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Differential Effects of Ro15-1788 in Actions of Chlordiazepoxide and Ethanol[1]

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CHAN, A. W. K., M. C. LANGAN, D. L. SCHANLEY, M. L. PENETRANTE, F. W. LEONG AND L. ALDRICH-CASTANIK. *Differential effects of Ro15-1788 in actions of chlordiazepoxide and ethanol.* PHARMACOL BIOCHEM BEHAV **29**(2) 315–320, 1988.—Three behavioral tests, namely, runway activity, horizontal dowel test and hypothermia, were used to compare the effects of Ro15-1788, a specific benzodiazepine antagonist, on the common neuropharmacological actions of chlordiazepoxide (CDP) and ethanol in C57BL/6J mice. Ro15-1788 completely reversed the CDP-induced inhibition of runway activity and incoordination on a horizontal dowel, but only partially antagonized the hypothermic effects of CDP. The latter phenomenon was likely to be due to the rapid elimination of Ro15-1788, but could also be due to the fact that hypothermia might not be a specific action of CDP. The sedative actions of ethanol were not antagonized at all by Ro15-1788. In fact, Ro15-1788 potentiated the incoordinating effect of ethanol as determined by the horizontal dowel test such that mice injected with Ro15-1788/ethanol had lower brain ethanol levels than mice injected with vehicle/ethanol when they fell off the dowel. In contrast, mice injected with Ro15-1788/CDP took longer to fall off and had significantly higher CDP levels at fall-off than mice injected with vehicle/CDP. The stimulatory effect of a low dose of ethanol on runway activity was reversed by Ro15-1788. These data are discussed in terms of the possible mechanisms of actions for CDP and ethanol.

Ethanol Chlordiazepoxide Ro15-1788 Behavioral tests

THE imidazodiazepine Ro15-1788 was first reported by Hunkeler *et al.* [20] to be a benzodiazepine (BZD) receptor antagonist lacking in intrinsic activity. Since then many studies have confirmed that Ro15-1788 has distinct antagonist properties against BZD in humans (e.g., [12, 13, 32, 36]) and animals (e.g., [15, 17, 34, 41]). This BZD antagonist also precipitated withdrawal reactions in animals chronically treated with BZD [6, 11, 25, 29]. Recent investigations have shown that Ro15-1788 actually possesses intrinsic activity in a variety of behavioral, neurological, electrophysiological and biochemical preparations in both animals and man [4, 14, 19]. Because of the general similarity of the CNS depressant effects induced by BZD and ethanol, investigators have also studied whether Ro15-1788 could antagonize the action of ethanol. Scollo-Lavizzari and Matthis [37] reported that intravenous administration of Ro15-1788 to ten patients intoxicated with ethanol caused marked clinical improvement in all cases, with onset of action within the first hour. The clinical effect of Ro15-1788 was independent of the blood alcohol level. Klotz *et al.* [23] studied the acute effects of Ro15-1788 on ethanol-induced sedation in six healthy male subjects. They found that while Ro15-1788 appeared to reverse transiently the ethanol-induced changes in EEG, the BZD antagonist had no effect on the marked sedative effects of ethanol or ethanol's prolongation of choice reaction time. In animal studies Ro15-1788 had no effects on the increased punishment response produced by ethanol [2,26], the ethanol-induced release of punished responding [24], the ethanol-stimulated $^{36}Cl^-$ uptake into brain vesicles [39], or the severity of alcohol withdrawal reactions [1,28]. These data suggest that there are different effects of Ro15-1788 in actions of CDP and ethanol.

The present study compared the effects of Ro15-1788 on several behavioral tests which are known to be affected by chlordiazepoxide (CDP) or ethanol in a similar fashion. These include the runway test, horizontal dowel test and hypothermia.

METHOD

Materials

CDP-hydrochloride, diazepam, and Ro15-1788 were kindly provided by Hoffmann-LaRoche, Inc. (Nutley, NJ). Ethanol, USP, was purchased from Aaper Chemical Co. (Shellbyville, KY). Diagnostic kits and reagents for ethanol analysis were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals

Male C57BL/6J mice (8–9 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME). They

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were housed singly in plastic cages in a controlled-environment room (21–22°C) on an 11/13 hr light/dark cycle, and received Teklad mouse diet (Teklad Mills, Winfield, IA) and tap water ad lib for at least one week before the beginning of an experiment. All behavioral tests were performed in the same room in which the mice were housed.

Runway Test

The runway apparatus was a modification of that described by Pohorecky [31] for determining withdrawal signs in rats: a wooden box (92 cm L × 6.4 cm W × 6.4 cm H) with a hinged Plexiglas top. At a selected time after drug injection (see the Results section), mice were placed on one end of the runway; the number of complete runs from one end to the other during a 5-minute test period was recorded. The time elapsed before the mouse completed its first run was also recorded.

Horizontal Dowel Test

This test was used by Goldstein and Zaechelein [16] for determining ethanol tolerance. The apparatus was a horizontal hardwood dowel, 2 cm in diameter and 30 cm long; it was located at 45 cm above a soft bed of shavings. Each end of the dowel had a cardboard shield to prevent distraction of the mouse. The mouse was gently restrained for 20 sec after the appropriate drug injection (see below) and was then placed on the rod. The fall-off time (seconds after injection) was recorded and the mouse was sacrificed by cervical dislocation immediately after falling. The whole brain was dissected and processed as described in Analytical Procedure. A fall-off time of 300 sec was assigned to the mouse if it did not fall off by 5 min after drug injection.

Hypothermia

Rectal temperature was determined in mice before and at selected intervals after the appropriate drug injection (see below) according to published procedures [7,33].

Drug Injections

All injections were done intraperitoneally. For most of the experiments the dose of Ro15-1788 was 25 mg/kg, although lower doses (1 and 5 mg/kg) were also used in the horizontal dowel test. An injectable, fine suspension of Ro15-1788 was prepared by shaking vigorously an aqueous suspension of the BZD antagonist containing Tween-80 (3 drops per 10 ml). The injection volume for Ro15-1788 was 0.01 ml/g body weight. In general, Ro15-1788 was injected either before or after drug (CDP or ethanol) injection. Control animals were injected with the vehicle for Ro15-1788 either before or after drug (CDP or ethanol) or saline injection. However, because it was very imprecise to determine fall-off time in mice injected with CDP (or ethanol) followed by vehicle injection 5 min later, Ro15-1788 or vehicle was not administered as a second injection for the horizontal dowel. In most instances these mice were not able to stay on the dowel for any length of time because of the incapacitating effect of CDP or ethanol.

The exact conditions of drug injection for each particular experiment are stated in the Results section. The doses of CDP varied with the type of test. For the runway test CDP doses of 15, 30 and 40 mg/kg were used; for the horizontal dowel test the dose was 160 mg/kg; for the hypothermia test the doses were 50, 80 and 120 mg/kg. The corresponding

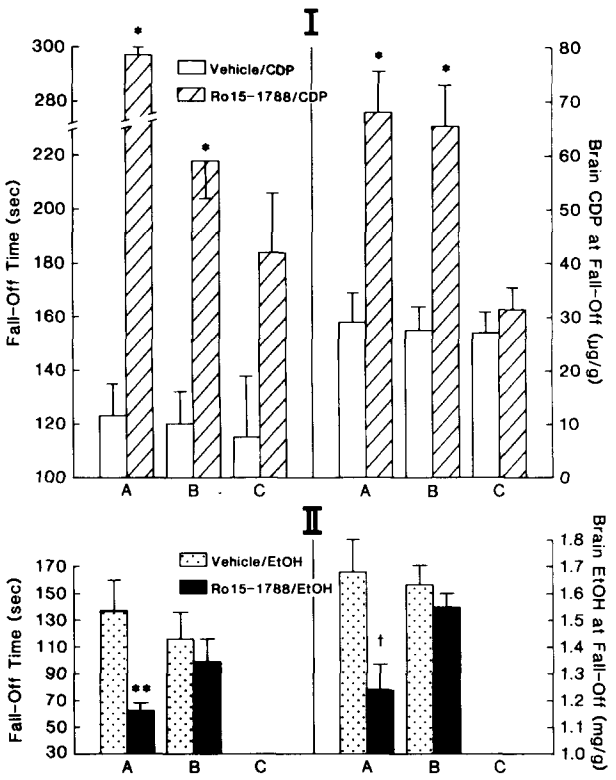

FIG. 1. Performance on horizontal dowel test after injections of Ro15-1788 plus CDP (Panel I), or with Ro15-1788 plus ethanol (Panel II). Mice were injected with the following: (a) vehicle followed by CDP (open bar); (b) Ro15-1788 followed by CDP (striped bar); (c) vehicle followed by ethanol (dotted bar); (d) Ro15-1788 followed by ethanol (solid bar). Doses for Ro15-1788, CDP and ethanol were 25 mg/kg, 160 mg/kg, and 1.85 g/kg, respectively. The time intervals (min) between the first and second injections were: A=5, B=20, C=60. N=10–11 in each treatment group and injection schedule. For mice treated with Ro15-1788/CDP in I.A, 10 out of 11 mice were each assigned a fall-off time of 300 sec. The brain CDP levels for these mice were those pertaining to the assigned time. Note the discontinuous scale for the fall-off time in Panel I. $^{*}p<0.001$; $^{**}p<<0.005$; $^{\dagger}p<0.05$.

doses for ethanol were 1.25 and 2.0 g/kg for the runway test, 1.85 g/kg for the horizontal dowel test, and 3 g/kg for the hypothermia test. The corresponding doses for the two drugs were selected (based on data from pilot studies) to give approximately equipotent effects for each drug in the runway and horizontal dowel tests, and to induce similar peak effects ($^1/_2$ hr after injection) in the hypothermia test. The use of multiple drug doses was to evaluate possible dose-related effects.

Analytical Procedure

Brain ethanol levels were analyzed enzymatically using an Ethanol Kit according to published procedures [9,16]. The whole brain was homogenized in 9 volumes of cold 3.4% perchloric acid and the precipitate was removed by centrifugation. The supernatant was used for ethanol analysis. Brain CDP and N-desmethyl-CDP (NDCDP) levels were determined by high pressure liquid chromatography according to previously published procedures [5,18]. Diazepam was used as an internal standard.

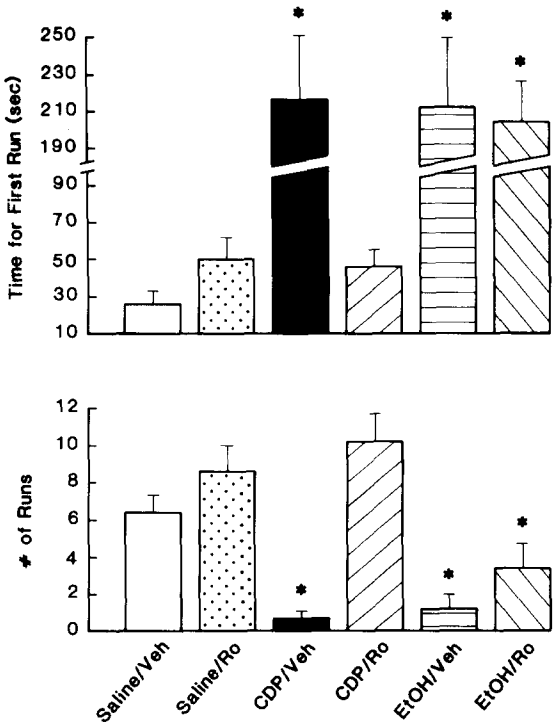

FIG. 2. Runway activity after injections of CDP/Ro15-1788 or ethanol/Ro15-1788. The doses for CDP, ethanol and Ro15-1788 were 40 mg/kg, 2 g/kg and 25 mg/kg, respectively. For the first injection, mice received one of the following: saline, CDP, or ethanol. The second injection was either Ro15-1788 or vehicle, and was given 15 min after the first injection. The mice were tested 5 min after the second injection. N=10–12 in each treatment group. Veh=vehicle; Ro=Ro15-1788. Note the discontinuous scale for the time for first run. *$p<0.001$ compared to respective control groups.

Statistical Analysis

Results were expressed as mean±S.E. Significance of the difference ($p<0.05$ being significant) was analyzed by ANOVA programs (Version 1.1, Human Systems Dynamics, Northridge, CA) with an Apple IIe computer.

RESULTS

Horizontal Dowel Test

Mice injected with Ro15-1788 behaved like mice injected with vehicle in that they could remain unaffected on the dowel for well over 5 min. The antagonism of CDP effects by Ro15-1788 is illustrated in Fig. 1, Panel I. Mice injected with CDP 5 min after vehicle injection had a mean fall-off time of 123.5 sec. In contrast, when CDP was injected 5 min after Ro15-1788 injection, 10 out of 11 mice stayed on the dowel for the allotted 300 sec (they were assigned a fall-off time of 300 sec and were sacrificed at that time), with only one mouse having a fall-off time of 271 sec. Therefore, the mean fall-off time (297.4 sec) for this group was actually an underestimation. These mice had a much higher brain CDP level at fall-off than mice injected with vehicle/CDP (Fig. 1), $F(1,20)=19.3$, $p<0.001$. In fact, there were detectable brain levels of N-desmethyl CDP (NDCDP; mean value 2.8±1.1 μg/g) in the mice which had been injected with Ro15-1788/CDP (injection schedule A). This was because some metabolism of CDP had taken place during the longer time that these mice stayed on the dowel. In contrast, no detectable brain NDCDP level was found in the mice injected with vehicle/CDP (injection schedule A). When CDP was injected 20 min after Ro15-1788 injection (Fig. 1, Panel IB) there was prolongation of the fall-off time, $F(1,20)=14.2$, $p<0.001$, as well as an increase in brain CDP level at fall-off, $F(1,20)=24.0$, $p<0.001$, compared to results in mice injected with vehicle/CDP. However, when CDP was injected 60 min after Ro15-1788 injection (Fig. 1, Panel IC), there were no significant differences in fall-off time, $F(1,19)=3.3$, $p=0.08$, or brain CDP levels, $F(1,19)=0.28$, N.S., compared to mice injected with vehicle/CDP. This indicates that the antagonistic effect of Ro15-1788 had dissipated within 1 hr. In a separate experiment one group of mice was injected with Ro15-1788 (1 mg/kg) followed by CDP injection (160 mg/kg) 5 min later. These mice had a mean fall-off time of 241±12 sec (two mice had fall-off times of 300 sec) and the mean brain CDP level at fall-off time was 66.8±6.2 μg/g. Therefore, the antagonistic effect of the 1 mg/kg dose of Ro15-1788 (5-min interval for subsequent CDP injection) was comparable to that of the 25 mg/kg dose in which the subsequent CDP injection was 20 min later (Fig. 1, Panel IB).

Contrary to the influence of Ro15-1788 on CDP actions, mice injected with ethanol 5 min after the injection of Ro15-1788 (Fig. 1, Panel IIA) had a significantly shorter fall-off time, $F(1,20)=8.9$, $p<0.005$, and a significantly lower brain ethanol level at fall-off, $F(1,20)=4.5$, $p<0.05$, compared to mice injected with vehicle/ethanol. Thus, Ro15-1788 rendered the ethanol-injected mice apparently more sensitive to ethanol. Mice injected with ethanol 20 min after the injection of vehicle or Ro15-1788 showed comparable fall-off time (Fig. 1, Panel IIB), $F(1,20)=0.43$, N.S., and brain ethanol level at fall-off, $F(1,20)=0.76$, N.S. This indicates that the effect of Ro15-1788 had dissipated.

Runway Test

Ro15-1788 (25 mg/kg) alone did not affect runway activity; mice injected with saline/vehicle did not differ from those injected with saline/Ro15-1788 in terms of the total number of runs, $F(1,21)=1.7$, $p>0.2$, and the time for first run, $F(1,21)=3.5$, $p>0.05$ (Fig. 2). These two groups were pooled in the subsequent statistical analyses involving other treatment groups. As depicted in Fig. 2, Ro15-1788, injected 15 min after CDP injection, antagonized the CDP-induced inhibition of runway activity. A comparison of the treatment groups, CDP/vehicle, CDP/Ro15-1788 and the pooled saline group yielded $F(2,42)=20.8$, $p<0.001$ for total number of runs, and $F(2,42)=33.6$, $p<0.001$ for time for first run. Mice treated with CDP/Ro15-1788 were also significantly different from those injected with CDP/vehicle [$F(1,20)=37.6$, $p<0.001$ for total runs, and $F(1,20)=22.0$, $p<0.001$ for time for first run]. Lower doses of CDP (5, 15 and 30 mg/kg) and Ro15-1788 (5 and 10 mg/kg) were also used, and similar results were obtained in regard to the inhibiting effect of CDP and the antagonism by Ro15-1788. Therefore, these data are not shown.

Ethanol (2 g/kg) also inhibited runway activity (Fig. 2). However, mice injected with ethanol/Ro15-1788 behaved similarly to those injected with ethanol/vehicle [$F(1,20)=2.4$, $p>0.1$ for total runs, and $F(1,20)=0.03$, N.S. for time for first run], indicating that Ro15-1788 did not antagonize the inhibiting action of ethanol. Both of these groups were significantly different from the pooled saline control group in terms of total number of runs, $F(2,42)=9.2$, $p<0.001$, and time for

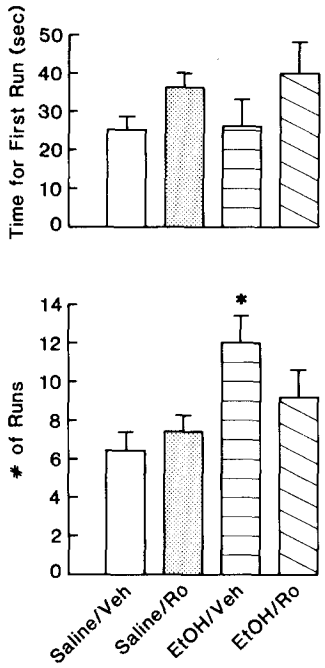

FIG. 3. Effect of Ro15-1788 (Ro) on the stimulatory action of ethanol on runway activity. The doses for Ro and ethanol were 5 mg/kg and 1.25 g/kg, respectively. The first injection was either saline or ethanol; the second injection, administered 15 min later, was either Ro or vehicle (veh). Mice were tested 5 min after the second injection. N=9–12. *$p<0.05$.

first run, $F(2,42)=22.2$, $p<0.001$.

A low dose of ethanol (1.25 g/kg) had a stimulatory effect on runway activity, causing a significant increase in the number of runs without affecting the time to complete the first run (Fig. 3). A comparison of mice treated with ethanol/vehicle with those treated with ethanol/Ro and the pooled control groups (saline/veh and saline/Ro) yielded $F(2,40)=3.3$, $p<0.05$ for the total number of runs. Injection of Ro15-1788 blocked the stimulatory action of ethanol; thus, there was no significant difference in the number of runs between mice treated with ethanol/Ro and the pooled control groups, $F(1,32)=0.03$, N.S.

Hypothermia

The hypothermic effect of CDP (80 mg/kg) was partially antagonized by Ro15-1788 (25 mg/kg), with a marked antagonism only at 1/2 hr (Fig. 4A). Although the rectal temperatures at 1/2 hr in mice injected with vehicle/CDP or Ro/CDP were both significantly lower than the zero hr readings, the former mice had a much more severe hypothermia at 1/2 hr than the latter mice. However, during the later time periods (e.g., 3, 4 and 5 hr; Fig. 4A), mice treated with Ro/CDP had significantly lower rectal temperature than mice treated with veh/CDP; e.g., for the 3 hr readings, $F(1,19)=9.6$, $p<0.01$. Similar results (not shown) were obtained when a lower dose of CDP (50 mg/kg) was used, or when the order of injections was reversed, i.e., CDP injection followed by Ro15-1788 injection. The transient antagonistic effect of Ro15-1788 (25 mg/kg) on CDP hypothermia could not be detected when a high dose of CDP (120 mg/kg) was used. Ro15-1788 alone did not have any hypothermic actions since mice injected with Ro/saline or vehicle/saline showed similar temperature

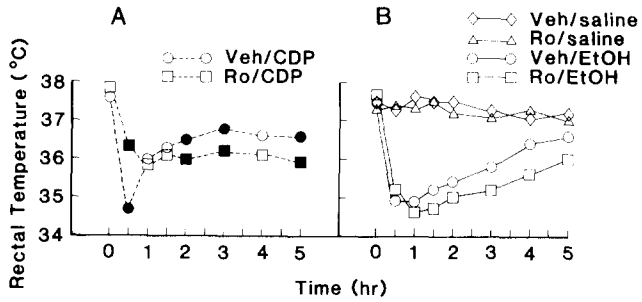

FIG. 4. Effect of Ro15-1788 on hypothermic responses to CDP (A) or ethanol (B). The first injection was either Ro15-1788 (Ro; 25 mg/kg) or vehicle (veh). The second injection, given 5 min after the first one, was one of the following: saline, ethanol (3 g/kg), or CDP (80 mg/kg). The time intervals were those after the first injection. N=9–11 in each treatment group. Values for SE were not shown because most of them were of small magnitude (less than 1%), except for larger SE values at 4 hr for A and B, and at 3 and 5 hr for B. Closed symbols indicate significant difference ($p<0.01$) between the two groups.

changes (Fig. 4B); this is in agreement with results reported by Taylor *et al.* [40], but not with those of Sugaya *et al.* [38] who found that Ro15-1788 alone had a hypothermic effect in a different strain of mice.

There was no antagonism of the hypothermic effect of ethanol by Ro15-1788 at 1/2 and 1 hr (Fig. 4B). In fact, mice injected with Ro15-1788/ethanol tended to have lower rectal temperature at the other time intervals than mice injected with vehicle/ethanol, but the differences were not significant, $F(1,19)=2.8$, $p>0.1$. Similar results were obtained when the order of injections was reversed, i.e., ethanol or saline injection before Ro15-1788 or vehicle injection.

DISCUSSION

Using the runway and horizontal dowel tests, we have shown that Ro15-1788 completely reversed the sedative action of CDP, consistent with findings of previous reports using other behavioral tests [4, 15, 17, 34, 41]. In the runway test, mice receiving the combination of CDP/Ro15-1788 consistently made more runs than mice receiving saline/vehicle or saline/Ro15-1788 (Fig. 2); however, the differences failed to reach significance. A similar phenomenon was also reported by Lister and File [27] who used head-dipping activity as the behavioral measure. As evident from the results of the horizontal dowel test (Fig. 1), the antagonistic effect of a single dose of Ro15-1788 dissipated within 1 hr. This was most likely due to the rapid metabolism of Ro15-1788. In man, the elimination half-life of Ro15-1788 has been estimated to be less than 1 hr [22,35]. We have done preliminary experiments in which serial blood samples from mice injected with a single dose of Ro15-1788 were analyzed by HPLC for the drug; from these data we estimate the plasma elimination rate of Ro15-1788 to be 3 to 8 minutes. However, it is conceivable that the antagonist can remain effectively bound to brain benzodiazepine receptors for longer durations. As a result of the antagonistic action of Ro15-1788, mice injected with Ro15-1788/CDP had significantly higher brain CDP levels when they fell off the dowel than those in mice injected with vehicle/CDP. In other words, the combination of Ro15-1788/CDP rendered the mice apparently less sensitive to the effect of CDP than mice injected with vehicle/CDP. Pharmacokinetic changes induced by Ro15-1788, if

any occurred at all, would not have contributed significantly to the observed results. This is because of the short testing time (5 min) in which the difference between the longest and the shortest fall-off times was only about 180 sec (Fig. 1, Panel I). Besides, investigators [12,23] have shown that Ro15-1788 did not affect the pharmacokinetics of BZD or ethanol.

There was only partial antagonism of the hypothermic action of CDP by Ro15-1788. The rapid metabolism of the specific benzodiazepine antagonist was probably responsible for the incomplete reversal of hypothermia. Another plausible explanation is that the hypothermic effect of CDP might not be a specific action of this drug or other benzodiazepines. The hypothermic response could be partly due to an indirect effect of CDP on other neurotransmitter systems which exert more direct control on thermoregulation. Numerous reviews (e.g., [10,21]) deal with the roles of neurotransmitters in thermal regulation. The mechanism for the hypothermic actions of BZD is yet unknown. The hypothermic effect of ethanol is essentially poikilothermic, i.e., an impairment of adaptation to both heat and cold [21]. Taylor *et al.* [40] have reported that mice injected with the combination of flurazepam and Ro15-1788 showed less hypothermia than mice injected with flurazepam alone only at 15 and 30 min after injection but not at later time periods. These findings are in agreement with our present results.

In contrast to its antagonistic action on CDP effects, Ro15-1788 did not antagonize the sedative and hypothermic effects of ethanol (Figs. 2–4). These results are in line with previous reports on the inability of Ro15-1788 to antagonize other CNS actions of ethanol [1, 2, 24, 26, 39]. In fact, our results on the horizontal dowel test indicate that the effect of ethanol on motor coordination was potentiated by Ro15-1788 (Fig. 1, Panel II). However, the stimulatory effect of a low dose of ethanol on runway activity was antagonized by Ro15-1788 (Fig. 3). We are not sure of the mechanism for this antagonistic effect of Ro15-1788 which is in contrast to its lack of antagonism of the increased punishment response produced by ethanol [21,26]. Perhaps more investigations of the effects of Ro15-1788 on other stimulatory actions of ethanol using other behavioral parameters may provide the necessary information. The lack of antagonism by Ro15-1788 on the sedative action of ethanol does not necessarily imply that the mechanisms of action of ethanol and CDP are different. A possible link between the mechanism of action of CDP and that of ethanol is the benzodiazepine receptor/GABA receptor/Cl^- ionophore complex [39]. Suzdak *et al.* [39] have postulated that ethanol's action at the level of the GABA-coupled Cl^- ion channel may underlie many of its behavioral properties. These investigators showed that ethanol stimulates GABA receptor-mediated uptake of $^{36}Cl^-$ into isolated brain vesicles; the stimulation by ethanol was not antagonized by Ro15-1788. This may be one of the reasons why we did not observe any antagonism of the sedative actions of ethanol by Ro15-1788. Further support of the involvement of GABA receptors in the actions of ethanol was provided by Martz *et al.* [30] who found that the GABA antagonists, picrotoxin and bicuculline, antagonized the incoordinating effects of ethanol.

Some investigators [3,39] have recently reported that the partial inverse benzodiazepine agonist, Ro15-4513, can antagonize certain acute effects of ethanol in mice and rats. The same agonist has been shown to specifically antagonize the ethanol-stimulated $^{36}Cl^-$ uptake into brain vesicles [39]. It remains to be investigated whether the behavioral actions of ethanol, as determined by the tests used in the present study, can be antagonized by Ro15-4513.

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