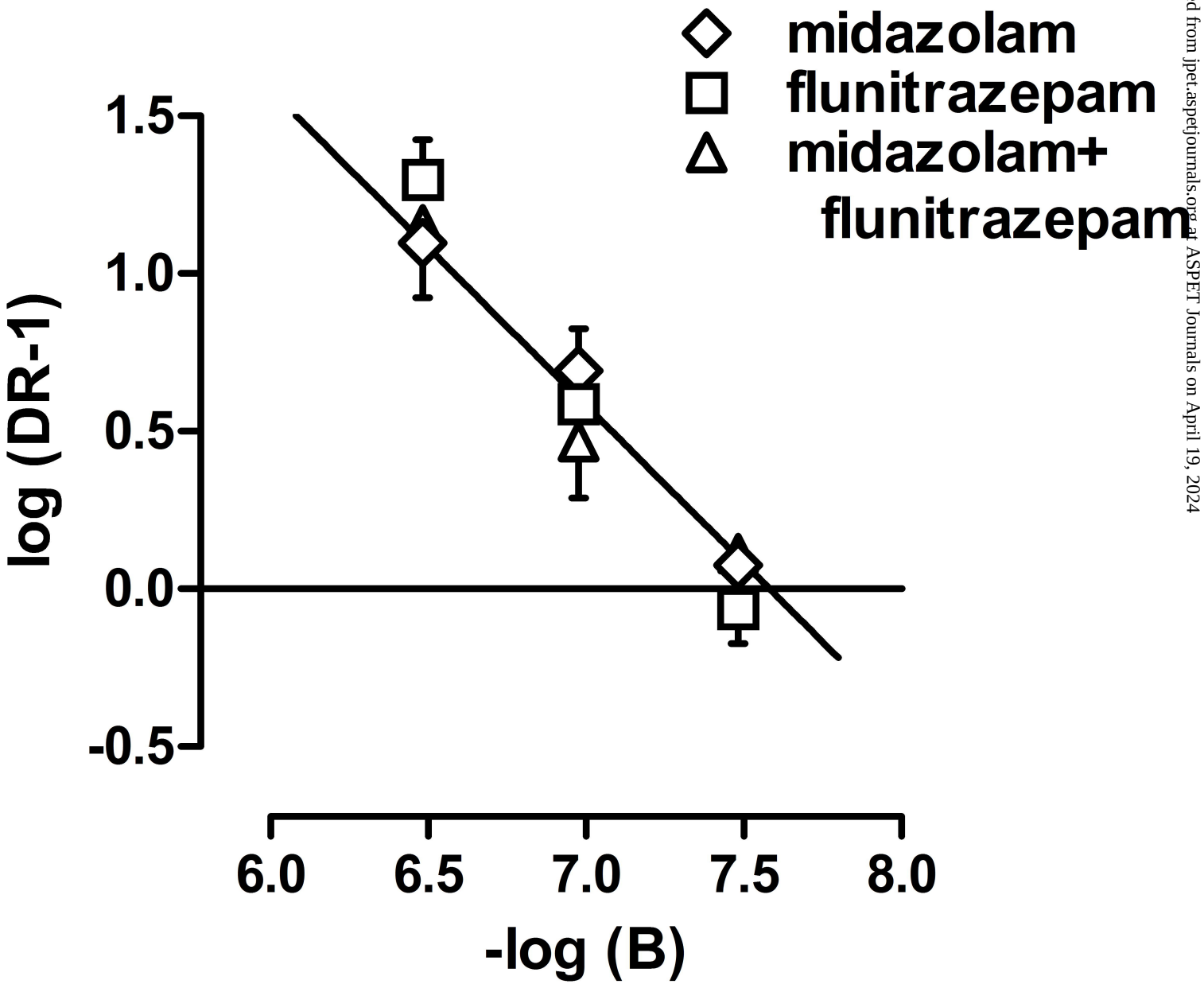
Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 3



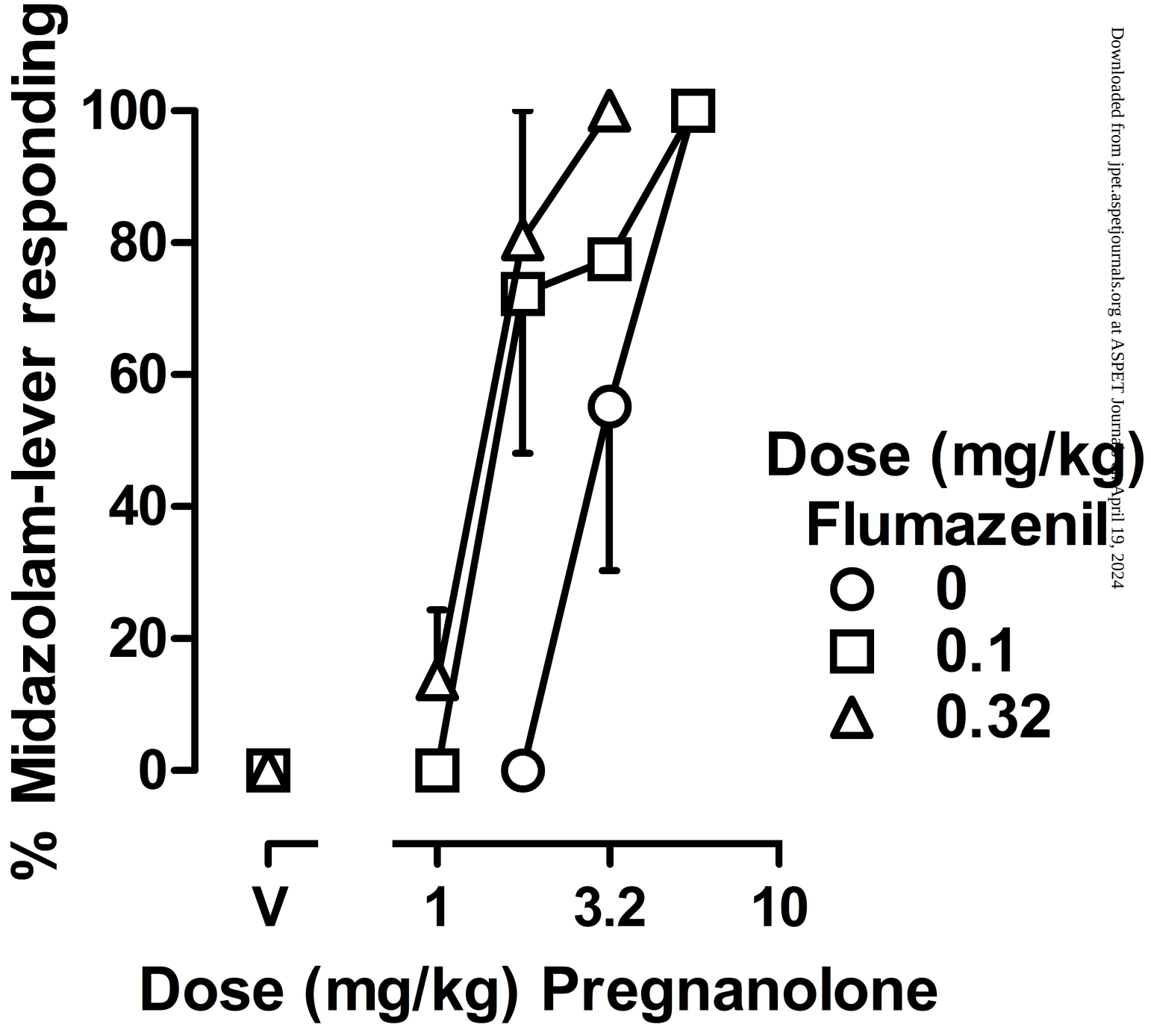
Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 4



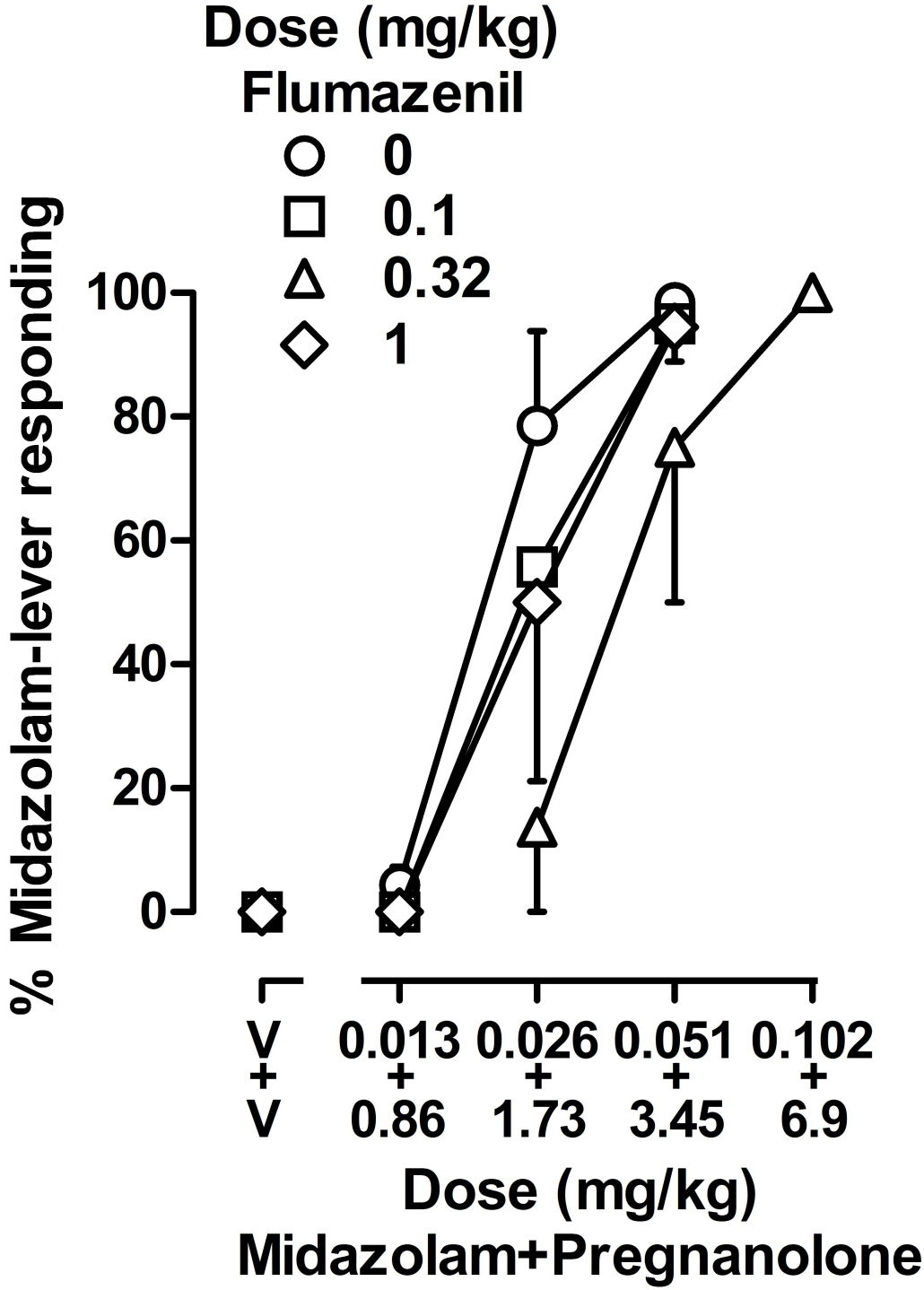
Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 5



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

Figure 6



Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024