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Flumazenil (Ro15-1788) Does Not Affect Ethanol Tolerance and Dependence[1]

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CHAN, A. W. K., F. W. LEONG, D. L. SCHANLEY, M. C. LANGAN AND F. PENETRANTE. *Flumazenil (Ro15-1788) does not affect ethanol tolerance and dependence.* PHARMACOL BIOCHEM BEHAV **39**(3) 659–663, 1991.—There are conflicting reports concerning whether flumazenil (Ro15-1788) can antagonize the central effects of ethanol and ethanol withdrawal reactions. C57BL/6J mice were treated chronically with an ethanol liquid diet. Control mice were pair fed an isocaloric diet containing no ethanol. These mice were injected with either Ro15-1788 (25 mg/kg) or vehicle immediately before, 14 h or 24 h before ethanol withdrawal. Under these conditions, no attenuation of the severity of handling-induced withdrawal seizures or of withdrawal hypothermia was observed in the ethanol-dependent mice injected with Ro15-1788. Likewise, there was no abolition or attenuation of tolerance to the ataxic effects (sleep time and horizontal dowel tests) and hypothermic effects of ethanol by Ro15-1788 when the mice were tested on day 3 of ethanol withdrawal. It is concluded that Ro15-1788 (25 mg/kg) has no effect on ethanol tolerance and dependence.

Ethanol Tolerance Dependence Withdrawal signs Ro15-1788

BECAUSE of the general similarity between the central pharmacological effects of ethanol and those of the benzodiazepines (BZD), investigators have studied whether the BZD receptor antagonist flumazenil (Ro15-1788) can also antagonize the central effects of ethanol. There have been anecdotal reports suggesting that Ro15-1788 could ameliorate the central depressant effects of ethanol (23,27). However, other human studies have reported that Ro15-1788 had no effect on the marked sedative effects of ethanol or ethanol's prolongation of choice reaction time (16), or on psychomotor functions in acute ethanol intoxication (10). Likewise, animal studies have shown that Ro15-1788 had no effects on the increased punishment response produced by ethanol (2,18), ethanol-stimulated $^{36}Cl^-$ uptake into brain vesicles (29), ethanol-induced release of punished responding (17), or sedative and hypothermic effects of ethanol, (5, 22, 30). In fact, there are reports of Ro15-1788 exacerbating the ethanol-induced motor incoordination (5,24) and hypothermia (24), and reduction in exploration (19). On the other hand, Belzung et al. (3) found that Ro15-1788 partly reversed some anxiolytic effects of ethanol in the mouse.

Based on the postulation that a putative endogenous inverse agonist of the BZD receptor may be responsible for the ethanol withdrawal syndrome, investigators have examined whether Ro15-1788 could antagonize the ethanol withdrawal reactions. The injection of Ro15-1788 in rats 4 to 7 h after the onset of ethanol withdrawal had no effect on the severity of withdrawal signs such as seizures and tremors (1,21). However, Ro15-1788 injected about 8 h after ethanol withdrawal in rats reversed the increased anxiety during ethanol withdrawal and the effect of Ro15-1788 appeared to be long-lasting (11). Recently, Buck reported that Ro15-1788 given 14 h before ethanol withdrawal attenuated withdrawal-related seizures and abolished tolerance to the ataxic effects of ethanol (abstract presented at the NATO Conference on the Molecular Pathology of Alcoholism, Il Ciocco, Italy, 1990). These data suggest that the time of injection of Ro15-1788 relative to the time of ethanol withdrawal may determine whether Ro15-1788 will have an effect on ethanol withdrawal symptoms. Because of its potential therapeutic usefulness, the findings of Buck need to be replicated, which is the aim of the present study.

METHOD

Animals

Male C57BL/6J mice (8 weeks old) were purchased from Jackson Laboratories, Bar Harbor, ME. They were housed singly in plastic cages in a controlled-environment room (21–22°C) on an 11/13 h light/dark cycle and received Teklad mouse diet (Teklad Mills, Winfield, IA) and tap water ad lib for 10–14 days before the beginning of an experiment.

Materials

Chocolate-flavored Sustacal liquid diet was purchased from Mead Johnson Nutritional Division (Evansville, IN), and vita-

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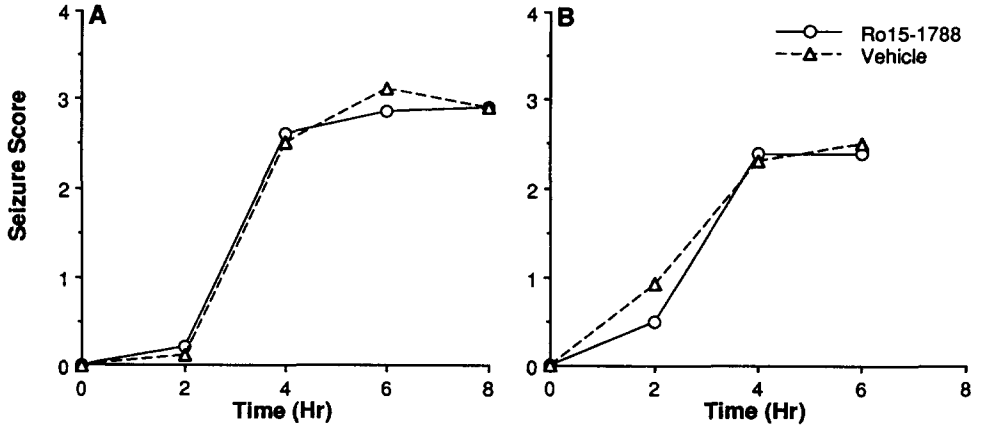

FIG. 1. Withdrawal seizure scores in ethanol-dependent mice which were injected with Ro15-1788 (25 mg/kg) or vehicle just before (A), or 24 h before (B), ethanol diet withdrawal. Zero hour was the time when the ethanol diet was withdrawn. Each group of mice had 15 mice. Because of the close proximity of data points for both groups, standard errors are not shown, but they are between 0.06 and 0.21.

min diet fortification mixture was from Nutritional Biochemicals (Cleveland, OH). Ninety-five percent ethanol, USP, was from Aaper Chemical Co. (Shelbyville, KY). Ro15-1788 was a gift from Hoffmann-La Roche, Inc. (Nutley, NJ). Diagnostic kits and reagents for ethanol analysis were purchased from Sigma Chemical Co. (St. Louis, MO).

Ethanol Diet Administration

Procedures for the preparation and administration of the ethanol diet were similar to those described previously (6,7). With this protocol, the ethanol concentration in the diet was gradually increased from 3.5% to 8% (v/v) and the diet period was 15 days. The mice had free access to the ethanol diet throughout each day. The mean ethanol intake ranged 15 to 22 g/kg/day. Control mice were pair fed an isocaloric diet (control diet) containing sucrose as a caloric substitute for ethanol. Depending on the type of tests performed after ethanol withdrawal, each subgroup of mice in the two diet treatment groups had 11–15 mice.

Ro15-1788 Injection and Ethanol Withdrawal

An injectable, fine suspension of Ro15-1788 was prepared by vigorously shaking an aqueous suspension of the BZD antagonist containing Tween-80 (3 drops per 10 ml) (5). The injection volume was 0.01 ml/g body weight. Ro15-1788 (25 mg/kg) or vehicle was injected intraperitoneally immediately before, 14 h, or 24 h before ethanol withdrawal. The dose of Ro15-1788 was chosen because it was used in our previous work involving the effects of Ro15-1788 on the actions of ethanol and chlordiazepoxide (CDP) (5). The same dose of Ro15-1788 was also found to be the most effective in precipitating CDP withdrawal in mice chronically treated with a CDP diet (8). At withdrawal the ethanol diet was replaced by the control diet, while the control mice continued to be pair fed the control diet. Rectal temperature (25) and handling-induced seizure score (7,12) were measured at the time of, and at every 2-h interval (for 8 h) after, ethanol withdrawal. Our previous work (7) has shown that maximal changes in rectal temperature and withdrawal seizure score occurred at 4 to 8 h after ethanol withdrawal. The rectal probe was inserted 2.5 cm into the rectum and a reading was taken when the temperature attained a steady level. The environmental temperature was held constant to within ± 1°C. The severity of handling-induced seizures was scored based on the numerical scoring system (from 0 to 4) described by Goldstein (12).

Test for Ethanol Tolerance

Ethanol injections were given intraperitoneally. Separate groups of mice were used for the tests described below. On day 3 of ethanol withdrawal, both the ethanol-diet and the control-diet mice, which had been injected with either Ro15-1788 or vehicle as described above, were tested for their responses to a challenge dose (see below for doses) of ethanol as follows: (a) Sleep

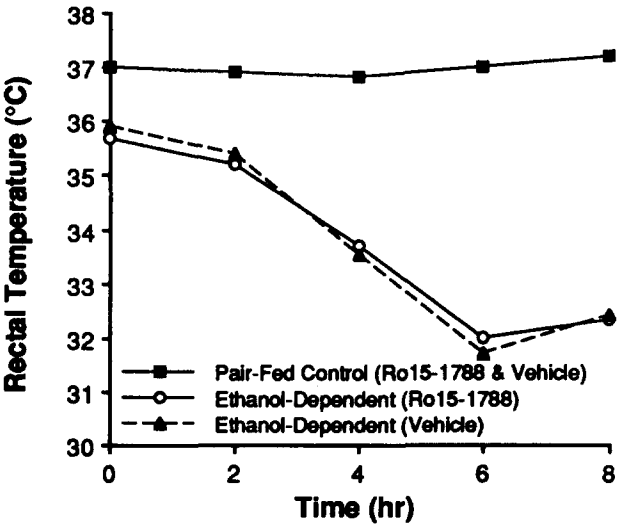

FIG. 2. Rectal temperature changes during withdrawal in ethanol-dependent mice which were injected with Ro15-1788 (25 mg/kg) or vehicle just before withdrawal of ethanol diet. Data are combined for the pair-fed control mice which were similarly injected with Ro15-1788 or vehicle. N = 15 in each group of the ethanol-dependent mice, and N = 24 in the pooled pair-fed control mice. Standard errors are in the range 0.11 to 0.40.

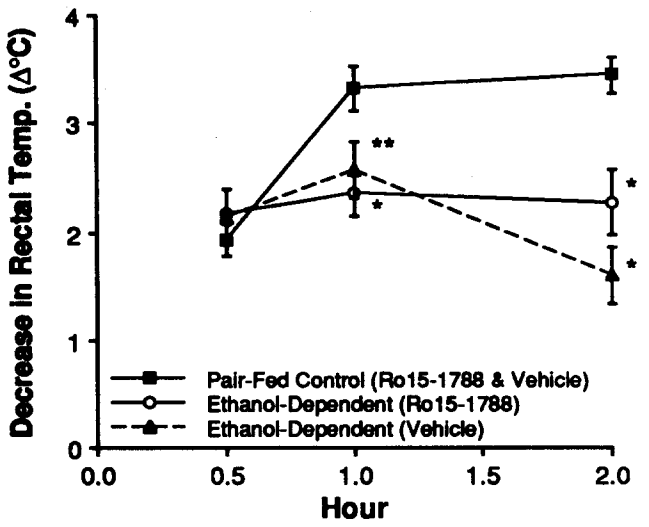

FIG. 3. Effects of prior Ro15-1788 injection (just before ethanol withdrawal) on hypothermic responses to ethanol. The mice were tested on day 3 of ethanol withdrawal with a dose of ethanol (3.5 g/kg). Values are mean decreases (relative to zero h values) ± S.E. Data for the pair-fed control groups, previously treated with Ro15-1788 or vehicle, are pooled. N = 15 and 11 for the ethanol-dependent mice injected with Ro15-1788 and vehicle, respectively. N = 24 for the pooled pair-fed control mice. *$p<0.01$, **$p<0.05$, compared to the pooled control group.

time: The mice were injected with 3.5 g/kg of ethanol. As described previously (28), sleep onset time was the interval between ethanol injection and loss of righting reflex, and sleep time was the interval between the loss and recovery of righting reflex. Each mouse was sacrificed at the time it regained its righting reflex, and the whole brain was homogenized and analyzed for ethanol levels according to published procedures (5,9); (b) Ethanol-induced hypothermia: The dose of ethanol was 3 g/kg. Rectal temperature was determined before and at 0.5, 1 and 2 h after ethanol injection; (c) Horizontal dowel test: The apparatus and testing procedure have been described previously (5,13). Briefly, the mouse was gently restrained for 20 s after ethanol injection (2.25 g/kg) and was then placed on the dowel. The fall-off time (seconds after injection) was recorded and the mouse was sacrificed by cervical dislocation immediately after falling. The whole brain was homogenized and analyzed for ethanol levels as described previously (5,9).

Statistical Analysis

Statistical significance was set at the 0.05 level. Comparison of withdrawal seizure score was done using the Mann-Whitney U-test. Other comparisons were analyzed by a computerized ANOVA program (Version 1.1, Human Systems Dynamics, Northridge, CA).

RESULTS

As shown in Fig. 1, there were no significant differences in the severity of handling-induced seizures in the ethanol-dependent mice which were injected with either Ro15-1788 or vehicle. The times of Ro15-1788 injection were just before, (Fig. 1A), or 24 h before (Fig. 1B), withdrawal of ethanol diet. Likewise, when the injections were done at 14 h before withdrawal, there were no differences in seizure scores in the two groups of mice (data not shown). The injection of Ro15-1788 also had no effect on the rectal temperature changes during ethanol withdrawal (Fig. 2). Only data for the mice injected with Ro15-1788 or vehicle just before withdrawal are shown, because the other injection schedules (14 or 24 h before withdrawal) yielded very similar results. Therefore, Ro15-1788 did not affect the severity of ethanol withdrawal.

TABLE 1

EFFECT OF Ro15-1788 ON HORIZONTAL DOWEL TEST

Treatment Group	N	Fall-Off Time (s)	Brain EtOH (mg/g)
Ethanol-dependent (Ro15-1788)	13	158.3 ± 17.5*	1.95 ± 0.07*
Ethanol-dependent (Vehicle)	12	156.4 ± 15.4*	1.99 ± 0.11*
Pair-fed Control (Ro15-1788)	12	80.3 ± 11.3	1.13 ± 0.16
Pair-fed Control (Vehicle)	12	62.8 ± 6.4	1.21 ± 0.15

Mice were injected with Ro15-1788 (25 mg/kg) or vehicle just before ethanol withdrawal. They were tested on day 3 of withdrawal. The dose of ethanol was 2.25 g/kg.

*Significantly different from the respective pair-fed control group, $p<0.001$.

Injection of Ro15-1788 at any of the three injection time points outlined in Method section also did not affect tolerance to ethanol. Therefore, only data from one injection schedule are presented. Table 1 shows that the ethanol-dependent mice injected with Ro15-1788 exhibited nearly identical tolerance to ethanol as the ethanol-dependent mice injected with vehicle, in that they had a longer fall-off time and a higher brain ethanol level at fall-off compared to the respective pair-fed controls. The

TABLE 2

EFFECT OF Ro15-1788 ON TOLERANCE TO THE ATAXIC ACTIONS OF ETHANOL

Treatment Group	N	Sleep Onset Time (min)	Sleep Time (min)	Brain EtOH at Awakening (mg/g)
Ethanol-dependent (Ro15-1788)	11	3.85 ± 1.79	41.2 ± 5.91†	3.33 ± 0.07*
Ethanol-dependent (Vehicle)	13	1.88 ± 0.08*	53.5 ± 7.98*	3.43 ± 0.07*
Pair-fed Control (Ro15-1788)	12	1.46 ± 0.05	80.9 ± 8.90	3.03 ± 0.09
Pair-fed Control (Vehicle)	12	1.66 ± 0.05	79.7 ± 9.70	3.05 ± 0.13

Mice were injected with Ro15-1788 or vehicle just before ethanol withdrawal. They were tested on day 3 of withdrawal. The dose of ethanol was 3.5 g/kg.

*Significantly different from the respective pair-fed control group, $p<0.05$.

†Significantly different from the respective pair-fed control group, $p<0.01$.

ethanol-dependent mice previously treated with either Ro15-1788 or vehicle also did not differ significantly in the magnitude of tolerance to the hypothermic effects of ethanol (Fig. 3); e.g., $F(1,38)=0.21$, for the 1 h data in the two groups of ethanol-dependent mice. There was significantly less hypothermic response in these mice compared to their respective controls at 1 or 2 h. The data for the pair-fed control mice previously injected with either Ro15-1788 or vehicle were pooled together because they showed similar hypothermic responses to the challenge dose of ethanol. For example, the mean decreases (°C) in rectal temperature at 1 h for the ethanol-dependent mice previously injected with Ro15-1788 or vehicle were 2.3 and 2.5, respectively, compared to a decrease of 3.5 for the pooled control mice, $F(1,37)=13.6$, $p<0.001$ and $F(1,33)=9.5$, $p<0.05$, respectively. Data from the sleep time test (Table 2) also support the conclusion that Ro15-1788 did not affect the expression of tolerance to the intoxicating effects of ethanol in the ethanol-dependent mice. Thus both groups of ethanol-dependent mice (Ro15-1788 or vehicle) had significantly shorter sleep times and higher brain ethanol levels at awakening than the respective pair-fed controls. For example, ANOVA of sleep time and brain ethanol data between the ethanol-dependent and pair-fed control mice (both previously injected with Ro15-1788) yielded, $F(1,21)=10.6$, $p<0.01$, and, $F(1,21)=6.1$, $p<0.05$, respectively.

DISCUSSION

Previous investigations in rats have shown that Ro15-1788 did not antagonize the ethanol withdrawal syndrome when it was injected 4–7 h after the onset of ethanol withdrawal (1,21). These results did not support the hypothesis that ethanol withdrawal reactions are produced by an endogenous ligand acting on the BZD receptors in the brain. Given the rapid metabolism of Ro15-1788 in men and animals (5, 15, 20, 26), the absence of an effect of Ro15-1788 on ethanol withdrawal signs is understandable. In the rat brain, the half-life of Ro15-1788 was only 16 min (20), and an even shorter half-life had been estimated in mice (5). Recently File et al. (11) reported that Ro15-1788, injected about 8 h after ethanol withdrawal in rats and 20 min before testing, significantly reversed the increased anxiety during ethanol withdrawal; such an effect appeared to be long-lasting. Another report (Buck, cited earlier in text) showed that Ro15-1788 given 14 h before ethanol withdrawal in mice attenuated withdrawal-related seizures, and abolished tolerance to the ataxic effects of ethanol, but did not alter tolerance to ethanol-induced hypothermia. These investigators suggested that the brief occupation of BZD receptors by Ro15-1788 resets the cellular mechanisms responsible for alcohol tolerance and dependence. However, results presented in this study did not support such a hypothesis. We found that Ro15-1788 given just before, and 14 or 24 h before ethanol withdrawal, had no effect on the severity of withdrawal handling-induced seizures and hypothermia or on the subsequent manifestations of ethanol tolerance on day 3 of withdrawal. These results do not support a possible role of Ro15-1788 in the prevention of the development of tolerance and physical dependence associated with chronic alcohol intake.

The neurochemical changes underlying the symptoms (e.g., seizures) of alcohol withdrawal are complex. The BZD are effective in suppressing withdrawal reactions because the BZD-induced neurochemical changes are opposite to many of those associated with alcohol withdrawal (4). Since Ro15-1788 is known not to have any effect on the ethanol-stimulated chloride ion uptake into brain vesicles (29), our present data are not contradictory to the suggestion that the GABA-activated chloride channel flux may mediate the development of ethanol tolerance and dependence (14). As Harris (14) has pointed out, we do not have a precise understanding of how ethanol and BZD act on GABA-activated chloride channels.

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