Apparent \( pA_2 \) Values of Benzodiazepine Antagonists and Partial Agonists in Monkeys

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ABSTRACT

Drugs that bind to benzodiazepine recognition sites of \( \gamma \)-aminobutyric acid type A receptor complexes may function as agonists in some behavioral assays and as antagonists in other behavioral assays. The present studies compared the effects of the benzodiazepines midazolam, flumazenil, bretazenil, Ro 41-7812, and Ro 42-8773 and the \( \beta \)-carboline, \( \beta \)-carboline-3-carboxylate-f-butyl ester (\( \beta \)-CCt) under two different types of schedule-controlled responding in squirrel monkeys. One group of monkeys responded under a fixed-ratio schedule of stimulus-shock termination, and a second group of monkeys responded under a multiple fixed-ratio schedule of food presentation involving suppressed and nonsuppressed behavior. Under the schedule of stimulus-shock termination, midazolam produced dose-related decreases in response rate, and these effects were surmountably antagonized by flumazenil, bretazenil, Ro 41-7812, Ro 42-8773, and \( \beta \)-CCt. Schild plot analysis of these data revealed the following mean \( pA_2 \) values: flumazenil, 7.18; bretazenil, 7.62; Ro 41-7812, 7.06; Ro 42-8773, 6.95. Apparent \( pA_2 \) values were not calculated for \( \beta \)-CCt because the CL of the slope of the Schild plot included positive values. Under the multiple schedule, midazolam, bretazenil, and Ro 42-8773 dose-dependently increased rates of suppressed responding, whereas flumazenil, Ro 41-7812, and \( \beta \)-CCt had no significant rate-altering effects. Flumazenil antagonized the antisuppressant effects of midazolam and bretazenil; however, individual variability in these effects prohibited the determination of apparent \( pA_2 \) values. These results indicate that in vivo \( pA_2 \) values may be determined for benzodiazepine-site ligands. These results further demonstrate that some benzodiazepine-site ligands, e.g., bretazenil and Ro 42-8773, may function as both agonists and as competitive antagonists in vivo.

Dose-ratio, or Schild, analysis can be used to identify competitive interactions between drugs, both in vitro and in vivo. If an antagonist produces dose-related, parallel, rightward displacements of an agonist dose-effect function, and if the slope of a Schild regression does not differ from unity, the relationship between drugs is presumed to be a competitive interaction at a homogeneous receptor population, and the resultant \( pA_2 \) value provides an estimate of the \( K_B \) of the antagonist. In studies with opioid drugs, the determination of \( pA_2 \) values under different conditions have proven useful in characterizing receptor mechanisms that underlie specific opioid actions in whole animals. For example, Schild analysis of studies with the opioid antagonist quazocine yields similar apparent \( pA_2 \) values, approximately 7.4 to 7.5 for both the ventilatory depressant and the analgesic effects of alfentanil, supporting the idea that both effects are mediated by \( \mu \)-opioid receptors in nonhuman primates (Butelman et al., 1993). In contrast, Schild analysis of the ventilatory depressant and analgesic effects of ethylketocyclazocine (EKC) results in different apparent \( pA_2 \) values (8.0 and 6.1, respectively), consistent with the hypothesis that \( \mu \)-opioid receptors mediate the ventilatory effects of EKC, whereas \( \kappa \)-opioid receptor mechanisms mediate the analgesic effects of EKC (Dykstra et al., 1987; Butelman et al., 1993).

Despite the success of Schild analysis in identifying receptor mechanisms that mediate the behavioral effects of opioids, apparent \( pA_2 \) values have been infrequently calculated for benzodiazepine-receptor ligands. This is surprising, in view of the proposed involvement of multiple subtypes of \( \gamma \)-aminobutyric acid type A (GABA\( _A \)) receptors in mediating the effects of benzodiazepines (Lippa et al., 1979; Shannon et al., 1984b; Sanger, 1995). A possible explanation for the infrequent use of Schild analysis with benzodiazepine agonists and antagonists is that benzodiazepine-site ligands do not conform to the requirements of Schild analysis. Unlike opioid agonists, benzodiazepine-site agonists do not produce

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ABBREVIATIONS: \( \beta \)-CCt, \( \beta \)-carboline-3-carboxylate-f-butyl ester; FR, fixed-ratio; GABA, \( \gamma \)-aminobutyric acid; EKC, ethylketocyclazocine.
direct cellular responses; rather, they modulate the effects of GABA at the GABA<sub>A</sub> receptor-channel complex. Thus, most measurable effects of benzodiazepines are indirect effects. Nonetheless, quantitative analysis has been applied to some data obtained with benzodiazepine-site ligands. For example, in one study of the antagonism of the anticonvulsant effects of diazepam in rats, an apparent pA<sub>2</sub> value of 5.28 for flumazenil was reported (Kunchandy and Kulkarni, 1986), whereas in a subsequent study of the pentobarbital-like discriminative stimulus effects of benzodiazepines in rhesus monkeys, a mean apparent pA<sub>2</sub> value of 6.56 for flumazenil was obtained with diazepam (Woolverton and Nader, 1995). More recently, studies of flumazenil in combination with midazolam in rhesus monkeys trained to discriminate midazolam from vehicle yielded two different apparent pA<sub>2</sub> values; a value of 7.55 was obtained for the discriminative stimulus effects of midazolam in the presence of flumazenil, whereas an apparent pA<sub>2</sub> value of 7.16 was calculated for response rate-decreasing effects (Lelas et al., 1999). Taken together, these results might suggest that different receptor subtypes mediate the anticonvulsant, discriminative stimulus and response rate-decreasing effects of benzodiazepine agonists. In a recent review, apparent pA<sub>2</sub> and pKB values were calculated post hoc in an effort to quantitatively analyze data obtained with benzodiazepine-site ligands across different studies (Rowlett and Woolverton, 1996). The results of these analyses also suggested that the population of receptors that mediate the effects of benzodiazepine-site ligands was heterogeneous. However, the data from these studies often resulted in Schild plots with slope values that differed from unity or that contained both positive and negative values within the 95% CL. A rigorous experimental examination of the applicability of in vivo Schild analysis for benzodiazepine-site ligands has, to date, not been fully explored.

The present studies were conducted, in part, to identify suitable conditions for quantitatively evaluating the behavioral effects of benzodiazepine-site antagonists. Using midazolam as a benzodiazepine-site full agonist, we compared the effects of flumazenil, the prototypical benzodiazepine antagonist, with the effects of β-carboline-3-carboxylate-t-butyl ester (β-CCT), an antagonist that may selectively bind GABA<sub>A</sub> complexes that contain α<sub>1</sub> subunits, and brevetizain, Ro 42-8773, and Ro 41-7812, three drugs previously described as benzodiazepine-site partial agonists, on different types of schedule-controlled responding. The effects of all drugs on rates of lever-press responding were determined under a simple fixed-ratio (FR) schedule of stimulus-shock termination, and, in a separate group of monkeys, under a multiple schedule involving suppressed and nonsuppressed food-maintained behavior. The antagonist effects of drugs that did not decrease response rates were next studied by determining how pretreatment modified the dose-effect function for midazolam under the schedule of stimulus shock-termination. Finally, the antagonist effects of flumazenil were studied by comparing its modification of the effects of midazolam and brevetizain under the multiple schedule of suppressed and nonsuppressed behavior. The results of these pretreatment studies were then evaluated by dose-ratio analysis and, when meeting the assumptions of Schild analysis, the calculation of apparent pA<sub>2</sub> values.

**Materials and Methods**

### Apparatus and Schedules

During experimental sessions, monkeys sat in Plexiglas chairs (Spealman et al., 1977) enclosed in ventilated, sound-attenuating chambers provided with white noise to mask extraneous sounds. While seated, monkeys faced a panel equipped with colored lights, a single response lever, and a food pellet dispenser. Each press of the lever with a force greater than 0.2 N produced an audible click and was recorded as a response. Before each session, a shaved portion of each monkey's tail was coated with electrode paste and place under brass electrodes for delivery of brief, low-intensity shock stimuli.

### Behavior Maintained by Stimulus-Shock Termination

Monkeys S-95, S-154, and S-347 were trained to respond under a FR-30 schedule of stimulus-shock termination. Under this schedule, a visual stimulus was associated with a program of brief, low-intensity electric shock (200 ms; 3 mA). Every 30th lever press terminated the visual stimulus and initiated a 10-s timeout period. Daily sessions consisted of five cycles, each comprising a 10-min timeout period followed by a 3-min period during which the fixed-ratio schedule was in effect. During the timeout periods, the chamber was dark, and responding had no programmed consequences. Under the schedule of stimulus-shock termination, shocks were rarely delivered under control conditions. Under all conditions, sessions were terminated after the delivery of five electric shocks.

Drug sessions were conducted once or twice per week, and training sessions were conducted on intervening days. The effects of individual drugs were studied using cumulative dosing procedures; drugs were administered 10 min before the start of each response period such that the total dose increased by 0.25 or 0.5 log<sub>10</sub> unit increments throughout the session. Antagonists were given as single injections 10 min before the first injection of an agonist. Generally, each set of experiments with a particular antagonist was bracketed by determination of control midazolam dose-effect functions; the effects of midazolam alone on responding maintained under a schedule of stimulus-shock termination were thus determined six times in the course of completing these studies.

### Multiple Schedule of Food-Maintained Behavior

Monkeys S-99, S-290, S-481, S-465, and S-436 were trained to respond under a multiple fixed-ratio schedule of food reinforcement. Under the multiple schedule, components during which white or red stimulus lights were illuminated alternated throughout the session. In the presence of the white stimulus lights (white light components), completion of 15 (S-290) or 30 (all other monkeys) lever-press responses (FR-15 or FR-30) resulted in the delivery of a 190-mg banana-flavored food pellet and initiated a 10-s timeout period during which the chamber was dark and responding had no programmed consequences. In the presence of the red stimulus lights (red light components), completion of the FR-30 response requirement resulted in the

### Materials and Methods

Eight adult male squirrel monkeys (Saimiri sciureus), weighing 0.7 to 1.0 kg, were studied in daily sessions (5–7 days/week). Between sessions, the monkeys were housed in individual cages in a climate-controlled vivarium. Three monkeys (S-95, S-154, and S-347) had unrestricted access to food (Purina Mills, Framingham, MA High Protein Diet and fresh fruit), and the remaining monkeys were maintained at approximately 90% of their free-feeding weights by adjusting their access to food daily. Water was available ad libitum. Monkey S-436 was untrained at the beginning of the study, and all other monkeys had responded previously under FR schedules of either food-presentation or stimulus-shock termination and had received benzodiazepines or other psychoactive drugs.

Animal maintenance and research were conducted in accordance with the guidelines provided by the National Institutes of Health Committee on Laboratory Animal Resources and protocols were approved by the Institutional Animal Care and Use Committee.
delivery of a food pellet and initiated a 10-s timeout period. Additionally, a second schedule was superimposed under which completion of every 25th (S-290) or 50th (other monkeys) response in the presence of red stimulus lights produced a brief, low-intensity electric shock to the monkey’s tail (200 ms; 0.5–2.0 mA). Shock intensity was adjusted for individual monkeys to levels that suppressed responding in the red light components to less than 10% of nonsuppressed response rates, i.e., response rates in the white light components. Daily sessions consisted of three cycles, each comprising a 10-min timeout period followed by presentation of the multiple schedule. Each component of the multiple schedule was in effect for 3 min, and components were separated by a 60-s timeout period during which the chamber was dark and responding had no consequences.

Drug sessions were conducted once or twice per week, and training sessions were conducted on intervening days. The effects of individual drugs were studied using cumulative dosing procedures similar to those described above, except that drugs were administered 5 min before the start of each white light component, and antagonists were administered 5 min before the first injection of an agonist. The effects of more than four doses of a drug were examined by administering overlapping ranges of cumulative doses in separate test sessions.

**Drugs**

Midazolam maleate, bretazenil, Ro 41-7812, Ro 42-8773, and flumazenil were gifts from Dr. James R. Martin (Hoffman LaRoche, Zurich, Switzerland). β-CCT was a gift from Dr. James M. Cook (University of Wisconsin, Milwaukee, WI). All drugs were initially dissolved in a vehicle of 20% ethanol 20% Alkamuls EL-620 (Rhône-Poulenc, Cranbury, NJ) and 60% saline and were diluted in saline. Midazolam, Ro 41-7812, and Ro 42-8773 were gifts from Dr. J.R. Martin.

**Data Analysis**

Rates of suppressed and nonsuppressed responding following drug injection were expressed as a percentage of control rates of nonsuppressed responding. Rates of response after drug injection in the stimulus shock-termination procedure were expressed as a percentage of control response rates. The effects of drugs on response rates were compared to those of vehicle using one-way repeated measures ANOVA followed by Dunnett’s method of multiple comparison procedures; significance was set at .05. The transformed values were used to calculate ED50 values and 95% CL by linear regression when more than two data points were available but were otherwise calculated by interpolation. In one instance, an ED50 was calculated by extrapolation from a linear line based on three points above 50%.

<table>
<thead>
<tr>
<th>Dose</th>
<th>Midazolam</th>
<th>Flumazenil</th>
<th>Bretazenil</th>
<th>Ro 42-8773</th>
<th>Ro 41-7812</th>
<th>β-CCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.03</td>
<td>109.6 ± 15.1</td>
<td>114.4 ± 8.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>113.5 ± 12.8</td>
<td>106.8 ± 0.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>83.4 ± 11.2</td>
<td>122.2 ± 7.9</td>
<td>100.5 ± 2.9</td>
<td>122.2 ± 7.9</td>
<td>101.5 ± 5.0</td>
<td>98.1 ± 9.5</td>
</tr>
<tr>
<td>1.0</td>
<td>146 ± 3.5*</td>
<td>118 ± 4.6</td>
<td>95.6 ± 0.8</td>
<td>118 ± 4.6</td>
<td>107 ± 10.6</td>
<td>92.9 ± 1.0</td>
</tr>
<tr>
<td>3.0</td>
<td>0.3 ± 0.3*</td>
<td>99.3 ± 5.7</td>
<td>91.8 ± 3.1</td>
<td>99.3 ± 5.7</td>
<td>99.8 ± 12.4</td>
<td>83.5 ± 11.5</td>
</tr>
<tr>
<td>10.0</td>
<td>101.4 ± 8.2</td>
<td></td>
<td></td>
<td>101.4 ± 8.2</td>
<td>130 ± 7.2</td>
<td>95.8 ± 8.5</td>
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<tr>
<td>30.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>94.6 ± 10.4</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from control levels of responding, p < .05.

**Results**

**Stimulus-Shock Termination Schedule**

Control response-rates under the schedule of stimulus-shock termination varied among subjects from 1.9 to 5.2 responses/s but were stable for each subject over the course of the present experiments. Response rates after vehicle administration did not differ from response rates on days during which no injections were given and data from vehicle and no-injection control days were combined to give the following mean (± S.D.) control rates of responding: S-95, 4.97 ± 0.19 responses/s; S154, 1.97 ± 0.07 responses/s; S-322, 1.96 ± 0.06 responses/s.

**Midazolam.** Midazolam produced significant rate-decreasing effects on responding under the schedule of stimulus-shock termination (Table 1). These effects were observed over the same range of doses in all monkeys and were relatively stable over time. For example, the ED50 for midazolam was 0.3 ± 0.1 mg/kg in the first determination and remained at 0.5 to 0.6 mg/kg in the second through the sixth determinations of the dose-effect function.

**Flumazenil.** Flumazenil, at doses that had no direct effects on response rates (Table 1), dose-dependently antagonized the response rate-decreasing effects of midazolam. Under the schedule of stimulus-shock termination, flumazenil produced parallel rightward displacements of the midazolam dose-effect function in each of three monkeys. A dose of 0.3 mg/kg flumazenil displaced the midazolam dose-effect function approximately 10-fold to the right, and the highest dose of flumazenil, 3.0 mg/kg, produced almost a 90-fold rightward displacement of the midazolam dose-effect function (Fig. 1). Schild analysis of these data revealed a slope near unity and an apparent pA2 value of 7.18 (Table 2; see also Fig. 6).  

**β-CCT.** Pretreatment with β-CCT, which did not have rate-altering effects under the schedule of stimulus shock-termination (Table 1), produced dose-dependent rightward displacements of the midazolam dose-effect function. However, as shown in Fig. 2, the antagonism produced by β-CCT was neither as uniform nor of the same magnitude as that produced by flumazenil. Pretreatment with 3, 10, and 30 mg/kg β-CCT resulted in, respectively, 5-, 12-, and 21-fold displacements of the midazolam dose-effect function. A lower dose of β-CCT, 1.0 mg/kg, had no antagonist effects, and higher doses could not be tested because of the limited solubility of the drug. Schild analysis of data obtained with β-CCT and midazolam revealed a slope value of −0.68 (see Fig. 6); however, the 95% CI contained negative and positive values (Table 2), and an apparent pA2 value was not derived.

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**TABLE 1**

Drug effects on responding maintained under a schedule of stimulus–shock termination, expressed as mean (± S.E.) percentage of control responding

<table>
<thead>
<tr>
<th>Dose</th>
<th>Midazolam</th>
<th>Flumazenil</th>
<th>Bretazenil</th>
<th>Ro 42-8773</th>
<th>Ro 41-7812</th>
<th>β-CCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1.0</td>
<td>146 ± 3.5*</td>
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<td>95.6 ± 0.8</td>
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<tr>
<td>3.0</td>
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<td></td>
<td></td>
<td></td>
<td>94.6 ± 10.4</td>
<td></td>
</tr>
</tbody>
</table>

*Significantly different from control levels of responding, p < .05.
Bretazenil. Bretazenil, up to a dose of 3.0 mg/kg, had no response rate-decreasing effects (Table 1). Pretreatment with bretazenil displaced the midazolam dose-effect function dose-dependently to the right (Fig. 3). Doses of 0.01, 0.1, 0.3, and 1.0 mg/kg bretazenil displaced the midazolam dose-effect function 2, 10-, 35-, and 90-fold to the right, and Schild analysis of these data yielded an apparent $pA_2$ value of 7.62 with a slope near unity (Table 2; see Fig. 6).

Ro 41-7812. Ro 41-7812 (0.3–10.0 mg/kg) did not alter response rates in any monkey (Table 1) but did dose-dependently antagonize the response rate-decreasing effects of midazolam, displacing the midazolam dose-effect function to the right (Fig. 5). A dose of 0.3 mg/kg Ro 41-7812 displaced the midazolam dose-effect function approximately 6-fold to the right, and the highest dose, 10 mg/kg, displaced the midazolam dose-effect function approximately 80-fold to the right (Fig. 6).

**Multiple Schedule of Food-Maintained Behavior**

Rates of nonsuppressed responding ranged from 2.1 to 3.9 responses/s for individual monkeys. In the presence of red stimulus lights, responding was suppressed to <10% of nonsuppressed response rates. Administration of vehicle had no appreciable effect on responding in either component of the schedule; therefore, data from vehicle and no-injection control days were combined to calculate control rates of responding, listed in Table 3.

**Midazolam.** Midazolam produced dose-related increases in suppressed responding over a limited dose-range in all monkeys (Fig. 7). Significant increases in suppressed responding were obtained at doses of 0.1 and 0.3 mg/kg. After administration of 0.3 mg/kg midazolam, mean rates of suppressed responding were $2.4 \pm 0.4$ responses/s, or $74 \pm 5\%$ of control rates of nonsuppressed behavior. Midazolam also produced dose-related decreases in rates of nonsuppressed responding and, after doses of midazolam higher than 0.1 mg/kg, rates of nonsuppressed responding were significantly below control values. Mean response rates during both components of the schedule were less than 0.6 responses/s after administration of 1.0 mg/kg midazolam.

**Bretazenil and Ro 42-8773.** Bretazenil and Ro 42-8773 both significantly increased rates of suppressed responding in all monkeys. The averaged dose-effect function for bretazenil appears to be biphasic; however, decreases in suppressed responding after high doses of bretazenil only were
observed in one monkey (S-99). Bretazenil produced a maximum increase in rates of suppressed responding to 96% of control response rates, and Ro 42-8773 increased rates of suppressed responding to 78% of control rates. The antisuppressant effects of bretazenil and Ro 42-8773 were apparent over 30- to 100-fold dose ranges; neither drug produced decreases in nonsuppressed response rates at any dose (Fig. 7). Notably, the antisuppressant effects of brezatelnil and Ro 42-8773 were apparent at the same doses that had antagonist effects (compare Fig. 7 with Figs. 4 and 5).

**β-CCT and Ro 41-7812.** β-CCT (0.3–3.0 mg/kg) had no apparent antisuppressant effects (Fig. 7). Higher doses of β-CCT, up to 18 mg/kg, were studied in two monkeys (S-99 and S-436, data not shown); these doses also did not increase suppressed behavior. β-CCT also did not decrease rates of nonsuppressed behavior in four of five monkeys; in one monkey (S-481) doses of 1.0 to 3.0 mg/kg β-CCT decreased nonsuppressed responding to less than 20% of control rates. Ro 41-7812 had neither antisupp-

### Table 2

<table>
<thead>
<tr>
<th>Drug</th>
<th>Slope</th>
<th>95% CL</th>
<th>pA2 value</th>
<th>95% CL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flumazenil</td>
<td>-0.85</td>
<td>(-1.05, -0.65)</td>
<td>7.18</td>
<td>(6.68,7.68)</td>
</tr>
<tr>
<td>Ro 41-7812</td>
<td>-0.80</td>
<td>(-1.43, -0.17)</td>
<td>7.06</td>
<td>(5.03,9.09)</td>
</tr>
<tr>
<td>Bretazenil</td>
<td>-1.02</td>
<td>(-1.43, -0.61)</td>
<td>7.62</td>
<td>(6.76,8.48)</td>
</tr>
<tr>
<td>Ro 42-8773</td>
<td>-1.06</td>
<td>(-1.11, -1.01)</td>
<td>6.95</td>
<td>(6.76,7.14)</td>
</tr>
<tr>
<td>β-CCT</td>
<td>-0.68</td>
<td>(-1.59,0.23)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Notably, the antisuppressant effects of bretazenil and Ro 42-8773 were apparent over 30- to 100-fold dose ranges; neither drug produced decreases in nonsuppressed response rates at any dose (Fig. 7). Notably, the antisuppressant effects of bretazenil and Ro 42-8773 were apparent at the same doses that had antagonist effects (compare Fig. 7 with Figs. 4 and 5).

**Flumazenil.** Flumazenil (0.3–3.0 mg/kg) had no antisuppressant effects and no rate-decreasing effects on nonsuppressed behavior in three of five monkeys; in two monkeys (S-99 and S-465), however, 3.0 mg/kg flumazenil decreased nonsuppressed responding to <40% of control rates.

As shown in Fig. 8, single injections of flumazenil (0.3–3.0 mg/kg) administered 5 min before cumulative dose-effect determinations for midazolam and bretazenil, antagonized the antisuppressant effects of both drugs. An evaluation of the group data (Fig. 8) using Schild analysis revealed a slope for flumazenil with midazolam of $-2.14$ ($-9.37, 5.08$); for flumazenil with bretazenil, the slope was $-0.83$ ($-2.43, 0.78$). Because the 95% CL of the slopes included both positive and negative values, apparent pA2 values were not determined for these group data.

Although the group data suggest dose-dependent antagonism of the antisuppressant effects of midazolam and bretazenil by flumazenil, inspection of the data for individual monkeys indicates that the effects of flumazenil on suppressed responding are not uniform. In two of four monkeys (S-99 and S-481), increasing doses of flumazenil produced parallel rightward displacements of the dose-effect functions for the antisuppressant effects of midazolam (Fig. 9). In the other two monkeys, antagonism by 3.0 mg/kg flumazenil was not surmounted by up to 30 mg/kg midazolam. Flumazenil produced orderly rightward displacements of the bretazenil dose-effect function for antisuppressant effects in only one monkey, S-481 (Fig. 10). In contrast, the effects of flumazenil were not dose-dependent in the other three monkeys, and the maximum antisuppressant effects of bretazenil were decreased (S-99 and S-465) or increased (S-436) after flumazenil.

### Discussion

Six different benzodiazepine-site ligands were characterized according to their effects on schedule-controlled responding in squirrel monkeys. Two of the drugs, midazolam and flumazenil, have been studied extensively and were included in the present study as reference compounds. Midazolam has actions characteristic of benzodiazepine agonists; that is, relatively low doses of midazolam produced increases in rates of suppressed responding whereas higher doses of midazolam (>0.3 mg/kg) produced decreases in rates of both nonsuppressed food-maintained responding and responding maintained under a schedule of stimulus shock-termination. The doses of midazolam that had rate-altering effects in the present studies, in which cumulative dosing procedures were used, are within the range of doses previously found to have
effects in squirrel monkeys using single dosing procedures (Spealman, 1985; Gleeson and Barrett, 1990). Cumulative dosing procedures have been widely used in many behavioral assays, for example, to study antinociceptive, discriminative stimulus, and rate-altering effects of drugs (Kelleher and Goldberg, 1979; Spealman, 1985; Paronis and Holtzman, 1992). The present results indicate that cumulative dosing techniques also may be applied to studies of the antisuppressant effects of drugs.

There is growing evidence that flumazenil is able to produce measurable effects in the absence of other drugs. Early reports suggested that flumazenil has weak anticonvulsant and myorelaxant effects (Marescaux et al., 1984; Kawasaki et al., 1984). More recently, flumazenil has been shown to produce discriminable effects in several species, including humans (Acri et al., 1995; Gerak and France, 1998; Smith et al., 1999). In the present studies, flumazenil was essentially devoid of agonist effects, because it had no antisuppressant effects and only mild and inconsistent rate-decreasing effects. Similar findings have been reported in previous studies of flumazenil on food-maintained responding in squirrel monkeys (Wettstein and Spealman, 1987; Wettstein, 1988). When given as a pretreatment, however, flumazenil profoundly antagonized both the antisuppressant and the response rate-decreasing effects of the benzodiazepine-site agonist, midazolam. The finding that flumazenil will antagonize midazolam has also been reported previously (Spealman, 1985), although the present results mark the first determination of an apparent pA2 value for flumazenil with midazolam in nonhuman primates (Paronis and Bergman, 1997).

Ro 41-7812, like flumazenil, also may produce modest agonist effects in mice, rats, and pigeons (Moreau et al., 1991; Martin et al., 1993; Witkin et al., 1996). Yet, in the present studies, Ro 41-7812 did not have either antisuppressant effects or response rate-decreasing effects in monkeys. Previous studies have also demonstrated that Ro 41-7812 will antagonize the sedative and motor-impairing effects of other benzodiazepine drugs (Martin et al., 1993, 1995). In the present studies, pretreatment with Ro 41-7812, like flumazenil, displaced the midazolam dose-effect function for rate-decreasing effects to the right in a parallel and dose-dependent manner. The current observations that flumazenil and Ro 41-7812 displace the midazolam dose-effect function in a parallel fashion and yield Schild plots with slopes near unity suggests that both drugs similarly act as competitive antagonists of midazolam.

Bretazenil and Ro 42-8773 are believed to be benzodiazepine-site partial agonists; i.e., drugs that produce some, but not all, of the behavioral effects associated with classical benzodiazepine-site agonists such as chlordiazepoxide and diazepam. In previous studies in pigeons, mice, and rats, bretazenil and Ro 42-8773, like other benzodiazepine agonists, increased suppressed responding but, unlike other benzodiazepine agonists, did not produce rate-decreasing or sedative-like effects (Moreau et al., 1991; Martin et al., 1993; Sanger, 1995; Witkin et al., 1996). The present results indicate that the antisuppressant effects of benzodiazepine agonists also may be produced independently of rate-decreasing effects on schedule-controlled responding in monkeys. In addition to their agonist effects, bretazenil and Ro 42-8773 each displaced the midazolam dose-effect function for rate-decreasing effects to the right in a parallel and dose-dependent manner. Importantly, the agonist (antisuppressant) effects and the antagonist effects of these drugs appeared at the same doses, consistent with their characterization as partial agonists. The observations that these drugs will dose-dependently displace a complete midazolam dose-effect function suggests that the antagonism of midazolam by bretazenil and Ro 42-8773 is competitive and surmountable over a range of doses.

Fig. 7. Effects of different benzodiazepine-site ligands on responding under the multiple fixed-ratio schedule. Top panels, responding during the suppressed components of the schedule; bottom panels, responding during nonsuppressed components. The effects of vehicle are shown in the leftmost panels. Abscissa, cumulative dose of each drug. Ordinate, response rates, expressed as a percentage of control rates of nonsuppressed responding. Each symbol represents the mean of four or five (noted in parentheses) monkeys, vertical lines are ±S.E.M. (*p < .05 compared with vehicle control).

Fig. 8. Flumazenil antagonism of the rate-increasing effects of midazolam (left panel) and bretazenil (right panel) on suppressed responding. Other details are as in Fig. 7.
Earlier reports on the effects of β-CCt indicated that it selectively antagonizes the anticonvulsant and antisu- pressant, but not the ataxic, effects of diazepam (Shannon et al., 1984b). In the present studies, β-CCt served to antagonize the response rate-decreasing effects of midazolam. However, compared to the antagonism produced by flumazenil and bretazenil, the effects of β-CCt were limited. It has been postulated that different subtypes of receptors mediate different effects of benzodiazepine-like drugs (Martin et al., 1995; Sanger, 1995). In this regard, β-CCt has a higher affinity for recombinant GABA<sub>A</sub> complexes that contain α<sub>1</sub> subunits than for those that do not contain α<sub>1</sub> subunits (Cox et al., 1995). The inability of the highest dose of β-CCt to displace the midazolam dose-effect function more than 20-fold to the right, as was seen with all other drugs tested, may suggest that midazolam is producing its response-rate decreasing effects, in part, through a population of receptors that is not available to β-CCt.

The determination of pA<sub>2</sub> values stipulates that drug concentrations at the receptor be proportional to administered doses, that measurements be taken at time of peak effect, and that the drugs interact in a competitive manner (Shannon et al., 1984a; Dykstra et al., 1988). In several instances, the data collected in the present studies appeared to satisfy these requirements insofar as the rightward displacements of the midazolam dose-effect functions were parallel and the slopes of the Schild plot did not differ from unity. Thus, it appears that Schild analysis may be appropriately applied in studying the antagonist effects of different benzodiazepine-site ligands in vivo. Based on their apparent pA<sub>2</sub> values, the present findings suggest that flumazenil, bretazenil, Ro 41-7812, and Ro 42-8773 all have very similar affinities for benzodiazepine receptors in vivo, with less than a 10-fold difference between them. Similar relevant potencies of these drugs were also noted in results from in vitro and ex vivo binding studies, which demonstrated that these four drugs encompass, at most, a 10-fold range of binding affinities for GABA<sub>A</sub> receptor complexes (Moreau et al., 1991; Martin et al., 1993; Witkin et al., 1996). The similarity in potency and the overlap in the 95% CL of the pA<sub>2</sub> values for the drugs precludes drawing conclusions about the rank order of potency of these drugs. However, the agreement between potencies in the present data and the agreement between affinities obtained in binding studies provides additional evidence that Schild analysis may be a useful tool for examining mechanisms of action underlying the behavioral effects of benzodiazepine-site ligands.

The relative conformity with unity for the slopes of the Schild analyses in these studies contrasts earlier attempts to analyze the antagonism of diazepam by either CGS 8216 or flumazenil using dose-ratio analysis (Herling and Shannon, 1982; Kunchandy and Kulkarni, 1986; Woolverton and Nader, 1995). The difficulty in obtaining reliable pA<sub>2</sub> values with CGS 8216 may be due to the pharmacological properties of that drug. CGS 8216 is an inverse agonist at benzodiazepine receptors and produces proconvulsant effects and alterations in schedule-controlled responding when given alone (File, 1983; Wettstein and Spealman, 1988). Such direct actions of CGS 8216 may limit the utility of Schild analysis for analyzing its antagonist actions. On the other hand, it is less clear why previous studies of the antagonism of diazepam by flumazenil also resulted in Schild plots with slopes that were steep or that contained both positive and negative values within their 95% CL. Possibly, procedural factors may have constrained the antagonism produced by flumazenil. In the present studies, for example, parallel displacements of the agonist dose-effect functions were obtained with flumazenil under the schedule of stimulus shock-termination but not under the multiple schedule of suppressed and nonsuppressed behavior. Under the latter schedule, flumazenil decreased the maximum effects obtained with agonist drugs in half of the monkeys, shifting the dose-effect functions down rather than to the right. Thus, methodological, as well as pharmacological, considerations may determine whether the assumptions required for Schild analysis can be met in vivo. It was unexpected that the rate-decreasing effects of midazolam under the schedule of stimulus shock-termination were antagonized in a more orderly manner than were its...
antisuressant effects under the multiple schedule of food presentation. Under the latter schedule, the rate-decreasing effects of high doses of midazolam were more pronounced than its rate-increasing effects and likely interfered with orderly displacements of the midazolam dose-effect function. Thus, the monotonic rate-decreasing effects of benzodiazepine-site agonists, although not qualitatively unique, may be more suitable for quantitative analysis than are their other, more pharmacologically characteristic effects, e.g., antisuressant actions.

In conclusion, the present data suggest that Schild analysis may be appropriately applied under some, but not all, conditions in studies of the behavioral effects of benzodiazepine-site ligands. Apparent \( pA_2 \) values have been very productively applied in identifying different receptor mechanism of drug action in both in vitro and in vivo studies with opioids and other classes of drugs. As more selective benzodiazepine-site ligands become available, Schild analysis may be a similarly powerful tool for the quantitative analysis of their effects.

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