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The effects of the novel benzodiazepine receptor inverse agonist Ru 34000 on ethanol-maintained behaviors

Harry L. June *, Mary W. Eggers, Connease Warren-Reese, Jana DeLong, Anila Ricks-Cord, La Franze Durr, Charity R. Cason

Department of Psychology LD 124, Indiana University-Purdue University, Purdue School of Science, 402 North Blackford Street, Indianapolis, IN 46202, USA

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Abstract

Ru 34000 [5-ethyl-7-methoxy-imidazo (1,2-a) pyrimidin-2-yl cyclopropyl methanone] is a novel imidazopyrimidine benzodiazepine inverse agonist that exhibits low affinity for central benzodiazepine receptors ($K_i \approx 0.98$ μM). The present study examined the in vivo actions of Ru 34000 (0.5–5 mg/kg) following intraperitoneal (i.p.), subcutaneous (s.c), oral (p.o.), and intraventral tegmental administration in alcohol-preferring (P) rats trained under a concurrent operant schedule (FR4–FR4) for ethanol (10% v/v) and a palatable saccharin (0.025% or 0.75% w/v) reinforcer. Ru 34000 (i.p., s.c., p.o.) markedly reduced ethanol responding by 28–96% of control levels without affecting saccharin responding, except for the highest dose level. Clear dose-dependent suppressant effects were observed with all routes of administration on ethanol responding. Flumazenil [ethyl-8-fluro-5, 6-dihydro-5-methyl-6-4*H*-imidazo [1,5-a]-[1,4]-benzodiazepine-3-carboxylate] (6 mg/kg; i.p.), a benzodiazepine receptor antagonist reversed the Ru 34000-reduction of ethanol responding, suggesting that the effects were mediated at the benzodiazepine receptor. Bilateral microinjections of Ru 34000 (50, 100, 200 ng) into the ventral tegmental area dose-dependently reduced ethanol responding by as much as 97% of control levels. The results suggest that the in vivo actions of Ru 34000 are determined not only by its binding affinity, but also by its bioavailability at active benzodiazepine sites and route of drug administration. Low affinity imidazopyrimidines may be useful pharmacological probes to further understand the role of the $GABA_A$-benzodiazepine receptor complex in ethanol motivated behaviors.

Keywords: Ethanol self-administration; $GABA_A$ receptor; Benzodiazepine; Ru 34000; Ru 40410; Alcohol-preferring (P) rat

1. Introduction

The abuse liability of alcohol is thought to derive in part from its reinforcing properties (Weiss and Koob, 1991). The neurotransmitter systems mediating the reinforcing properties of alcohol, however, are not well understood. Recently, a series of molecular studies (Yang et al., 1995; Zhang et al., 1995; Gunnersen et al., 1996) have elucidated the pharmacological profiles and subunit heterogeneity of a variety of benzodiazepine receptor inverse agonists and antagonists with different chemical structures (e.g., imidazobenzodiazepines, β-carbolines, pyrazoloquinolines). This work has led to an increasing literature demonstrating the role of the $GABA_A$-benzodiazepine receptor complex in alcohol reinforcement (Cason et al., 1996; June et al., 1996a,b, 1997, 1998a,b,c).

In recent years, a series of imidazopyrimidine and quinoline ligands have been developed and pharmacologically characterized by the Roussel Uclaf Laboratories (Romainville, France) (Gardner et al., 1987, 1991; Bagust et al., 1990; Gardner, 1992). Unlike many of the imidazobenzodiazepines and β-carbolines, these agents have good absorption characteristics and are not rapidly metabolized to inactive molecules (Gardner, 1988, 1992). However, their capacity to antagonize the reinforcing and other behavioral properties of ethanol is not yet known. Given the unique pharmacokinetics of the imidazopyrimidine and quinoline ligands, their bioavailability may be a major factor in their in vivo activity and capacity to function effectively as alcohol antagonists.

* Corresponding author. Tel.: +1-317-274-6755; fax: +1-317-274-6756; e-mail: hjune@iupui.edu

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Ru 34000 is a highly potent imidazopyrimidine inverse agonist in vivo, but it exhibits very low affinity at central benzodiazepine receptors ($K_i \sim 0.98$ μM) (Gardner, 1992; Bagust et al., 1990). Ru 34000 does not displace [^{3}H]RO15-4513 from cerebellar diazepam insensitive binding sites (Korpi et al., 1992), suggesting that it is unlike the well-known imidazobenzodiazepine ethanol antagonist RO15-4513 (Sudzak et al., 1986; Wong and Skolnick, 1992). Both the behavioral and neurochemical effects of Ru 34000, however, have been reported to be antagonized by the benzodiazepine receptor antagonist flumazenil (Bagust et al., 1990; Gardner et al., 1991; Gardner, 1992); hence, the benzodiazepine site is the likely receptor through which Ru 34000 mediates its neurobehavioral effects.

The objective of the present study was to determine the capacity of Ru 34000 to antagonize the reinforcing properties of ethanol. Second, the contribution of route of administration was also examined. Ru 34000 was administered i.p., s.c., and orally (gavaged/per oral; p.o.). The third objective was to determine if Ru 34000 has its behavioral effects via the benzodiazepine receptor. To accomplish this goal, flumazenil and Ru 40410 (Hunt et al., 1984; Laurent, 1984; Gardner et al., 1987) (two competitive benzodiazepine receptor antagonists) were administered to block/attenuate the Ru 34000-induced reduction of ethanol responding. The fourth objective was to determine whether the ventral tegmental area (a hypothesized ethanol reward substrate) (Koob and Bloom, 1988; Koob, 1992a,b) is a possible neuroanatomical site that mediates the Ru 34000-induced reduction of ethanol responding. To accomplish this goal, the effects of bilateral infusions of Ru 34000 into the ventral tegmental area were evaluated on ethanol responding.

2. Materials and methods

2.1. Subjects

Male selectively bred alcohol-preferring (P) rats ($N = 17$) from the S40 generation (Lumeng et al., 1995) were used. The rats were approximately 2–3 months of age and weighed between 260 and 310 g at the beginning of the experiment. Animals were individually housed in wire-mesh stainless-steel cages at an ambient temperature of 21°C on a normal light cycle. All rats were provided ad libitum access to both food and water.

2.2. Drugs and solutions

The ethanol (10% v/v) and saccharin solutions (0.025% or 0.075% w/v) were prepared as previously described (June et al., 1995; June et al., 1996b). All i.p. and s.c. administered drugs were prepared as an emulsion in 1% Tween-80 vehicle (Sigma, St. Louis, MO) and mixed with a 0.90% sodium chloride solution to a fixed volume (for more details see June et al., 1996b). For p.o. administration, drugs were dissolved in deionized water using Tween-80 as a wetting agent when necessary (Gardner et al., 1987). Drugs were administered in a volume of 1 ml/kg body weight. Drugs were sonicated to aid in dissolving the compounds. The inverse agonist Ru 34000 [5-ethyl-7-methoxy-imidazo (1,2-a) pyrimidin-2-yl cyclopropyl methanone] and the selective quinoline benzodiazepine receptor antagonist Ru 40410 [7-chloro *N*-(4, 5-dihydro 2-thiazolyl) 4-hydroxy 3-quinolein carboxamine] were tested at 0–5 mg/kg and 10 mg/kg, respectively. Both compounds were donated by Roussel Uclaf Laboratories. RO15-1788 (flumazenil) [ethyl-8-fluro-5,6-dihydro-5-methyl-6-4*H*-imidazo[1,5-a]-[1,4]-benzodiazepine-3-carboxylate] was tested alone and in combination with Ru 34000 (1 mg/kg) at the 6 mg/kg dose level. Flumazenil was donated by Hoffmann–La Roche (Nutley, NJ). All drug solutions were made immediately prior to injection. When Ru 34000 was microinjected into the ventral tegmental area, it was dissolved in artificial cerebrospinal fluid (aCSF) consisting of 120.0 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, 2.5 mM $CaCl_2$ and 10.0 mM D-glucose. One drop of Tween-80 was also added to the aCSF. When necessary, HCl acid was added to solutions to adjust pH levels to 7.3 ± 0.2. All doses of Ru 34000 and Ru 40410 were selected based on a preliminary study from our lab. The flumazenil dose was selected based on previous work by June et al., 1994a, 1996b.

2.3. Surgery

A second group of male P rats ($n = 7$) from the S40 generation and from a previous ethanol self-administration experiment served as subjects for the ventral tegmental area infusion study. The rats weighed between 400–520 g at the beginning of the experiment. After stable ethanol responding was reestablished using the modified saccharin fading procedure (Samson, 1986, 1987; Hyytiä and Koob, 1995; June et al., 1998a) (see Section 2.4), rats were anesthetized with sodium pentobarbital (45 mg/kg) and stereotaxically implanted with bilateral 26-gauge guide cannulas (Plastics One) aimed at the ventral tegmental area as previously described (Gatto et al., 1995). The coordinates were: anteroposterior (A-P) -5.3 mm, mediolateral (M-L) ± 2.8 mm from bregma, and dorsoventral (D-V) -7.4 mm from the surface of the skull at a 14° angle from the vertical plane. Stereotaxic coordinates were based on the atlas of Paxinos and Watson (1986). Animals were allowed at least 7 days to recover from surgery before operant testing resumed.

2.4. Behavioral training phase and procedures

All animals were trained to initiate ethanol responding in the operant chamber (Coulbourn Instruments) using a

modification of the sucrose fading technique originally developed by Samson (1987). The only difference was that the session was extended from 30 to 60 min, and saccharin was used in place of sucrose (see June et al., 1998a,b). Following training, animals were then maintained on a concurrent FR4–FR4 schedule for ethanol (10% v/v) and saccharin (0.025% or 0.075% w/v). Animals were stabilized on a 0.025% and 0.075% saccharin concentration instead of water to increase the number of responses relative to ethanol. To assure animals were consuming pharmacologically relevant amounts of ethanol, tail blood (100 μl) was taken after the initial 30 min of the operant session and evaluated using previously published procedures (Lumeng et al., 1982).

2.5. Treatment phase

2.5.1. Systemic injections

When Ru 34000 (1–5 mg/kg) or a Tween-80 vehicle injection was given alone by the i.p. and s.c. routes, it was administered 5 min prior to the operant session. The benzodiazepine receptor antagonist flumazenil (6 mg/kg), Ru 40410 (10 mg/kg), or Tween-80 vehicle given by the i.p. route was injected 20 min prior to the testing session when given alone and in combination with Ru 34000 (1 mg/kg). Animals administered Ru 34000 by the p.o. route received their drug treatment 30 min prior to the operant sessions via a stainless-steel feeding tube. A minimum of 5 days were allotted between all drug injections.

2.5.2. Ventral tegmental infusions

Ru 34000 (50, 100, 200 ng) or aCSF was infused into the ventral tegmental area for 5 min at a rate of 0.5 μl/5 min using 33-gauge injector cannulas that were connected via polyethylene tubing to a 10-μl Hamilton microsyringe. Microinjections were delivered immediately before the operant session with a Harvard infusion pump in the rat's home cages. A minimum of 5 days were allotted between microinjections. After the completion of the behavioral testing, animals were killed by CO_2 inhalation. Black India ink (0.5 μl) was injected into the infusion site, and the brain was removed and frozen. The frozen brains were sliced on a microtome at 14-μm sections and the sections were stained with cresyl violet acetate. Infusions sites were examined under a light microscope and indicated on drawings adapted from the rat brain atlas of Paxinos and Watson (1986). Rats with improper placements were excluded from the final data analysis.

2.5.3. Data analyses

A two-factor repeated measures ANOVA for consumption type (ethanol/saccharin) and drug dose were conducted on the experimental treatments. Post-hoc comparisons between individual drug treatments were made using the Newman–Keuls or Duncan Tests. The Clear Lake ANOVA statistical program package was used for all data analyses.

3. Results

3.1. Blood ethanol concentration determinations

The blood alcohol concentration data for the P rats in the present study ($n = 10$) were evaluated with other P rats ($n = 16$) of similar age and alcohol experience to perform correlational analyses. Blood alcohol concentration determinations were conducted prior to any experimental drug treatments. Following the initial 30 min of the operant session P rats consumed amounts of the 10% (v/v) ethanol solution to yield intakes of absolute ethanol of 0.78 to 1.57 g/kg under a FR4–FR4 schedule when concurrently presented with a 0.025% (w/v) saccharin solution. Ethanol responding ranged from 140–374 responses/30 min, while blood alcohol concentrations ranged from 0 to 42 mg%. The blood alcohol concentrations correlated significantly with ethanol responding ($r = 0.65$, $P < 0.001$) and ethanol intake (g/kg) ($r = 0.64$), $P < 0.001$.

3.2. Computation of baseline measurements and data analysis for drug treatments

When rats were given i.p. injections of Tween-80 vehicle, their control ethanol responding rates were similar to animals infused with deionized water (i.e., p.o. condition). Thus, these data were averaged and used as the baseline control condition throughout the experiment. The baseline for the saccharin data was computed in a similar manner.

3.3. Ru 34000 effects on ethanol and saccharin responding: comparison using different routes of drug administration

Fig. 1 shows that i.p. Ru 34000 dose dependently reduced ethanol responding by approximately 28–96% of control levels. Significant decreases were obtained with doses ≥ 1.0 mg/kg ($P < 0.01$), but not with the 0.5 and 0.75 mg/kg doses (data not shown). In contrast, no significant effects were seen for saccharin responding, except for the 5.0 mg/kg dose condition. These response profiles resulted in a significant drug dose X response type interaction ($F(6,54) = 6.756$, $P < 0.001$). When given s.c. and p.o. Ru 34000 produced a similar profile of effects as was seen with the i.p. doses; hence, these data will not be presented. The effects on ethanol and saccharin responding with the i.p. doses were confirmed by post-hoc analyses ($P < 0.01$) (see Fig. 1).

3.3.1. Cumulative response profiles for ethanol and saccharin responding following i.p. administration of Ru 34000

Fig. 2a–b show that during the initial 10-min interval, despite the palatable saccharin reinforcer (0.025% w/v),

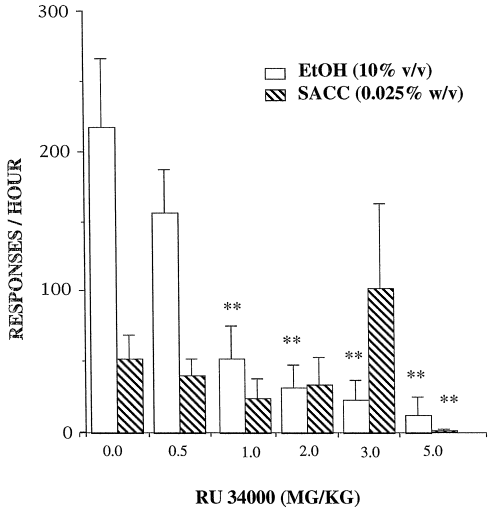

Fig. 1. Dose–response of intraperitoneal (i.p.) administration of the imidazopyrimidine Ru 34000 (0–5 mg/kg) on ethanol (10% v/v) and saccharin-reinforced (0.025% w/v) responding ($n = 10$). The Ru 34000 injections were given 5 min prior to the 1-h operant session. $* P < 0.05$ and $** P < 0.01$ vs. control vehicle values by ANOVA and post hoc Newman–Keuls test.

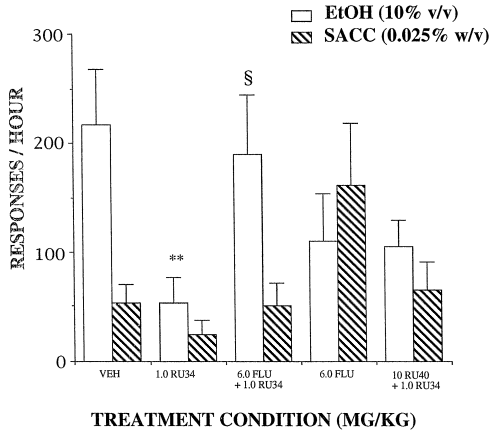

Fig. 3. Effects of i.p. Ru 34000 (1.0 mg/kg) alone, and in combination with the benzodiazepine receptor antagonists flumazenil (FLU) (6 mg/kg) and Ru 40410 (RU40) (10 mg/kg) on ethanol (10% v/v) and saccharin-reinforced (0.025% w/v) responding ($n = 10$). Flumazenil and Ru 40410 were given 20 min prior to the Ru 34000. Five minutes after the Ru 34000, animals were placed in the operant chamber for the 1-h test session. Flumazenil attenuated the Ru 34000 reduction of ethanol responding, while Ru 40410 was not effective. $** P < 0.01$ vs. control vehicle values by ANOVA and post hoc Newman–Keuls test. $\S P < 0.05$ vs. Ru 34000 (1.0 mg/kg) values by ANOVA and post hoc Newman–Keuls test, indicating reversal of the Ru 34000 reduction of ethanol responding.

the response profile for P rats under control conditions was characterized by high initial responding for the 10% (v/v) ethanol solution. Fig. 2a shows that the 0.5 mg/kg dose produced a mild suppressant effect on EtOH and saccharin responding across the 60-min session. Fig. 2a also shows a profound suppression on ethanol responding when the 1.0–5.0 mg/kg doses of Ru 34000 were given i.p. This suppression was sustained throughout the 10–60 min period. The 1.0 mg/kg dose mildly suppressed saccharin responding across the 60-min interval, while profound

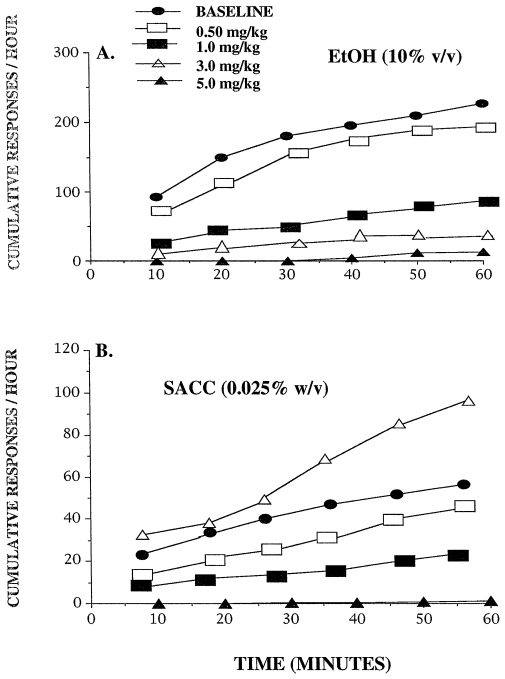

Fig. 2. (a)–(b). Cumulative responses for ethanol (10% v/v) (A) and saccharin (0.025% w/v) (B) responding following Ru 34000 (0.0 (Baseline)—5.0 mg/kg) given i.p. for rats included in Fig. 1 ($n = 10$). The effects of Ru 34000 were observed primarily on the initiation of ethanol responding (i.e., first 10 min interval).

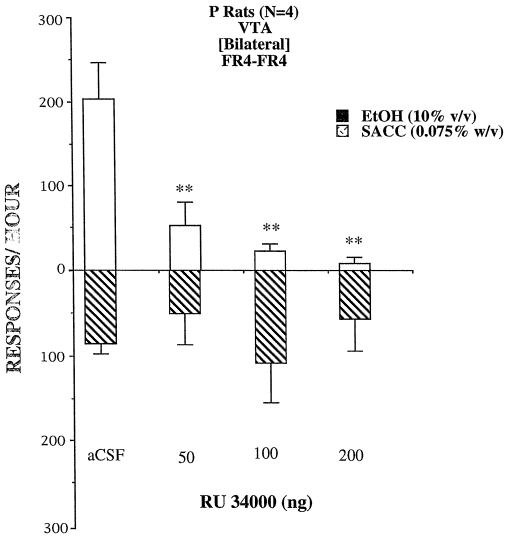

Fig. 4. Effects of bilateral intraventral tegmental area infusions of Ru 34000 (aCSF (0.0), 50, 100, 200 ng) given acutely on ethanol (10% v/v) and saccharin-reinforced (0.075% w/v) responding ($n = 4$). $** P < 0.01$ vs. aCSF (control condition values) by ANOVA and post hoc Duncan test. Similar to the systemic administrations (Fig. 1), Ru 34000 markedly suppressed ethanol responding given centrally with no effects on saccharin responding.

suppression on saccharin responding was seen with the 5.0 mg/kg dose.

3.4. Effects of flumazenil and Ru 40410 on the Ru 34000-induced suppression of ethanol responding

To determine if the Ru 34000-induced reduction was mediated via the benzodiazepine component of the $GABA_A$-benzodiazepine receptor complex, the selective antagonists flumazenil (6.0 mg/kg) and Ru 40410 (10 mg/kg) were given 20 min prior to the $\approx ED_{50}$ dose of Ru 34000 (1 mg/kg). Following the combination treatments, a significant drug dose X response type interaction ($F(4,32) = 5.34$, $P < 0.001$) emerged. Fig. 3 shows that flumazenil completely reversed the Ru 34000- induced suppression of responding ($P < 0.01$). However, the Ru 40410 treatment was not effective. Flumazenil alone did not significantly alter ethanol or saccharin responding relative to control levels ($P > 0.05$).

3.5. Effects of the ventral tegmental infusions of Ru 34000 on ethanol and saccharin responding

Fig. 4 shows data for P rats ($n = 4$) with the correct placements following direct infusions of Ru 34000 (50, 100, 200 ng) into the ventral tegmental area. Compared with the aCSF condition, Ru 34000 dose-dependently reduced ethanol responding resulting in a significant drug dose X response type interaction ($F(4,12) = 4.25$, $p < 0.0205$). Ethanol responding was reduced by 80%, 92%, and 97% of control levels with the 50, 100, and 200 ng doses, respectively. Saccharin responding was not altered by any of the drug doses. The Duncan post-hoc test confirmed that all of the Ru 34000 infusions significantly decreased ethanol responding compared with the aCSF condition.

Fig. 5 shows the histological verification for the subjects included in the data presentation of Fig. 4. Only 4/7 subjects had guide cannulas accurately placed in the ven-

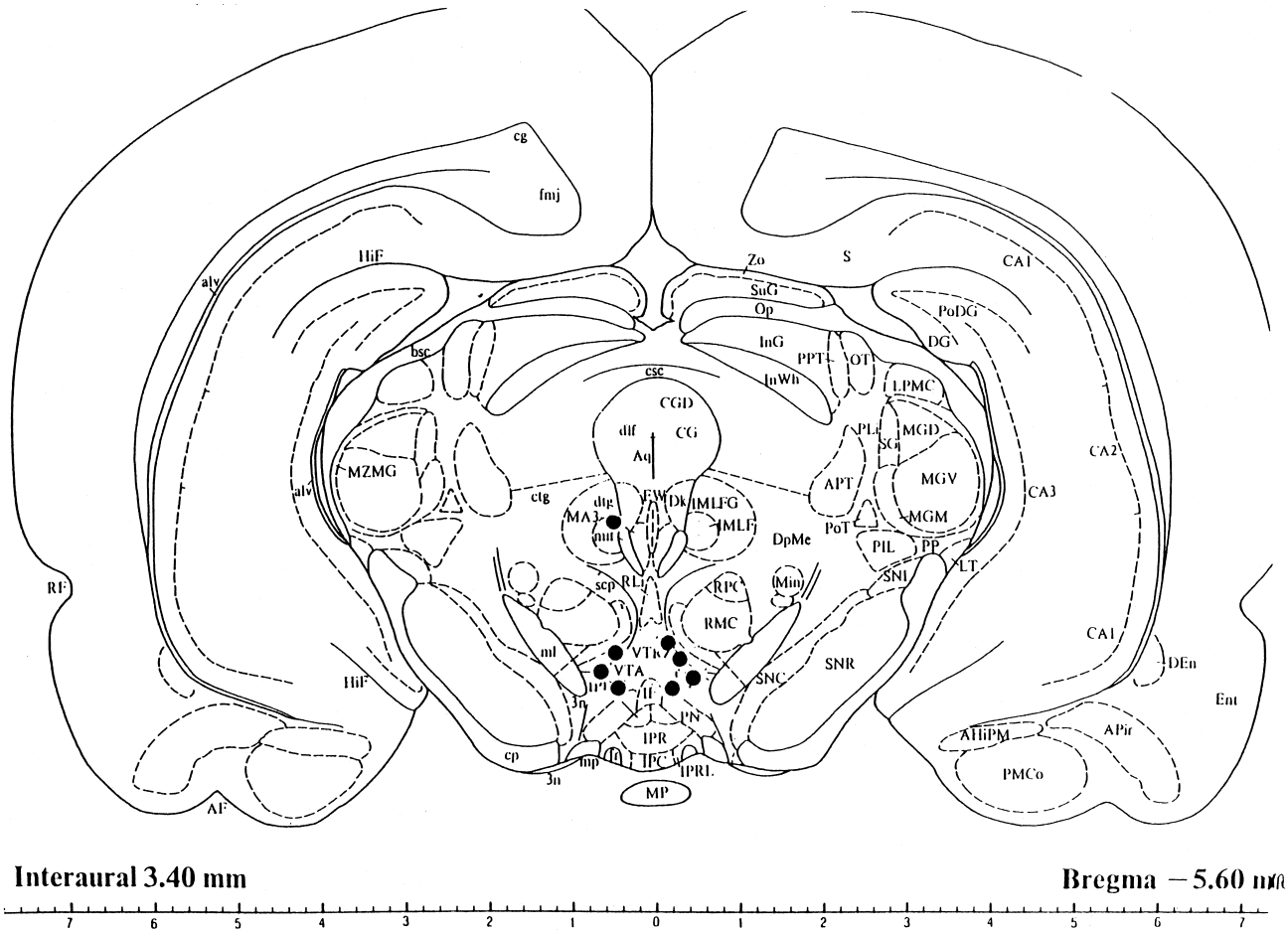

Fig. 5. Location of bilateral 28-gauge injection cannula tips for rats ($n = 4$) included in the data analyses for the intraventral tegmental area injections. Solid circles indicate placements within the ventral tegmental area. Each pair of dots represent the bilateral placement of one rat. All placements were found in the dorsal portion of the ventral tegmental area (i.e., −5.60 mm from bregma). Of the four subjects, one rat had the left guide cannulae improperly implanted dorsal to the target site, while the right guide was implanted correctly. However, the performance for this animal after drug treatment was similar to that of the three rats with the correct placements in both hemispheres. [Coronal sections are adapted from the rat brain atlas of Paxinos and Watson (1986); reproduced with permission from Academic Press].

tral tegmental area. The placements were found in the dorsal portion of the ventral tegmental area (i.e. −5.60 mm from bregma). Of these subjects, one rat had the left guide cannula improperly implanted dorsal to the target site, while the right guide was implanted correctly. However, the performance for this animal after drug treatment was similar to that of the three rats with the correct placements in both hemispheres.

4. Discussion

The major finding of the present study was that the novel imidazopyrimidine inverse agonist Ru 34000 was capable of attenuating the reinforcing properties of ethanol in an 'established animal model' of alcohol-seeking behavior (Li et al., 1991). The effects were observed independent of the drug-delivery route; hence, the bioavailability for i.p. and oral Ru 34000 appears to be relatively similar. Thus, similar to the imidazobenzodiazepine inverse agonists (e.g., RO15-4513, RO19-4603) (for review see June et al., 1994b, 1996a, 1998a), with high affinities at central benzodiazepine receptors ($K_i \leq 5.0$ nM) (Wong and Skolnick, 1992) the imidazopyrimidne Ru 34000 was found to be a potent antagonist of ethanol motivated behavior despite its relatively low binding affinity ($K_i \sim 0.98$ μM) (Bagust et al., 1990; Gardner, 1992).

In agreement with the systemic administration studies, Ru 34000 (50, 100, and 200 ng) microinjected into the ventral tegmental area dose-dependently suppressed ethanol responding. Moreover, Ru 34000 failed to alter responding for a 0.075% w/v saccharin concentration given simultaneously with ethanol. Recently, Bockstaele and Pickel (1995) demonstrated that a reciprocal GABAergic projection exists from the ventral tegmental area to the nucleus accumbens. It is likely that the reciprocity of this circuitry may play an important role in mediating ethanol reinforcement. Further, it has been demonstrated that P rats will learn an operant task to infuse ethanol directly into the ventral tegmental area (Gatto et al., 1995). Thus, the data of the present study combined with those of previous research (Bockstaele and Pickel, 1995; Gatto et al., 1995; also see Hodge et al., 1996) suggest that the $GABA_A$-benzodiazepine receptors in the ventral tegmental area may play an important role to initiate or maintain ethanol motivated behaviors.

Flumazenil (6 mg/kg) produced a complete reversal of the Ru 34000-reduction in responding in the absence of producing statistically significant effects of its own relative to the control condition. In contrast to flumazenil, Ru 40410 did not significantly attenuate the Ru 34000-reduction in responding. Thus, while Ru 34000 exhibits low affinity at central benzodiazepine receptors, its suppressant actions on ethanol responding appear to be mediated via the benzodiazepine receptor complex. The failure of Ru 40410 to reverse Ru 34000 effects may be due in part to its pharmacokinetics (Hunt et al., 1984; Gardner et al., 1987). Gardner et al. (1987) have demonstrated that the bioavailability of Ru 40410 is optimized via the i.v. route.

Previous investigators (Clements-Jewery et al., 1987; Gardner, 1992) have reported that several of the imidazo [1,2-a] pyrimidines exhibit exceptional efficacy in a wide range of behavioral tests following oral and s.c. administration. In the present study, Ru 34000 also displayed exceptional efficacy in suppressing ethanol responding via the i.p., s.c., and p.o. routes. However, across the doses tested, greater suppression was seen on saccharin responding when Ru 34000 was given i.p. These response profiles suggest that while the bioavailability with Ru 34000 may be optimized with i.p. administration, optimal selectivity for ethanol responding appears to be achieved with the oral route. However, it is important to note that the ethanol and saccharin response rates were not equated at basal levels; thus, it remains to be determined whether decreases even following oral injections are selective for ethanol.

Taken together, these findings suggest that route of administration and bioavailability are important factors in determining the ability of Ru 34000 to reduce ethanol responding. Further, previous kinetic studies on several imidazopyrimidines have shown that, while some of these agents have low affinity at central benzodiazepine receptors, this low affinity is often countered by high drug levels in the brain (in the order of 10 μg/ml) (Ager et al., 1991). Unlike Ru 34000, Ru 40410 has been shown to exhibit poor bioavailability (Gardner et al., 1987). This was further demonstrated by the fact that only the 50 and 70 mg/kg i.p doses significantly reduced ethanol responding in our preliminary research (data not shown), albeit the 70 mg/kg dose nonselectively suppressed responding for both ethanol and saccharin (data not shown). The exact mechanism(s) by which benzodiazepine receptor antagonists reduce ethanol self-administration, however, remains unclear (for review see June et al., 1996b, 1998a,b,c).

In summary, the present study extends our previous research in demonstrating that antagonism of the reinforcing properties of ethanol is not specific to inverse agonist and antagonist ligands having 'only' the imidazobenzodiazepine chemical structure. In addition, the in vivo activity of certain benzodiazepine ligands in attenuating ethanol responding is determined not only by their binding affinity and intrinsic efficacy, but also by their absorption, bioavailability at active sites, and route of administration. Thus, the different efficacies for Ru 34000 and Ru 40410 seen with the different routes of administration on ethanol responding may result from a delicate 'balance' between pharmacokinetic and pharmacodynamic factors (Gardner, 1988). The current findings also demonstrate that $GABA_A$ receptors in the ventral tegmental area may mediate in part, the Ru 34000 reduction in ethanol responding. Low affinity imidazopyrimidine ligands may be used as pharmacological probes to further understand the role of the

$GABA_A$-benzodiazepine receptor complex in mediating ethanol motivated behaviors.

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