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Differential behavioral effects of low efficacy positive $GABA_A$ modulators in combination with benzodiazepines and a neuroactive steroid in rhesus monkeys

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1 In the clinic, low efficacy positive $GABA_A$ modulators might be preferred to high efficacy positive modulators insofar as low efficacy modulators might have comparatively less abuse and dependence liability.

2 Drug discrimination was used to examine the behavioral effects of L-838,417 and bretazenil, two low efficacy positive $GABA_A$ modulators that act at benzodiazepine sites, alone and in combination with benzodiazepines and a neuroactive steroid (alfaxolone). In rhesus monkeys ($n=5$) discriminating midazolam, alfaxolone substituted for midazolam. In four monkeys, L-838,417 and bretazenil did not substitute for, but rather dose-dependently antagonized, midazolam; L-838-417 and bretazenil, as well as flumazenil, enhanced the midazolam-like effects of alfaxolone. L-838,417 and bretazenil substituted for midazolam in a fifth monkey. In a separate group of rhesus monkeys ($n=3$) that received 5.6 mg kg^{-1} per day of diazepam and that discriminated flumazenil, L-838,417 and bretazenil substituted for flumazenil.

3 These results demonstrate that L-838,417, bretazenil, and flumazenil can have agonist or antagonist actions in the same animal depending upon whether they are studied in combination with a higher efficacy positive $GABA_A$ modulator acting at the same (benzodiazepine) or a different (neuroactive steroid) site. Thus, combinations of low efficacy positive modulators acting at different sites on the $GABA_A$ receptor complex could yield drug mixtures with significant therapeutic effects and with reduced abuse and dependence liability, as compared to higher efficacy positive modulators such as currently available benzodiazepines.
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Abbreviations: CL, confidence limit; FR, fixed ratio; GABA, γ-aminobutyric acid

Introduction

Benzodiazepines that positively modulate $GABA_A$-mediated Cl^- flux (i.e. positive $GABA_A$ modulators) have diverse therapeutic effects including anxiolytic, hypnotic, and anticonvulsant effects, and are used widely in medicine, especially for the treatment of anxiety (Rickels & Rynn, 2002). Despite their therapeutic utility and safety, relative to other positive $GABA_A$ modulators (e.g. barbiturates), benzodiazepines can impair memory and motor function, can have abuse liability and, when used repeatedly, can result in tolerance and dependence (Woods *et al*., 1987; Griffiths & Weerts, 1997). Benzodiazepine dependence can be evidenced by a variety of adverse consequences that emerge upon discontinuation of use including anxiety, insomnia, and, in some cases, convulsions. Treatment of anxiety and other neuropsychiatric disorders involving $GABA_A$ transmission could be improved by the development of ligands that have fewer of the adverse effects of currently available benzodiazepines.

Agonist efficacy is one important determinant of therapeutic effect for drugs from other pharmacologic classes (e.g. opioids) and appears to be important for the therapeutic effects of positive $GABA_A$ modulators (e.g. Bergman *et al*., 2000). Most benzodiazepines in clinical use facilitate $GABA_A$-mediated Cl^- flux with high efficacy, and do so nonselectively at four subtypes of the $GABA_A$ receptor complex that contain different α-subunits (α_1-, α_2-, α_3- and α_5; Smith *et al*., 2001). The unwanted effects of clinically used benzodiazepines might be due to their high efficacy or to their lack of selectivity for $GABA_A$ receptor subtypes. For example, studies that compare responding for food (i.e. unpunished responding) to responding for food and shock (i.e. punished responding) demonstrate that both low and high efficacy positive $GABA_A$ modulators increase punished responding (e.g. have anxiolytic activity). In contrast, at doses that increase punished responding, high efficacy positive $GABA_A$ modulators can also decrease unpunished responding, whereas low efficacy positive $GABA_A$ modulators do not (Kleven & Koek, 1999; Paronis & Bergman, 1999), presumably because higher efficacy is required to decrease responding.

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Drug discrimination is sensitive to differences in positive $GABA_A$ modulation. For example, consistent with it being a low efficacy positive modulator at α_1-, α_2-, α_3- and α_5 subunits (Facklam *et al*., 1992; Smith *et al*., 2001), bretazenil does not substitute for, but rather attenuates, the discriminative stimulus effects of the higher efficacy positive $GABA_A$ modulator midazolam (Lelas *et al*., 1999). Moreover, consistent with it being a low efficacy modulator at α_2-, α_3- and α_5 subunits and a neutral modulator at α_1 subunits (McKernan *et al*., 2000), L-838,417 attenuates the discriminative stimulus effects of the higher efficacy positive $GABA_A$ modulator triazolam (Rowlett *et al*., 2005). Thus, antagonism of high efficacy positive modulatory benzodiazepine-site ligands is consistent with bretazenil and L-838,417 having low efficacy positive modulatory activity, and it is not clear whether these ligands can have agonist activity in these assays.

Three approaches were used in this study to examine the relationship between apparent efficacy and the behavioral effects of bretazenil, L-838,417 and clinically used benzodiazepines. First, L-838,417 and bretazenil were studied alone (i.e. for agonist activity) and in combination with midazolam (i.e. for antagonist activity) in monkeys discriminating midazolam. Second, L-838,417 and bretazenil were studied in diazepam-treated monkeys discriminating flumazenil, a discrimination assay that is especially sensitive to low efficacy positive $GABA_A$ modulators at benzodiazepine sites inasmuch as they substitute for flumazenil (i.e. precipitate diazepam withdrawal). Since bretazenil and L-838,417 had antagonist actions in attenuating the effects of midazolam in untreated monkeys and in substituting for flumazenil in diazepam-treated monkeys, in a third study these drugs were combined with a positive $GABA_A$ modulator (alfaxolone), which does not act at benzodiazepine sites, to test the hypothesis that a low efficacy positive $GABA_A$ modulator at one site enhances the effects of a positive $GABA_A$ modulator acting at another site.

Methods

Subjects

Five adult (four female and one male) rhesus monkeys (*Macaca mulatta*) were used for the midazolam discrimination study, and three adult (one female and two male) rhesus monkeys were used for the flumazenil discrimination study. Monkeys were housed individually on a 14-h light/10-h dark schedule, were maintained at 95% free-feeding weight (range 3.8–11.5 kg) with a diet comprising primate chow (High Protein Monkey Diet, Harlan Teklad, Madison, WI, U.S.A.), fresh fruit, and peanuts, and were provided water in the home cage. Monkeys received $GABA_A$ receptor ligands in previous studies (McMahon & France, 2003; 2005). The animals 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, and with the 1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources on Life Sciences, National Research Council, National Academy of Sciences).

Apparatus

During experimental sessions, monkeys were seated in chairs (Model R001, Primate Products, Miami, FL, U.S.A.) that provided restraint at the neck, and were placed in ventilated, sound-attenuating chambers equipped with two response levers, stimulus lights, and a food cup to which pellets (BioServ, Frenchtown, NJ, U.S.A.) could be delivered from a dispenser. For monkeys discriminating midazolam under a schedule of stimulus-shock termination, feet were placed in shoes containing brass electrodes through which a brief electric stimulus (3 mA, 250 ms) could be delivered from an A/C generator. An interface (MedAssociates, St Albans, VT, U.S.A.) connected the chambers to a computer that controlled and recorded experimental events.

Midazolam discrimination procedure

Monkeys had been trained to discriminate midazolam in multiple-cycle sessions. Each cycle comprised a 10-min time-out, during which responses had no programmed consequence, followed by a 5-min response period, during which illumination of red lights signaled a pending electric stimulus (every 15 s). The correct lever was determined by an injection (saline or midazolam) during the first minute of the cycle; determination of correct levers (e.g. left, saline; right, midazolam) varied among monkeys and remained the same for an individual throughout the study. Ten consecutive responses (fixed ratio (FR) 10) on the correct lever extinguished the red lights and postponed delivery of the schedule for 30 s. Responding on the incorrect lever reset the response requirement on the correct lever. Response periods ended after 5 min or after the delivery of four electric stimuli, whichever occurred first.

Saline training comprised administration of saline or a sham injection during the first minute of each of no more than eight cycles. Midazolam training comprised administration of midazolam (0.32 mg kg^{-1}, s.c.) during the first minute of a cycle followed by saline or sham during the first min of a second cycle; completion of the FR on the midazolam lever was required for a reinforcer during both of these cycles. On some training days, two to six saline or sham training cycles preceded a midazolam training cycle followed by a single sham cycle. Monkeys had previously satisfied the criteria for testing defined as 5 consecutive or 6 of 7 days in which at least 80% of the total responses occurred on the correct lever and fewer than 10 responses (one FR) occurred on the incorrect lever prior to completion of the FR on the correct lever. Tests were conducted every third day as long as performance during intervening training sessions satisfied the same criteria described above. Prior to tests, these criteria had to be satisfied for one midazolam and one saline training session, consecutively. The type of training session preceding test sessions varied nonsystematically. Test sessions were identical to training sessions except that 10 consecutive responses on either lever postponed the shock schedule and animals received single or cumulative doses of midazolam, alfaxolone, L-838,417, bretazenil and ketamine. Tests were conducted by injecting the appropriate vehicle solution during the first minute of the first cycle followed by increasing doses of drug during the first minute of subsequent cycles with the cumulative dose increasing by 0.25 or 0.5 log unit per cycle. On separate occasions, a dose of L-838,417 (0.32–3.2 mg kg^{-1}),

bretazenil (0.032–0.32 mg kg^{-1}) or flumazenil (0.1 mg kg^{-1}) was administered during the first cycle followed by cumulative doses of midazolam or alfaxolone. On separate occasions in one monkey, cumulative doses of midazolam were administered following pretreatment with a dose of flumazenil (0.032–0.32 mg kg^{-1}); in addition, cumulative doses of L-838,417 were administered following pretreatment with 0.1 mg kg^{-1} of flumazenil in this monkey. Test sessions ended when $\geqslant$80% of the total responses occurred on the midazolam lever or when response rate decreased sufficiently to result in the delivery of electric stimuli.

Flumazenil discrimination procedure

Diazepam was administered 3 h prior to experimental sessions consisting of multiple 15-min cycles. Each cycle consisted of a 10-min timeout, during which responses had no programmed consequence, and a 5-min response period signaled by illumination of green lights. Five consecutive responses (FR5) on the lever designated correct by the injection (vehicle or flumazenil) administered during the first minute of the cycle resulted in delivery of one 300-mg banana-flavored food pellet. Responding on the incorrect lever reset the response requirement on the correct lever. A maximum of 10 food pellets was available during a cycle; when the maximum number of food pellets was obtained in less than 5 min, the remainder of the response period was a timeout. The selection of vehicle- and flumazenil-appropriate levers varied among monkeys and remained the same for an individual throughout the study.

Vehicle training comprised administration of vehicle or a sham injection during the first minute of each of no more than eight cycles. Flumazenil training comprised administration of a dose of flumazenil (0.32 mg kg^{-1} for one monkey and 0.1 mg kg^{-1} for two monkeys) during the first minute of a cycle followed by a vehicle or sham injection during the first minute of a second cycle; completion of the FR on the flumazenil lever was required for a reinforcer during both of these cycles. On some training days, two to six vehicle–sham training cycles preceded two flumazenil–sham training cycles. Test sessions were conducted when animals satisfied the criteria specified above for monkeys discriminating midazolam. Cumulative dose–effect tests with flumazenil, L-838,417 and bretazenil were conducted by injecting the appropriate vehicle solution during the first minute of the first cycle followed by increasing doses during the first minute of subsequent cycles with the cumulative dose increasing by 0.5 log unit per cycle. Test sessions ended when $\geqslant$80% of the total responses occurred on the flumazenil-appropriate lever or when response rate was less than 20% of the control response rate.

Drugs

The vehicle for oral administration of diazepam was fruit punch combined with Suspending agent K (Bio-Serv, Frenchtown, NJ, U.S.A.) in a concentration of 1 g suspending agent per liter of fruit punch. Tablets containing 10 mg diazepam (Zenith Laboratories, Inc., Northvale, NJ, U.S.A.) were dissolved in vehicle, mixed in a blender, and administered using a 12-G drinking needle attached to a 60-cc syringe. To obtain a dose of 5.6 mg kg^{-1} of diazepam, a standard concentration of diazepam was administered in a volume adjusted to individual body weights. The diazepam mixture was prepared immediately before administration.

The following drugs were administered s.c. in a volume of 0.01–0.1 ml kg^{-1} body weight expressed in terms of the forms listed below: alfaxolone (Cyclodextrin Technologies Development, Inc., High Springs, FL, U.S.A.); bretazenil and flumazenil (gifts from F. Hoffmann LaRoche Ltd., Basel, Switzerland); ketamine hydrochloride (Fort Dodge Laboratories, Fort Dodge, IA, U.S.A.); L-838,417 (gift from Merck Sharpe and Dohme, U.K.) and midazolam hydrochloride (Roche Pharma Inc., Manati, Puerto Rico). Alfaxolone was purchased as a commercially prepared mixture of alfaxolone and hydroxypropyl-β-cyclodextrin and was dissolved with saline. Bretazenil and flumazenil were dissolved in a vehicle comprising 40% propylene glycol (Sigma, St Louis, MO, U.S.A.), 50% saline and 10% ethanol. L-838,417 was dissolved in a vehicle comprising 50% ethanol and 50% emulphor (Rhone-Poulenc Inc., Princeton, NJ, U.S.A.). Midazolam and ketamine were purchased as commercially-prepared solutions in concentrations of 5 and 100 mg ml^{-1}, respectively, and were diluted with saline.

Data analyses

Drug discrimination data were expressed as the percentage of total responses occurring on the drug-appropriate lever averaged among monkeys ($\pm$s.e.m.) and plotted as a function of dose. The dose of a compound required to produce 50% drug-appropriate responding (ED_{50}) was estimated using linear regression by using more than two appropriate data points, otherwise by interpolation. ED_{50} values were determined for individual animals and the 95% confidence limit (CL) was determined from the *t*-statistic. For antagonism of midazolam by L-838,417, bretazenil, or flumazenil, dose–effect curves did not deviate from parallelism and, therefore, pA_2 analyses were carried out with the Pharm/PCS Pharmacologic Calculation System (version 4.2) based on Tallarida & Murray (1987). The slope of the Schild plot was considered to conform to unity when the 95% CLs included -1 and did not include 0 (e.g. Paronis & Bergman, 1999). The pA_2 was obtained when the slope of the Schild plot was not different from unity. For antagonism of L-838,417 by flumazenil, a single-dose affinity estimate was calculated with an equation modified from Tallarida *et al.* (1979), where $pK_B = -\log (B \text{ (dose ratio} - 1)^{-1})$ with B expressed in mol kg^{-1} body weight.

Control response rate represents the average of the five vehicle training sessions before the test. Response rate was calculated as a percentage of control for individual animals, then averaged among subjects ($\pm$s.e.m.) and plotted as a function of dose. Discrimination data were not included for analysis when response rate for a particular monkey was less than 20% of control for that monkey; however, all response rate data were included in the group average.

Results

Effects of L-838,417, bretazenil, and flumazenil in monkeys discriminating midazolam

Midazolam dose-dependently increased drug-lever responding with a dose of 0.32 mg kg^{-1} of midazolam occasioning

predominantly midazolam-lever responding (Figures 1 and 2, top left); doses of midazolam up to 0.32 mg kg^{-1} did not alter response rate (Figures 1 and 2, bottom left). The control ED_{50} values (95% CLs) derived from the midazolam dose–effect curves in Figures 1 and 2 (top left) were 0.14 (0.11–0.18) and 0.12 (0.11–0.13) mg kg^{-1}, respectively.

In four of five monkeys discriminating midazolam, L-838,417 did not substitute for, but rather antagonized, midazolam (Figure 1, top left); doses of 0.32, 1.0, and 3.2 mg kg^{-1} of L-838,417 increased the ED_{50} of midazolam 2.1-, 4.7- and 17-fold, respectively. These doses of L-838,417 administered prior to midazolam did not modify response rate (Figure 1, bottom left, V), and doses of midazolam greater than 0.56 mg kg^{-1} in combination with L-838,417 decreased response rate (Figure 1, bottom left). In the same four monkeys, bretazenil also did not substitute for, but rather antagonized, midazolam (Figure 2, top left); doses of 0.032, 0.1, and 0.32 mg kg^{-1} of bretazenil increased the ED_{50} of midazolam 2.3-, 5.0-, and 11-fold, respectively. These doses of bretazenil administered prior to midazolam did not modify response rate (Figure 2, bottom left, V), and doses of midazolam greater than 1.0 mg kg^{-1} in combination with bretazenil decreased response rate (Figure 2, bottom left). A dose of 0.1 mg kg^{-1} of flumazenil also attenuated the midazolam discriminative stimulus in these four monkeys (Figure 3, top left), increasing the ED_{50} of midazolam 9.8-fold.

In the four monkeys for which L-838,417, bretazenil, and flumazenil attenuated the discriminative stimulus effects of midazolam, these compounds also were studied in combination with the neuroactive steroid alfaxolone. In these monkeys, alfaxolone dose-dependently increased responding on the midazolam lever with the largest doses (3.2 and 5.6 mg kg^{-1}) occasioning high levels of midazolam-lever responding (Figures 1 and 2, top right, closed circles). In three monkeys, 3.2 mg kg^{-1} of alfaxolone substituted for midazolam and also decreased response rate to 76% of control. In a fourth monkey, a larger dose (5.6 mg kg^{-1}) of alfaxolone substituted for midazolam; response rate was decreased to 26% of control at this dose of alfaxolone (Figures 1 and 2, bottom right, closed circles). The control ED_{50} (95% CLs) derived from the alfaxolone dose–effect curve shown in Figures 1 and 2 (top right) was 2.41 (1.65–3.52) mg kg^{-1}.

The same doses of L-838,417, bretazenil, and flumazenil that antagonized the discriminative stimulus effects of midazolam enhanced the midazolam-like discriminative stimulus effects of alfaxolone (compare top left and right, Figures 1–3). At doses of 1.0 and 3.2 mg kg^{-1}, L-838,417 decreased the ED_{50} of alfaxolone 2.0- and 5.1-fold, respectively. Similarly, at doses of 0.1 and 0.32 mg kg^{-1}, bretazenil decreased the ED_{50} of alfaxolone 4.6- and 8.0-fold, respectively. A dose of 0.1 mg kg^{-1} of flumazenil also decreased the ED_{50} of alfaxolone 3.0-fold. At the largest doses studied, L-838,417 and bretazenil slightly decreased response rate, and response rate was further decreased when these doses were combined with alfaxolone (Figures 1 and 2, bottom right). Response rate was not decreased by flumazenil (0.1 mg kg^{-1}) alone; in one of four monkeys, response rate was decreased by this dose of flumazenil in combination with the largest dose (3.2 mg kg^{-1}) of alfaxolone (Figure 3, bottom right).

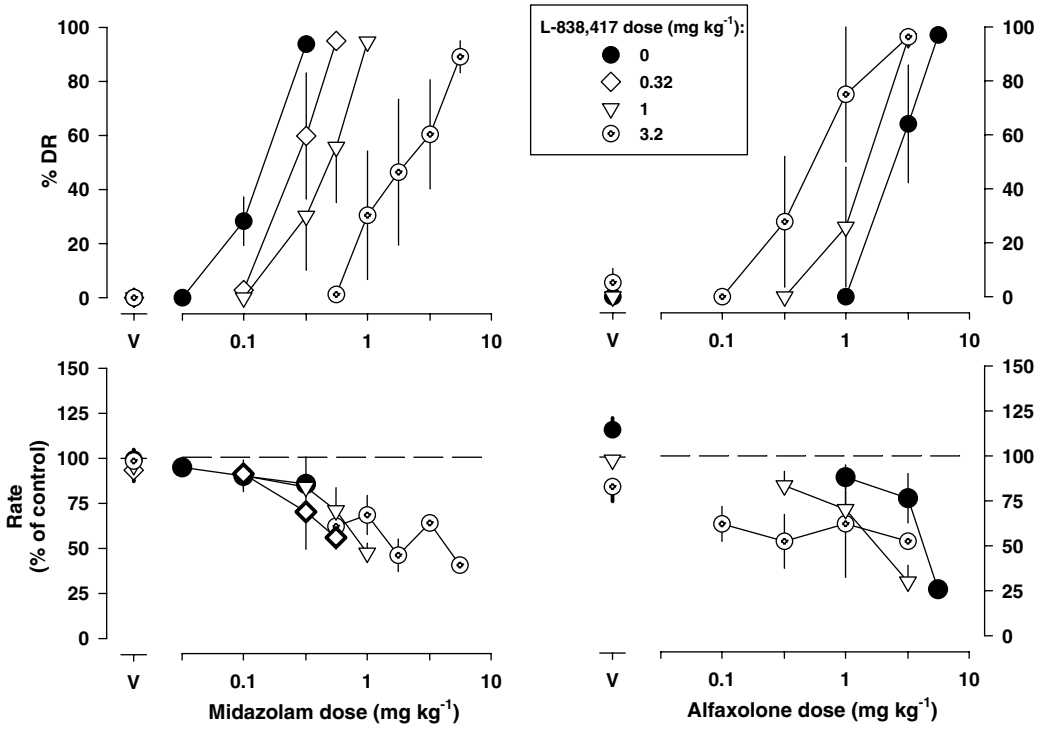

Figure 1 Differential effects of L-838,417 in combination with midazolam (left) and alfaxolone (right) in monkeys discriminating midazolam. Abscissae: dose in mg kg^{-1} body weight; V, vehicle. Ordinates: mean (±s.e.m.) percentage of responding on the drug-appropriate lever (%DR = drug responding, top) and mean (±s.e.m.) response rates expressed as percentage of control (vehicle training days) rates (rate (% of control), bottom). L-838,417 (0.32, 1.0, or 3.2 mg kg^{-1}) was administered 15 min prior to the first dose of midazolam and alfaxolone. Data represent average values from four monkeys.

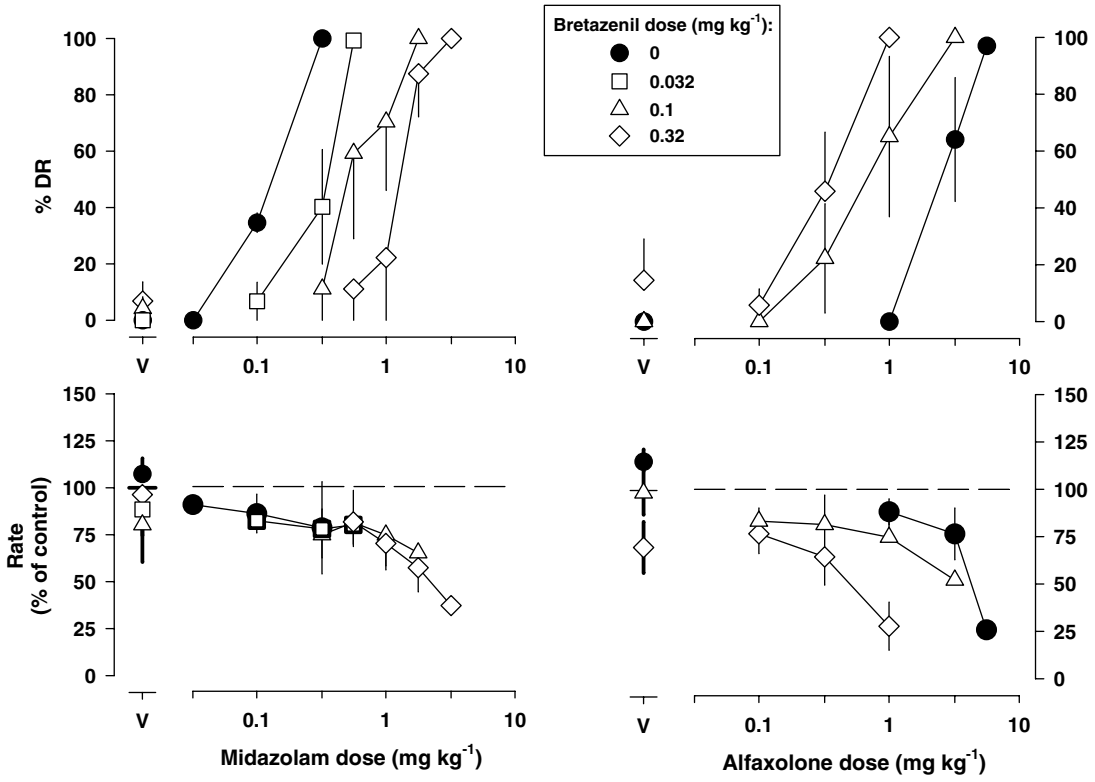

Figure 2 Differential effects of bretazenil in combination with midazolam (left) and alfaxolone (right) in monkeys discriminating midazolam. Bretazenil (0.032, 0.1, or 0.32 mg kg^{-1}) was administered 15 min prior to the first dose of midazolam and alfaxolone. The control alfaxolone dose–effect curve is replotted from Figure 1. Data represent average values from four monkeys. See Figure 1 for other details.

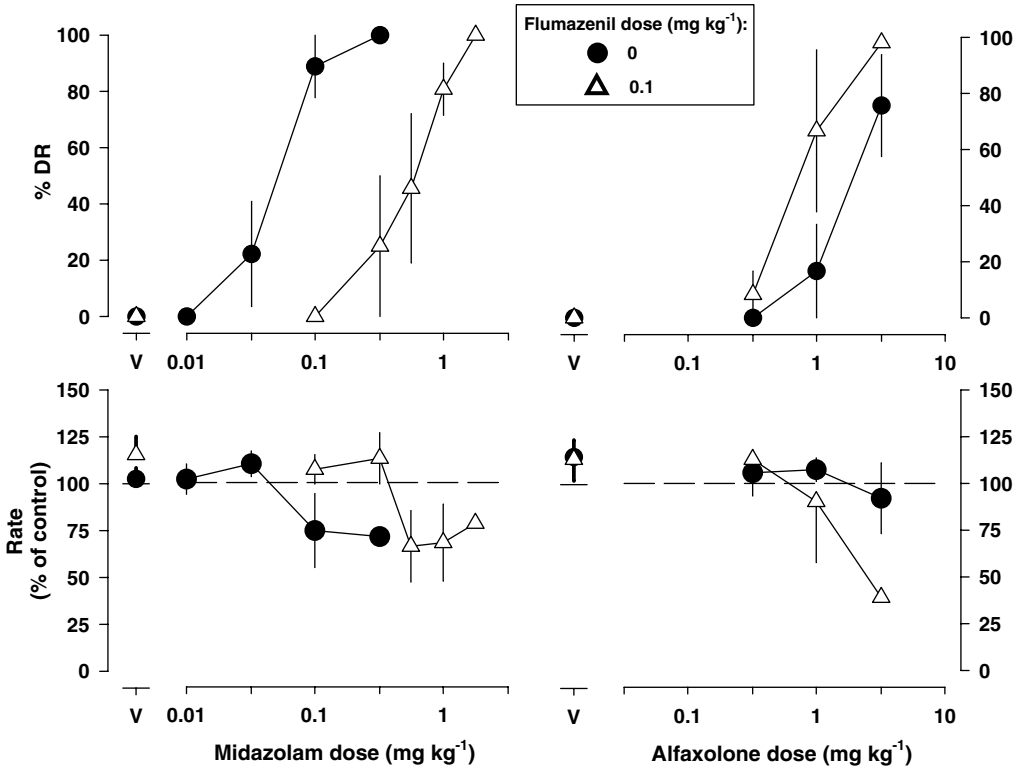

Figure 3 Differential effects of flumazenil in combination with midazolam (left) and alfaxolone (right) in monkeys discriminating midazolam. Flumazenil (0.1 mg kg^{-1}) was administered 15 min prior to the first dose of midazolam and alfaxolone. Data represent average values from four monkeys. See Figure 1 for other details.

Schild analysis of midazolam in combination with L-838,417 or bretazenil revealed a coefficient of determination (r^2) of 1.00 in both cases and slopes (95% CLs) that were not significantly different from unity (−1.17 (−0.43 to −1.90) for L-838,417 and −0.89 (−0.22 to −1.57) for bretazenil (Figure 4)). Apparent pA_2 values (95% CLs) were 6.12 (5.70–6.53) for L-838,417 and 7.26 (6.69–7.84) for bretazenil.

In a fifth monkey in the midazolam discrimination study, bretazenil, alfaxolone, and L-838,417 substituted for midazolam (data not shown); the ED_{50} values were 0.0060 mg kg^{-1} for bretazenil, 0.021 mg kg^{-1} for midazolam, 0.56 mg kg^{-1} for alfaxolone, and 0.59 mg kg^{-1} for L-838,417. At doses occasioning predominantly midazolam-lever responding, each compound increased response rate. Flumazenil antagonized midazolam with doses of 0.032, 0.1, and 0.32 mg kg^{-1} of flumazenil increasing the ED_{50} of midazolam 2.0-, 6.4-, and 26-fold, respectively. Response rate was increased by some doses of flumazenil alone or in combination with midazolam. Schild analysis of midazolam in combination with flumazenil revealed a coefficient of determination (r^2) of 1.00 and a slope (95% CLs) that was not significantly different from unity (−1.41 (−0.90 to −1.92)). The apparent pA_2 value (95% CLs) of flumazenil was 7.19 (6.68–7.70).

In the monkey in which L-838,417 substituted for midazolam, a dose of 0.1 mg kg^{-1} of flumazenil attenuated the midazolam-like effects of L-838,417, as evidenced by a 6.4-fold increase in the ED_{50} of L-838,417 (data not shown). A single-dose apparent affinity estimate for flumazenil in combination with L-838,417 yielded a pK_B of 7.20.

In all five monkeys that discriminated midazolam, a compound that does not act directly at $GABA_A$ receptors (e.g. ketamine) occasioned predominantly saline-lever responding up to a dose (3.2 mg kg^{-1}) that eliminated responding (data not shown).

Flumazenil-like effects of L-838,417 and bretazenil in diazepam-treated monkeys

Cumulative doses of flumazenil, bretazenil, and L-838,417 increased drug-lever responding with doses of 0.1 mg kg^{-1}, of flumazenil, 0.1 mg kg^{-1} of bretazenil, and 3.2 mg kg^{-1} of L-838,417 occasioning predominantly flumazenil-lever responding (Figure 5, top). Doses of flumazenil and L-838,417 occasioning flumazenil-lever responding also decreased response rate, whereas doses of bretazenil occasioning flumazenil-lever responding did not alter response rate (Figure 5, bottom). The ED_{50} values (95% CLs) derived from the dose–effect curves in Figure 5 (top) were 0.02 (0.01–0.03) mg kg^{-1} for flumazenil, 0.06 (0.01–0.39) mg kg^{-1} for bretazenil, and 0.89 (0.39–2.02) mg kg^{-1} for L-838,417.

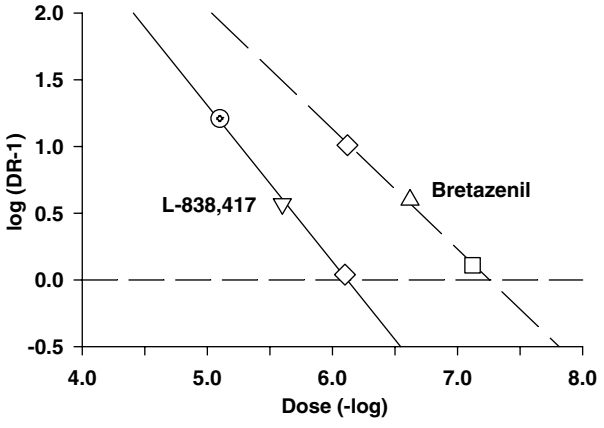

Figure 4 Schild plots constructed from the mean data shown in Figure 1 (L-838,417; solid line) and Figure 2 (bretazenil; dashed line). Abscissa: negative logarithm of the dose in mol kg^{-1}. Ordinates: logarithm of the dose ratio −1.

Discussion

In addition to differences in selectivity for $GABA_A$ receptor subtypes that vary in α subunit composition, differences in efficacy among positive $GABA_A$ modulators at benzodiazepine sites are important for their therapeutic profile. Drug discrimination was used to compare the behavioral effects of L-838,417, a positive $GABA_A$ modulator with low efficacy at some (α_2-, α_3-, or α_5-subunit-containing) and no efficacy at other (α_1-subunit-containing) benzodiazepine sites, to bretazenil, a positive modulator with low efficacy at each of these sites (McKernan *et al*., 2000; Smith *et al*., 2001). In four of five monkeys discriminating midazolam, L-838,417, bretazenil, and flumazenil did not substitute for, but rather antagonized, midazolam and the interactions of each compound with midazolam were consistent with simple, competitive antagonism. In these monkeys, alfaxolone substituted for midazolam

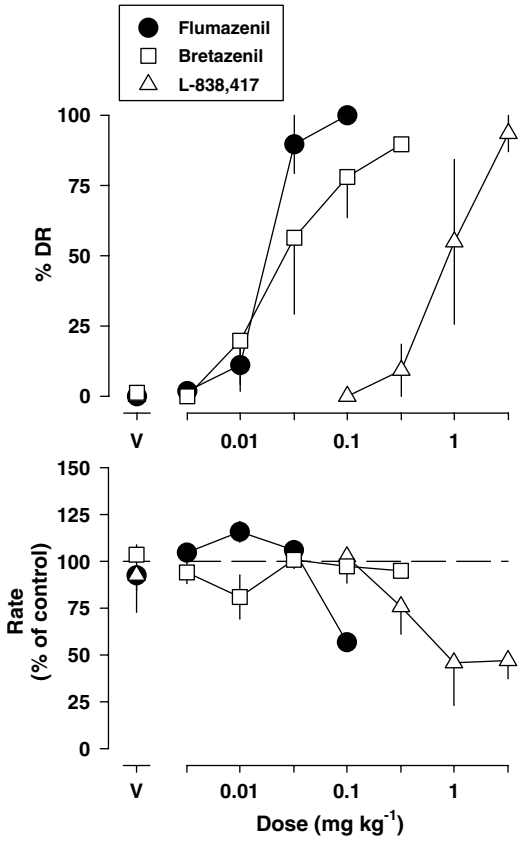

Figure 5 Substitution of L-838,417 and bretazenil for flumazenil in diazepam-treated monkeys discriminating flumazenil. Data represent average values of three monkeys. See Figure 1 for other details.

and the midazolam-like discriminative stimulus effects of alfaxolone were enhanced by L-838,417, bretazenil, and flumazenil. In a fifth monkey, L-838,417 and bretazenil substituted for midazolam, and flumazenil similarly antagonized midazolam and L-838,417. In a separate group of monkeys receiving diazepam (5.6 mg kg^{-1} per day), L-838,417 and bretazenil substituted for a flumazenil discriminative stimulus. This study provides evidence that L-838,417, bretazenil, and flumazenil had low efficacy positive modulatory actions that became evident when they were combined with a positive $GABA_A$ modulator acting at a non-benzodiazepine (neuroactive steroid) site. In addition to having therapeutic effects on their own, low efficacy positive modulators acting at one site on the $GABA_A$ receptor complex might augment the therapeutic actions of high efficacy positive $GABA_A$ modulators acting at a different site.

Positive $GABA_A$ modulators that act at benzodiazepine sites are used to treat anxiety, insomnia, and convulsions. In addition to therapeutic effects, these drugs can impair memory and motor function, they are used recreationally by some, and when taken repeatedly, tolerance and dependence can develop (Woods *et al*., 1987; Griffiths & Weerts, 1997). Based on the results of studies in genetically modified mice, it appears that the various effects of benzodiazepines are differentially mediated by benzodiazepine sites on $GABA_A$ receptors containing different α subunits (α_1, α_2, α_3, and α_5). Although some clinically available positive $GABA_A$ modulators bind differentially to benzodiazepine sites, most of these drugs have similar binding affinity and efficacy at all benzodiazepine sites (Smith *et al*., 2001). Drug development towards isolating specific actions of positive $GABA_A$ modulators through selective binding to different receptor subtypes has had limited success. As an alternative to differential binding affinity, an emerging concept for isolating specific actions of positive $GABA_A$ modulators is that of having nonselective binding and varying degrees of efficacy at receptor subtypes. For example, while L-838,417 has similar binding affinity at all benzodiazepine sites, electrophysiological data in recombinant cells expressing only one subtype indicate that L-838,417 facilitates GABA-mediated Cl^- flux with low efficacy at $GABA_A$ receptors containing α_2, α_3, or α_5 subunits while having little or no efficacy at receptors containing α_1 subunits (McKernan *et al*., 2000). That L-838,417 has anxiolytic and not sedative effects in rodents is consistent with the notion that the sedative and anxiolytic effects of benzodiazepines are mediated by $GABA_A$ receptors containing α_1- and α_2-subunits, respectively (McKernan *et al*., 2000). However, these effects of L-838,417 also are consistent with the notion that less efficacy (positive modulation) at $GABA_A$ receptors is necessary for anxiolytic as compared to sedative effects. Therefore, it is not clear whether selectivity for $GABA_A$ receptor subtypes (i.e. different efficacy at different sites) is more important than overall efficacy (regardless of site) for the behavioral effects of positive $GABA_A$ modulators.

Drug discrimination can provide a useful measure of the relationship between efficacy and the behavioral effects of drugs. In rhesus monkeys that discriminated the benzodiazepine midazolam, L-838,417 had behavioral effects that were consistent with low efficacy positive modulatory actions insofar as L-838,417 did not substitute for but, rather, antagonized midazolam in four of five monkeys (L-838,417 substituted for midazolam in a fifth monkey). Dose–ratio (Schild) analysis of the antagonism data was consistent with L-838,417 acting in a simple, competitive manner at the same (benzodiazepine) site(s) that mediates the discriminative stimulus effects of midazolam. The behavioral profile of bretazenil, another low efficacy positive modulator acting at benzodiazepine sites, was similar to that of L-838,417 in these monkeys. The low efficacy positive modulatory actions of these compounds were confirmed by data obtained in diazepam-treated rhesus monkeys that discriminated flumazenil (also Gerak & France, 1999). In these monkeys, both L-838,417 and bretazenil substituted for flumazenil and their relative antagonist potency in diazepam-treated monkeys was similar to their relative antagonist potency in untreated monkeys that discriminated midazolam. Collectively, these results indicate that the site(s) of action for L-838,417 and bretazenil overlap, at least in part, with the site(s) of action of nonselective benzodiazepines and further indicate that L-838,417 and bretazenil have lower efficacy than most currently available benzodiazepines.

In addition to benzodiazepine binding sites, the $GABA_A$ receptor complex contains other sites where drugs can facilitate GABA-mediated Cl^- flux. For example, neuroactive steroids are a class of naturally occurring and synthetic $GABA_A$ modulators, some of which substitute for the discriminative stimulus effects of midazolam in rhesus monkeys (McMahon *et al*., 2001). In light of the multiple distinct sites on the $GABA_A$ receptor complex through which drugs can produce qualitatively similar effects, low efficacy positive modulatory actions of benzodiazepine-site ligands might be evidenced by enhancement of the positive modulatory (i.e. midazolam-like discriminative stimulus) effects of neuroactive steroids. To test this possibility, L-838,417 and bretazenil were studied in combination with alfaxolone; that the midazolam-like effects of alfaxolone were enhanced by L-838,417 and bretazenil was consistent with these compounds having low efficacy positive modulatory actions at benzodiazepine sites on the $GABA_A$ receptor complex. Collectively, these studies underscore the importance of both efficacy and site of action in determining the nature of the interaction among positive $GABA_A$ modulators. Thus, the positive $GABA_A$ modulation by L-838,417 and bretazenil at benzodiazepine sites is not adequate for either drug to substitute for midazolam, yet their binding to these sites is evident by their ability to antagonize the higher efficacy positive $GABA_A$ modulator and benzodiazepine midazolam. In contrast, the limited positive modulatory actions of L-838,417 and bretazenil at benzodiazepine sites enhances the positive modulatory actions of a drug acting at a different site(s) on the $GABA_A$ receptor complex.

Bretazenil is reported to have similar low efficacy at all $GABA_A$ receptor subtypes that bind benzodiazepines (Facklam *et al*., 1992; Smith *et al*., 2001), whereas L-838,417 is reported to have low efficacy at receptor subtypes containing α_2-, α_3-, or α_5-subunits and no efficacy at receptors containing α_1-subunits (McKernan *et al*., 2000). Antagonism of midazolam by L-838,417 and bretazenil is consistent with the nonselective binding of these compounds at different $GABA_A$ receptor subtypes. On the other hand, the possibility of differential efficacy of L-838,417 and bretazenil at $GABA_A$ receptor subtypes cannot be readily examined under conditions that require high efficacy (positive modulation) for a compound to have behavioral activity (e.g. midazolam

discriminative stimulus). Differences in the mechanism of action of L-838,417 and bretazenil might become evident under conditions requiring comparatively lower efficacy (positive modulation) for behavioral activity or by combining them with receptor subtype-selective antagonists.

Generally, flumazenil does not share behavioral effects with positive $GABA_A$ modulators in drug discrimination procedures (however, see Gerak & France, 1999). Nonetheless, at relatively high concentrations, flumazenil can facilitate GABA-mediated Cl^- flux (Mehta & Ticku, 1989) and at large doses can attenuate seizures induced by direct-acting $GABA_A$ receptor antagonists (Nutt *et al*., 1982; Vellucci & Webster, 1983). That flumazenil enhanced the midazolam-like discriminative stimulus effects of alfaxolone is consistent with flumazenil having some, albeit limited, positive modulatory actions under these conditions. The relative efficacy of flumazenil, as well as bretazenil and L-838,417, might be estimated by comparing the doses of each compound that antagonize the discriminative stimulus effects of midazolam (apparent affinity) to the doses of each that shift the alfaxolone dose–effect curve a fixed amount leftward (an estimate of positive modulation). For example, a dose of $0.1\,mg\,kg^{-1}$ of flumazenil shifted the alfaxolone dose–effect curve three-fold leftward; this dose of flumazenil is 10-fold larger than the apparent affinity estimate for flumazenil in antagonizing midazolam (Lelas *et al*., 2000). The doses of bretazenil and L-838,417 necessary to shift the alfaxolone dose–effect curve three-fold leftward (estimated by linear regression on the two empirically derived shifts presented in upper right of Figures 1 and 2) are 0.058 and $1.46\,mg\,kg^{-1}$, respectively. These doses are only 2.9- and 4.9-fold larger than the apparent affinity estimates for bretazenil and L-838,417, respectively. As compared to their respective apparent affinities, a larger dose of flumazenil is required to enhance the discriminative stimulus effects of alfaxolone, indicating the need for greater receptor occupancy by flumazenil (as compared to L-838,417 and bretazenil) to exert positive modulatory actions. These differences are consistent with flumazenil having very low efficacy (positive modulation) at the $GABA_A$ receptor complex; moreover, the difference in relative potency between the two studies is greater for L-838,417 than for bretazenil (i.e. L-838,417 appears to have lower efficacy than bretazenil).

In summary, L-838,417 antagonized the discriminative stimulus effects of midazolam, consistent with L-838,417 and midazolam binding to the same benzodiazepine sites on the $GABA_A$ receptor complex. L-838,417 was demonstrated to have low efficacy insofar as L-838,417 antagonized a benzodiazepine and enhanced a neuroactive steroid in the same monkeys and also substituted for flumazenil in diazepam-treated monkeys. In these particular discrimination assays, the behavioral effects of L-838,417 were strikingly similar to bretazenil, another low efficacy positive modulator acting at benzodiazepine sites on the $GABA_A$ receptor complex. Drugs with low efficacy at one or more sites or receptor subtypes on the $GABA_A$ receptor complex could be useful alternatives to the clinical use of high efficacy benzodiazepines, especially when low efficacy is sufficient to achieve a therapeutic effect. Alternatively, a low efficacy positive modulator acting at one site on the $GABA_A$ receptor complex might enhance the actions of a high efficacy positive $GABA_A$ modulator acting at a different site.

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