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Pharmacology, Biochemistry and Behavior 75 (2003) 619–625
PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR
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NPI-031G (puerarin) reduces anxiogenic effects of alcohol withdrawal or benzodiazepine inverse or 5-HT_{2C} agonists

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Received 11 November 2002; received in revised form 24 February 2003; accepted 12 March 2003

Abstract

Because extracts of kudzu have been used as a hangover remedy in China for many centuries, we tested the ability of NPI-031G (puerarin), an isoflavone isolated from kudzu, to counteract anxiogenic effects associated with withdrawal from chronic alcohol exposure. NPI-O31G (50 and 150 mg/kg ip) significantly increased the social interaction and locomotor activity reduced by withdrawal from 17 days of alcohol (7%) diet. The effects of NPI-031G resembled those of the benzodiazepine antagonist, flumazenil (5 mg/kg), and the 5-HT_{2C} antagonist, SB 242084 (1 mg/kg). In a separate study, control rats were pretreated with NPI-031G (30 min) and then given the anxiogenic compounds DMCM, a benzodiazepine inverse agonist, or Ro 60 0175, a 5-HT_{2C} agonist. NPI-031G significantly counteracted the reduction in social interaction induced by either compound. To identify a potential mechanism of action of NPI-031G, synaptoneurosomes were isolated from the cerebral cortex of untreated rats and chloride uptake assays were carried out. NPI-031G did not have any effect on the stimulation of chloride uptake by muscimol, a GABA(A) agonist. However, it reduced the potentiation of muscimol-stimulated chloride uptake by flunitrazepam, a benzodiazepine agonist, at a concentration of 100 μM. A reduction in [^{3}H]flunitrazepam binding was also seen at this concentration. These findings are consistent with NPI-031G being a weak benzodiazepine site antagonist.

Keywords: NPI-031G; Alcohol withdrawal; Benzodiazepine; GABA(A) and 5-HT_{2C} receptors; Social interaction test; Anxiety-like behavior; Chloride uptake

1. Introduction

Chinese herbal remedies have been used for over 400 years to control various problems associated with alcohol drinking. Among these is their use as hangover remedies (Li, 1596). One of the most common herbs used for this purpose is an extract of kudzu (*Pueraria lobata*). Despite this long history of use to control alcohol problems, there are essentially no studies that have examined the ability of kudzu extracts or pure compounds isolated from kudzu to counteract symptoms of alcohol withdrawal in rodent models. This presentation aims to fill this gap by reporting on the effects of NPI-031G (puerarin), the major bioactive isoflavone in kudzu, on anxiety-like behavior in the social interaction test induced by alcohol withdrawal or the benzodiazepine inverse agonist, DMCM, or 5-HT_{2C} agonist, Ro 60 0175.

A number of laboratories have recently published evidence that isoflavonoids isolated from the kudzu plant are effective in reducing alcohol intake. Keung et al. demonstrated that daidzin and daidzein were the active herbal components isolated from *Radix pueraria* (kudzu) that suppressed alcohol intake in Syrian Golden hamsters (Keung, 1993, 2001; Keung and Vallee, 1993; Keung et al., 1995). Daidzin also decreases blood alcohol levels and shortens sleep time induced by alcohol (Xie et al., 1994). In a recent study, daidzin, daidzein, and NPI-031G isolated from kudzu and given orally suppressed voluntary alcohol consumption by alcohol-preferring P rats (Lin et al., 1996). These findings, together with our reports of the effective reduction of drinking by an herbal extract containing kudzu (NPI-028; Overstreet et al., 1996, 1998), provide a strong

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doi:10.1016/S0091-3057(03)00114-X

scientific basis for the traditional use of kudzu in the treatment of alcohol problems in China. However, none of these investigators nor anyone else has examined the potential anxiolytic effects of these isoflavones during alcohol withdrawal. Such information is important because if these agents do have anxiolytic properties during withdrawal, they may be useful adjuncts in detoxifying alcoholics, particularly if benzodiazepines can be avoided (Myrick et al., 2001).

2. Methods

2.1. Animals

Male Sprague–Dawley rats (Charles River) were used in these experiments. They were initially housed in groups of three or four in cages made of polycarbonate. They were housed in a standard animal room, with 22 °C, 50% humidity and 12:12 light/dark cycle (lights on at 0700). They had free access to food and water, except as described below. These experiments followed the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

2.2. Drugs

Alcohol (95%) was obtained from the University stores and was used to make up liquid diet containing 7% (w/v) alcohol (see below). NPI-031G was isolated from kudzu by countercurrent chromatography (Lee et al., 1989) and suspended in a vehicle of carboxymethylcellulose (CMC) at a concentration of 50 mg/ml. DMCM (methyl 6,7-dimethoxy-4-ethyl-*B*-carboline-3-carboxylate), a benzodiazepine inverse agonist, was obtained from Sigma (St. Louis, MO) and dissolved in a few drops of 1 N HCl; an anxiogenic dose of 0.5 mg/kg was used. Ro 60 0175, a 5-HT_{2C} agonist (Kennett et al., 2000), was obtained from Roche (Basel, Switzerland) and suspended in CMC; it was tested at an anxiogenic dose of 0.3 mg/kg. Flumazenil, a benzodiazepine antagonist, was obtained from Roche and suspended in CMC; it was tested at an anxiolytic dose of 5 mg/kg (Moy et al., 1997, 2000). SB 242084, a 5-HT_{2C} antagonist (Kennett et al., 1997), was obtained from Glaxo-Smith-Kline (Harrow, England) and suspended in CMC; it was injected at an anxiolytic dose of 1 mg/kg (Bagdy et al., 2001; Kennett et al., 1997; Knapp et al., 2000).

2.3. Alcohol diet

Following the short period of adaptation, all rats were placed on nutritionally complete liquid diets similar to those used previously in this laboratory (e.g. Frye et al., 1983; Moy et al., 1997, 2000; Overstreet et al., 2002). Briefly, the diet was a lactalbumin/dextrose-based, nutritionally complete diet (with concentrations of vitamins, minerals and other nutrients derived from ICN Research Diets). Dextrose calories in the control diet were equated with alcohol calories in the alcohol diet. After 3 days on the control diet, approximately 75% of the rats were placed on a diet containing alcohol (7%, w/v) continuously for 17 days.

A modified pair-feeding design was used. The rats maintained on the control diet were given a volume of diet equivalent to the average volume consumed the previous day by the rats maintained on the alcohol diet. The rats were weighed at weekly intervals and volumes of diet were adjusted to insure that the groups had similar body weights. Behavioral assessments were conducted after 17 days of exposure to the alcohol diet between 5 and 6 h after the removal of the alcohol. This time point was selected on the basis of previous observations of anxiety-like behavior in our laboratory (e.g. Knapp et al., 1998; Moy et al., 1997, 2000; Overstreet et al., 2002).

2.4. Anxiolytic effects in alcohol-withdrawn rats

In this experiment, rats were exposed to control or alcohol-containing diets for 17 days and then withdrawn. Approximately 4.5 h later and 30 min before the behavioral test, they received injections of one of the following treatments: CMC vehicle (12 controls and 12 ethanol diet rats), NPI-031G (10 rats at 50 mg/kg and 10 rats at 150 mg/kg) or SB 242084 (8 rats at 1 mg/kg). Flumazenil (8 rats at 5 mg/kg) is very short acting, so it was injected just 7 min before the behavioral test. Doses were based on preliminary studies (Knapp et al., 2000, 2001) and reports in the literature (Bagdy et al., 2001, Kennett et al., 1997, 2000; Moy et al., 1997, 2000). Pairs of rats with the same treatment were placed into the social interaction arena (see below) for a 5-min session 5 h after alcohol was withdrawn.

2.5. Counteraction of anxiogenic effects of DMCM and Ro 60 0175

Male Sprague–Dawley rats were adapted to the laboratory for 1 week and then assigned one of the following treatment groups: VV (vehicle followed by another vehicle 30 min later); VD (vehicle followed by 0.5 mg/kg DMCM 30 min later); PD (150 mg/kg puerarin [NPI-031G] followed by 0.5 mg/kg DMCM 30 min later); VR (vehicle followed by 0.3 mg/kg Ro 60 0175 30 min later); PR (150 mg/kg puerarin [NPI-031G] followed by 0.3 mg/kg Ro 60 0175 30 min later). Pairs of rats were injected in parallel and placed in the social interaction arena 30 min after the second injection. There were eight rats (four pairs) in each group.

2.6. Social interaction test

The social interaction test was used in these studies because it can detect the anxiolytic and anxiogenic effects

of serotonergic agents (e.g. Bagdy et al., 2001; Bristow et al., 2000; Gonzalez et al., 1998). A modification of the standard social interaction test was used to conserve animals. According to File (1980), the most sensitive procedure is to match up pairs of rats that had the same treatment on the basis of their body weights and then treat the total number of interactions by the pair as the unit of measure. However, for other experiments where the index rat may have an implanted cannula (Gonzalez et al., 1998, Irvine et al., 2001), an untreated dummy partner is used and only the interactions of the index rat are recorded. In the present studies, pairs of rats with the same treatment were placed in the arena and the social interactions initiated by each member of the pair were recorded, thereby requiring fewer rats (e.g. Overstreet et al., 2002). Statistical analyses of several data sets revealed that this approach provided the same statistical outcome as treating the scores of the pair as a unit. Furthermore, in a study of 25 pairs of rats maintained on control diet and 25 on ethanol diet, the rats exhibited essentially independent behavior, as there was no significant within-pair correlation in either group (.03 for control diet, −.13 for ethanol diet).

Experienced observers who were blind to the experimental condition carried out the social interaction test in a square open field (60 × 60 cm, with 16 squares marked out on the floor). The rats were unfamiliar with the open field and the lighting conditions were low in order to generate an intermediate level of social interaction behavior. Rat pairs were matched on the basis of alcohol intakes, body weights, and treatment conditions and placed simultaneously in the open field. During the 5-min session, line crosses (by two forepaws) and time spent in social interaction (grooming, sniffing, following, crawling over or under) were scored individually for each rat (Kampov-Polevoy et al., 2000; Overstreet et al., 2002).

2.7. Chloride uptake assays

Following decapitation of untreated, naïve rats, brains were immediately removed and placed in ice-cold saline. Cerebral cortices of four male rats were pooled for each experiment. Synaptoneurosomes were prepared and Cl^- uptake was conducted as previously described (Morrow et al., 1988; Kralic et al., 2002). The synaptoneurosomal pellet was resuspended in 6.6 volumes of ice-cold assay buffer (20 mM Hepes, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, pH 7.4) for a final protein concentration of approximately 1 mg/tube. The homogenate was aliquoted 200 μl per assay tube and preincubated at 30 °C for 12 min. In the first set of experiments, the effects of NPI-031G on muscimol-stimulated chloride uptake were investigated: Chloride uptake was initiated by addition of 0.2 μCi $^{36}Cl^-$ (NEN, Boston, MA) in the presence of muscimol (0, 3 or 100 μM) and NPI-031G (10 nM–100 μM). In the second set of experiments, the ability of 3 μM flunitrazepam to potentiate muscimol (3 μM)-stimulated chloride uptake was measured in the presence of NPI-031G (0.1–100 μM) or flumazenil (2 μM). The solution was vortexed and uptake terminated after 5 s by the addition of 4 ml of ice-cold assay buffer containing 100 μM picrotoxin with rapid vacuum filtration over G6 filters (Fisher Scientific, Pittsburgh, PA) using a single manifold filter apparatus (Hoeffer, San Francisco). Following two more washes, filters were allowed to dry and radioactive counts were determined by liquid scintillation spectroscopy. Chloride uptake measured in the absence of muscimol was subtracted from all tubes to determine muscimol-stimulated chloride. Net potentiation by flunitrazepam ± NPI-031G or flumazenil was obtained by subtracting muscimol-stimulated chloride uptake from total uptake.

2.8. Competitive radioligand binding

Following decapitation of untreated, naïve rats, brains were immediately removed and placed in ice-cold saline from which cerebral cortices were rapidly dissected over ice. [^{3}H]flunitrazepam (specific activity: 71 Ci/mmol; 2 nM, New England Nuclear, Boston, MA) competition binding assays were conducted in quadruplicate using membranes prepared by homogenization of pooled cerebral cortex from three male rats in 50 volumes wash buffer (50 mM Tris–HCl, pH 7.4). Samples were centrifuged at 40,000 × *g* for 15 min and resuspended in wash buffer twice before freezing the pellets at −80 °C overnight. Pellets were washed twice more before resuspension in assay buffer (50 mM Tris–HCl, pH 7.4, 120 mM NaCl and 5 mM KCl). The final tissue concentration was ∼1 mg/ml. Non-specific binding was determined using 10 μM flumazenil. Flumazenil (0.3 nM–100 μM) and NPI-031G (0.3 nM–100 μM) were used to compete for binding by 2 nM [^{3}H]flunitrazepam. The final assay volume of 500 μl incubated for 60 min at 0 to 4 °C. The reaction was terminated by rapid filtration under vacuum (<25 in. Hg) using Whatman GF/B filter strips. Samples were washed twice with 3-ml aliquots of assay buffer at 4 °C. Filters were dried, added to liquid scintillation cocktail and counted in a liquid scintillation counter. One site binding was evaluated by nonlinear regression analysis using Prism GraphPad (San Diego, CA) to obtain the apparent affinities.

2.9. Data analysis

Statistical analyses of the behavioral data were carried out using the GBStat software package. One-way ANOVAs initially analyzed the data. If the main effects were statistically significant, post hoc analyses were performed using Tukey's protected *t* tests. The social interaction test has a measure that is reflective of the general activity level of the rats (line crosses) as well as a measure that is reflective of the anxiety state (time spent in social interaction). Therefore, these analyses determined whether there was a selective effect of the drugs on the anxiety measure.

For chloride uptake concentration–response curves were evaluated using computerized nonlinear regression Prism Graphpad and compared using one-way ANOVA with Bonferroni's post hoc test.

3. Results

As can be seen in Fig. 1, both doses of NPI-031G partially counteracted the reduced time spent in social interaction, as did flumazenil and SB 242084 (Fig. 1, upper panel). There were highly significant differences among the groups [$F(5,54) = 13.82$, $P < .0001$], with the alcohol-withdrawn rats spending very little time in social interaction. In separate studies, it was determined that flumazenil and SB 242084 did not increase social interaction in control rats (Knapp et al., in press; Overstreet et al., 2003), and NPI-031G also did not alter time spent in social interaction (23.8 ± 3.2 s) in control rats.

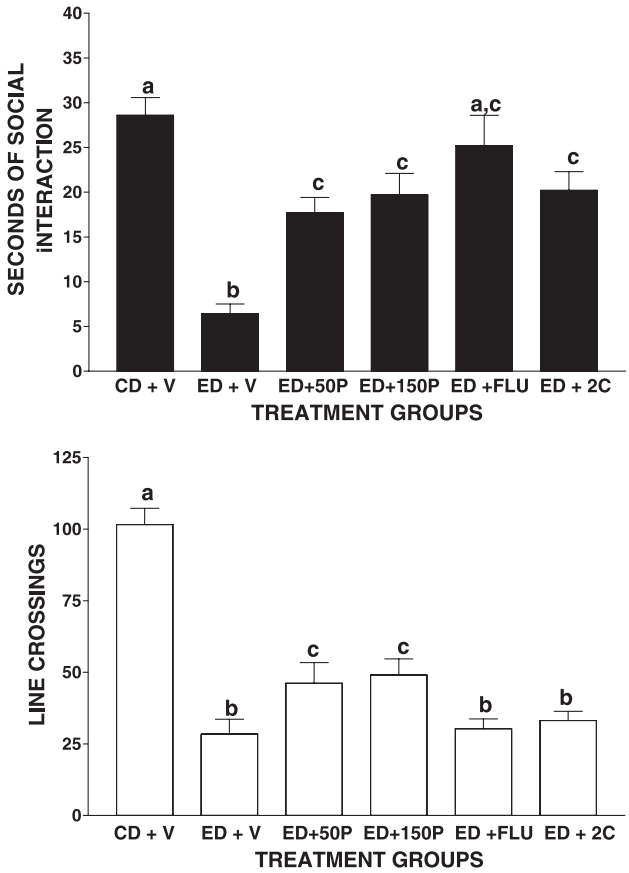

Fig. 1. Effects of NPI-031G, flumazenil, and SB 242084 on social interaction behavior in alcohol-withdrawn rats. Sprague–Dawley rats were exposed to control or alcohol-containing liquid diets for 17 days and then withdrawn for 5 h. Vehicle, NPI-031G, and SB 242084 were injected 30 min and flumazenil, 7 min, before the social interaction test. Treatments: CD + V = control diet treated with vehicle (CMC); ED + V = ethanol diet treated with vehicle; ED + 50 P = ethanol diet treated with 50 mg/kg puerarin (NPI-031G); ED + 150 P = ethanol diet treated with 150 mg/kg puerarin; ED + FLU = ethanol diet treated with 5 mg/kg flumazenil; ED + 2C = ethanol diet treated with SB 242084, a 5-HT_{2C} antagonist. Data represent the mean ± S.E.M. seconds spent in social interaction (upper panel) or line crossings (lower panel) for 8–12 rats. Groups with different letters are significantly different according to Tukey's *t* tests. There were no significant differences in the terminal alcohol intakes (mean g/kg/day ± S.E.M.) of the groups: 12.5 ± 0.7 for ED + V; 13.2 ± 0.3 for ED + 50 P; 12.7 ± 0.6 for ED + 150 P; 12.5 ± 0.6 for ED + FLU; 11.9 ± 0.4 for ED + 2C.

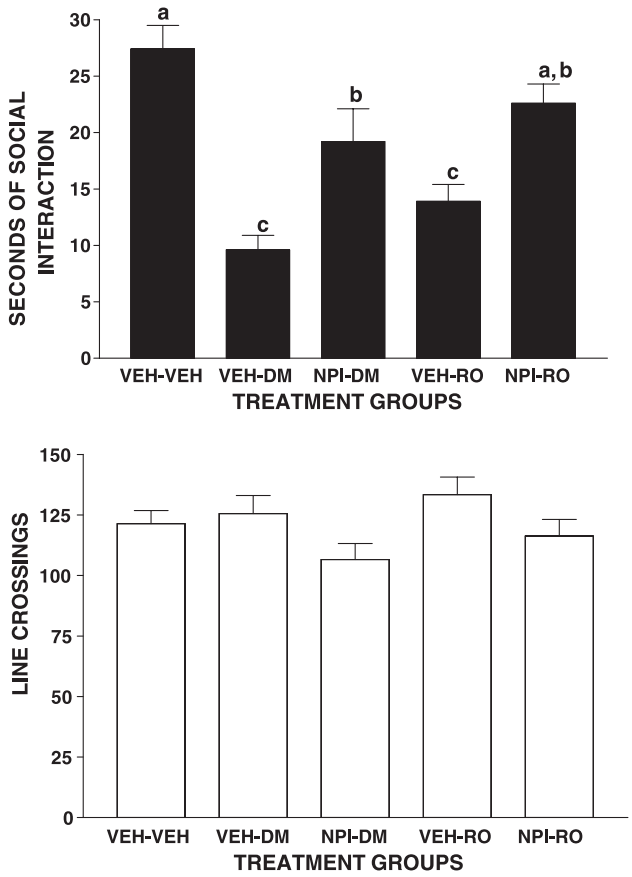

Fig. 2. Effects of pretreatment with NPI-031G on anxiogenic effects induced by DMCM or Ro 60 0175 in the social interaction test. Sprague–Dawley rats were injected with vehicle or NPI-031G 30 min prior to the injections of DMCM or Ro 60 0175. The rats were placed into the social interaction arena 30 min after these latter injections. Treatment groups: VV = vehicle given 30 min before vehicle; VD = vehicle given 30 min before DMCM (0.5 mg/kg); VR = vehicle given 30 min before Ro 60 0175 (0.3 mg/kg); PD = NPI-031G (150 mg/kg) given 30 min before DMCM; PR = NPI-031G given 30 min before Ro 60 0175. Data represent the mean ± S.E.M. seconds spent in social interaction for 8–14 rats for the upper panel and number of line crossings for the lower panel. Groups with different letters are significantly different according to Tukey's *t* tests.

Interestingly, NPI-031G also partially counteracted the reduction in line crossings induced by alcohol withdrawal, while flumazenil or SB 242084 did not (Fig. 1, lower panel). The one-way ANOVA indicated significant differences among the groups [$F(5,54) = 16.87$, $P < .0001$], with the alcohol-withdrawn rats exhibiting the fewest line crossings. Thus, the isoflavone NPI-031G partially counteracts two of the major behavioral symptoms of alcohol withdrawal.

NPI-031G could have counteracted the reduced social interaction exhibited by alcohol withdrawn rats by blocking

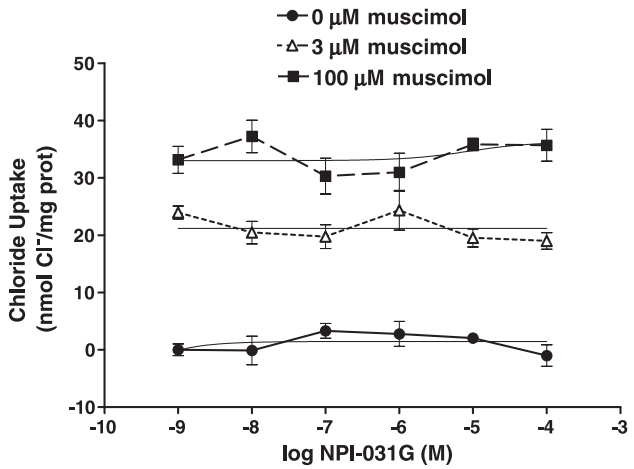

Fig. 3. Effects of NPI-031G on stimulation of chloride uptake by the GABA(A) agonist, muscimol. Synaptoneurosomes were prepared from the cerebral cortex obtained from untreated Sprague–Dawley rats. They were incubated with three concentrations of muscimol and multiple concentrations of NPI-031G. Despite substantial differences in chloride uptake by the different concentrations of muscimol, there were no differences due to the different concentrations of NPI-031G.

either benzodiazepine or 5-HT_{2C} receptors or both. As illustrated in the upper panel of Fig. 2, NPI-031G partially counteracted the reduced social interaction behavior generated by either DMCM, the benzodiazepine inverse agonist, or Ro 60 0175, a 5-HT_{2C} agonist [$F(4,67) = 13.65$, $P < .001$]. In a separate group of rats, it was established that partial counteraction of the anxiogenic effects of DMCM and Ro 60 0175 by NPI-031G cannot be explained by nonspecific additive effects. In contrast, none of the treatments

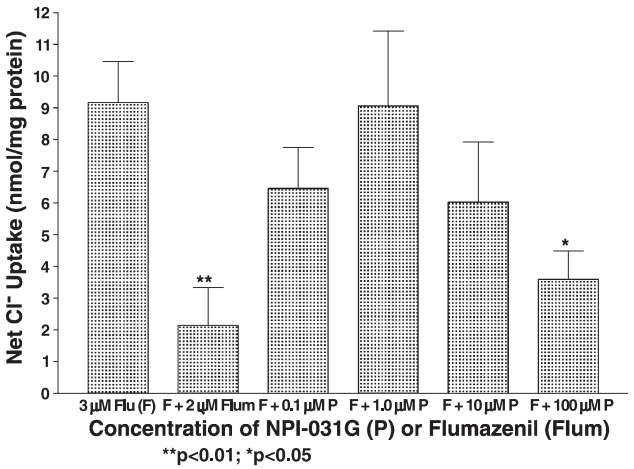

Fig. 4. Effects of NPI-031G (P) and flumazenil (Flum) on potentiation of muscimol-stimulated chloride uptake by flunitrazepam (F). Synaptoneurosomes were prepared from the cerebral cortex obtained from untreated Sprague–Dawley rats. They were incubated with standard concentrations of muscimol (3 μM) and flunitrazepam (3 μM) and varying concentrations of NPI-031G (P). The data present the net potentiation of muscimol-stimulated Cl^- uptake by flunitrazepam in the presence of flumazenil (Flum) or NPI-031G (P). ** Significant at $P < .01$ according to ANOVA and Bonferroni correction. * Significant at $P < .05$ according to ANOVA and Bonferroni correction.

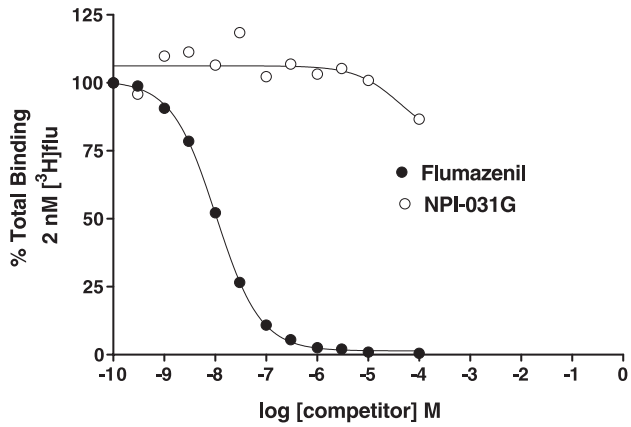

Fig. 5. Effects of NPI-031G and flumazenil on [^{3}H]flunitrazepam binding to cortical membranes. Homogenates of cerebral cortices from untreated Sprague–Dawley rats were incubated with [^{3}H]flunitrazepam and varying concentrations of flumazenil or NPI-031G.

significantly altered line crossings [$F(4,67) = 2.01$, $P > .05$], as illustrated in the bottom panel of Fig. 2.

NPI-031G did not alter stimulation of chloride uptake by muscimol, a GABA(A) receptor agonist, even at particularly high concentrations, as illustrated in Fig. 3. Different concentrations of muscimol were used so that potentiating or blocking effects could be detected, if they were present. However, NPI-031G did not influence the effects of muscimol at any concentration.

In contrast to its lack of effects on stimulation of chloride uptake by muscimol, NPI-031G did produce a small but significant inhibition of the potentiation of chloride uptake induced by flunitrazepam, a benzodiazepine agonist (Fig. 4). However, this inhibition was seen only at the highest concentration used (100 μM), whereas flumazenil, the benzodiazepine antagonist, inhibited the potentiation of chloride uptake by flunitrazepam at a much lower concentration (Fig. 4). Thus, NPI-031G appears to be acting as a weak benzodiazepine antagonist.

The data illustrated in Fig. 5 are consistent with the suggestion that NPI-031G is a weak benzodiazepine antagonist. Flumazenil significantly inhibited [^{3}H]flunitrazepam binding at low to intermediate concentrations, while NPI-031G produced a small but significant inhibition only at a concentration of 100 μM.

4. Discussion

The present study was stimulated in part by the lengthy anecdotal reports of kudzu's usefulness as a hangover remedy (Li, 1596). However, it should be stressed that hangover and withdrawal from alcohol in humans are not identical states and caution should be taken in relating these findings directly to hangover. Nevertheless, these findings indicate that NPI-031G has anxiolytic effects in alcohol-withdrawn rats and they also suggest potential mechanisms

of action. NPI-031G (puerarin) is the major bioactive isoflavone in kudzu (*pueraria lobata*) and because its sugar moiety is a C-glycoside, it is more resistant to metabolism than daidzin, another isoflavone in kudzu that has been shown to reduce alcohol intake in alcohol-preferring rats and hamsters (Keung and Vallee, 1993; Rooke et al., 2000). To date, there are no data on the ability of daidzin to counteract the anxiety-like behavior induced by alcohol withdrawal, so it is not possible at this stage to assess the relative contributions of NPI-031G and daidzin to the effects of kudzu. Nevertheless, the present findings clearly establish that NPI-031G is effective in ameliorating some of the symptoms of alcohol withdrawal in rats.

Because NPI-031G partially counteracted the reduced social interaction induced not only by alcohol withdrawal but also by injections of DMCM and Ro 60 0175, it may be acting as a benzodiazepine or 5-HT_{2C} antagonist. The fact that NPI-031G did not influence social interaction by itself under the conditions used suggests that it may be directly antagonizing the effects of DMCM and Ro 60 0175. Further study of the effects of NPI-031G on social interaction under bright light conditions that elicit anxiety-like behavior could provide useful information. It is unlikely that NPI-031G could be counteracting the effects of both compounds by interacting only with the benzodiazepine or 5-HT_{2C} receptor because we have found that these two systems that modulate anxiety are under independent control. In other words, the anxiogenic effects of DMCM, the benzodiazepine inverse agonist, and Ro 60 0175, the 5-HT_{2C} agonist, are not affected by pretreatment with SB 242084, the 5-HT_{2C} antagonist, and flumazenil, the benzodiazepine antagonist, respectively (unpublished study, 2000).

The effects of NPI-031G on chloride uptake support the hypothesis that it has benzodiazepine antagonist properties. If NPI-031G had an agonist or inverse agonist action at the benzodiazepine receptor, it would have altered muscimol-stimulated chloride uptake, but a benzodiazepine antagonist would not alter chloride uptake (Kralic et al., 2002). Like flumazenil, the classic benzodiazepine antagonist, NPI-031G significantly inhibited the potentiation of muscimol-stimulated chloride uptake by muscimol, but at a much higher dose (Fig. 4). These data are consistent with NPI-031G being a weak antagonist at benzodiazepine receptors. There has been one report indicating that puerarin (NPI-031G) inhibits [^{3}H]flunitrazepam binding (Shen et al., 1996). However, our findings demonstrated a very small though significant inhibition of binding at the highest concentration (Fig. 5). Whether NPI-031G inhibits [^{3}H]flunitrazepam binding cannot be conclusively answered by our data.

These in vitro findings, when coupled with the very robust anxiolytic effects of NPI-031G in alcohol-withdrawn and DMCM-treated rats, suggest that NPI-031G might preferentially bind to benzodiazepine inverse agonist sites on GABA(A) receptors. This hypothesis is currently being investigated.

There have been a number of sporadic reports of other biochemical actions of NPI-031G that could be relevant to its counteracting effect on the reduced social interaction seen in rats withdrawn from alcohol. For example, it has been reported that NPI-031G may inhibit calcium channels (Qian et al., 1999) and calcium channel inhibitors are known to modulate the consequences of alcohol withdrawal (Little et al., 1986; Colombo et al., 1995; Watson and Little, 2002). However, several attempts to alter the anxiogenic effects associated with alcohol withdrawal with calcium channel inhibitors have failed (File et al., 1989, 1991, 1992). Also, there has been a report indicating that NPI-031G can counteract neurotoxicity induced by kainic acid, *N*-methyl-D-aspartate (NMDA), and glutamate (Dong and Wang, 1998), a property it shares with acamprosate (Al Qatari et al., 2001). In general, however, glutamate or NMDA antagonists are not very effective in counteracting the anxiety-like behavior associated with alcohol withdrawal (Knapp et al., 2001), so it is unlikely that this mechanism could account for the anxiolytic effects of NPI-031G. Therefore, the most likely mechanism underlying the effects of NPI-031G is as a weak antagonist at the benzodiazepine receptors.

Acknowledgements

These studies were supported, in part, by funding from NIAAA (N44A02006, AA11605 and AA09013). We wish to thank Qi Yu for technical assistance.

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