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Interactions of Ro 15-4513 With Diazepam, Sodium Pentobarbital and Ethanol in a Holeboard Test

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LISTER, R. G. *Interactions of Ro 15-4513 with diazepam, sodium pentobarbital and ethanol in a holeboard test.* PHARMACOL BIOCHEM BEHAV **28**(1) 75–79, 1987.—Ro 15-4513 (1.5 mg/kg) decreased the exploratory activity of mice in a holeboard test. This effect was reversed by diazepam (1 mg/kg), ethanol (1 g/kg) and sodium pentobarbital (15 mg/kg). Higher doses of these three agents reduced the number of exploratory head-dips, and Ro 15-4513 antagonised these effects. These observations are consistent with the suggestion that Ro 15-4513 is a partial inverse agonist at benzodiazepine receptors and acts by reducing the efficacy of GABA. Ro 15-4513's interaction with ethanol in the holeboard closely resembled its interaction with the barbiturate.

Alcohol Barbiturate Benzodiazepine Ro 15-4513 GABA Motor activity Exploration Mouse

RO 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo-[1,5-a][1,4] benzodiazepine-3-carboxylate) has a high affinity for central benzodiazepine receptors and has been used to photoaffinity label these receptors [22,29]. It possesses intrinsic behavioral effects when administered alone. For example, it lowers seizure threshold to bicuculline and pentylenetetrazole, and reduces exploratory activity in a holeboard test [1, 16, 18]. The results of these studies are consistent with the suggestion that Ro 15-4513 is a benzodiazepine receptor inverse agonist. Recently it has been reported that Ro 15-4513 is capable of antagonising some of the behavioral effects of ethanol [2, 19, 31]. The available data are unclear as to whether the antagonism is specific to alcohol or is observed when Ro 15-4513 is combined with other CNS depressants [1, 2, 19, 24, 31].

The aim of the present study was to compare the interaction of Ro 15-4513 with three agents that produce similar behavioral effects: diazepam, sodium pentobarbital and ethanol. A holeboard test was used to assess the effects of these drugs. This test allows an animal's exploration to be assessed independently of its locomotor activity [5]. The dose of Ro 15-4513 was chosen on the basis of previous experiments using the holeboard and was one that is active in other behavioral paradigms [1, 18, 31]. Two doses of diazepam, ethanol and sodium pentobarbital were used. The lower dose was aimed at producing a slight stimulant effect, and the higher doses were chosen to reduce exploration.

METHOD

Ro 15-4513 and diazepam were suspended in distilled water to which a drop of Tween 20/10 ml had been added. Sodium pentobarbital was dissolved in distilled water to which a drop of Tween 20/10 ml had been added. These drugs were administered intraperitoneally (IP) in a volume of 10 ml/kg. Ethanol was prepared as 10% and 20% (w/v) solutions in distilled water to which a drop of Tween 20 was added. All injections were made IP using an injection volume of 10 ml/kg.

Male NIH Swiss mice, weighing approximately 24 g were housed in groups of 10, maintained on a 12 hr light:12 hr dark cycle and allowed ad lib access to food and water.

The holeboard apparatus was made of Plexiglas (40×40×30 cm) and had four holes 3 cm in diameter equally spaced in the floor. Infrared photocells in the walls of the box and directly beneath each hole provided automated measures of locomotor activity, of the number of exploratory head-dips made and the duration of head-dipping.

In experiment 1, 140 mice were divided into 7 equal groups receiving either: diazepam (1 or 2 mg/kg), ethanol (1 or 2 g/kg), sodium pentobarbital (15 or 30 mg/kg) or the water/Tween vehicle. Twenty minutes after treatment half the animals in each group received an IP injection of Ro 15-4513 (1.5 mg/kg) and the remaining animals were given the vehicle. Eight minutes later each animal was placed individually in the centre of the holeboard for an 8 min test. At the end of each test the animal was removed and the box was cleaned.

In experiment 2 the interactions of a higher dose of Ro 15-4513 with the high doses of ethanol and sodium pentobarbital were investigated. Fifty-six mice were divided into 3 approximately equal groups receiving either ethanol (2 g/kg), sodium pentobarbital (30 mg/kg) or the vehicle. Twenty minutes later half the animals in each group received

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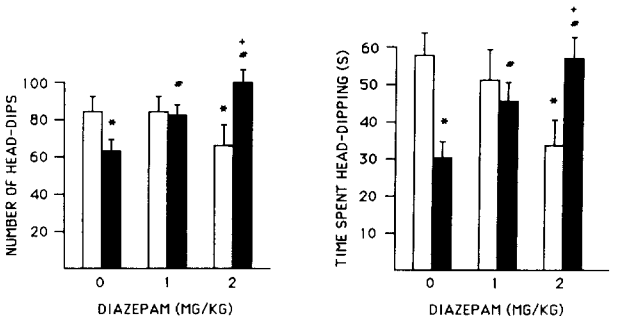

FIG. 1. The number of head-dips (left) and the time spent head-dipping (right) by mice 28 min after treatment with diazepam (1 or 2 mg/kg), or vehicle, and 8 min after treatment with Ro 15-4513 (1.5 mg/kg) or its vehicle. Scores are means±SEM, n=9 or 10 per group. *Significantly different from animals receiving the drug vehicles. # Significantly different from animals receiving Ro 15-4513 alone. + Significantly different from animals receiving diazepam (2 mg/kg) alone. □ + vehicle, ■ + Ro 15-4513 (1.5 mg/kg).

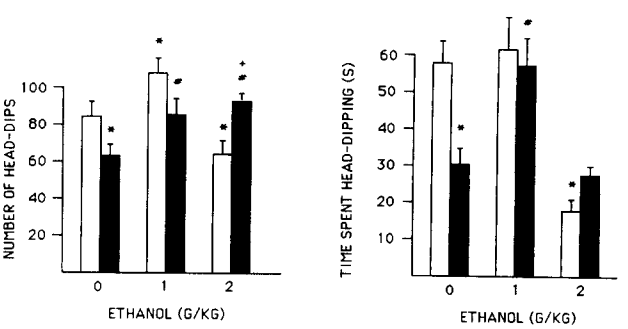

FIG. 2. The number of head-dips (left) and the time spent head-dipping (right) by mice 28 min after treatment with ethanol (1 or 2 g/kg) or vehicle, and 8 min after treatment with Ro 15-4513 (1.5 mg/kg) or its vehicle. Scores are means±SEM, n=9 or 10 per group. *Significantly different from animals receiving the drug vehicles. # Significantly different from animals receiving Ro 15-4513 alone. + Significantly different from animals receiving ethanol (2 g/kg) alone. □ + vehicle, ■ + Ro 15-4513 (1.5 mg/kg).

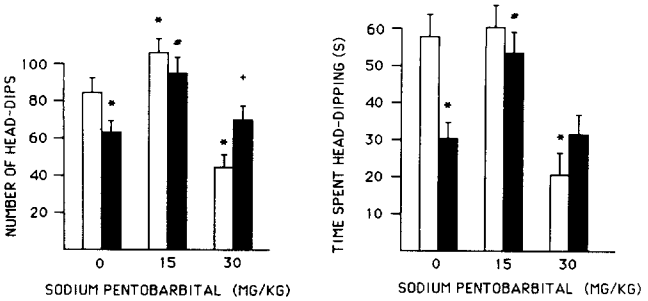

FIG. 3. The number of head-dips (left) and the time spent head-dipping (right) by mice 28 min after treatment with sodium pentobarbital (15 or 30 mg/kg), or vehicle, and 8 min after treatment with Ro 15-4513 (1.5 mg/kg) or its vehicle. Scores are means±SEM, n=9 or 10 per group. *Significantly different from animals receiving the drug vehicles. # Significantly different from animals receiving Ro 15-4513 alone. + Significantly different from animals receiving sodium pentobarbital (30 mg/kg) alone. □ + vehicle, ■ + Ro 15-4513 (1.5 mg/kg).

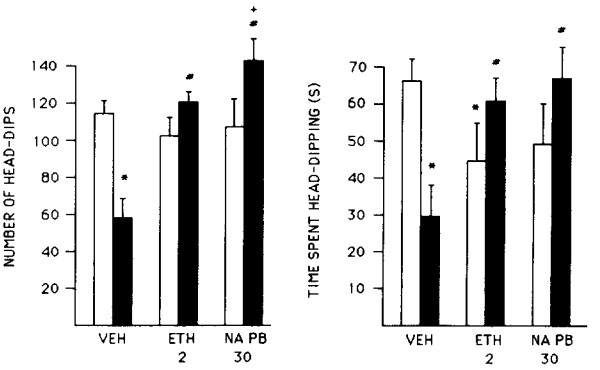

FIG. 4. The number of head-dips (left) and the time spent head-dipping (right) by mice 28 min after treatment with ethanol (ETH 2 g/kg), sodium pentobarbital (NA PB 30 mg/kg) or vehicle, and 8 min after treatment with Ro 15-4513 (3.0 mg/kg) or its vehicle. Scores are means±SEM, n=9 or 10 per group. *Significantly different from animals receiving the drug vehicles. # Significantly different from animals receiving Ro 15-4513 alone. + Significantly different from animals receiving sodium pentobarbital (30 mg/kg) alone. □ + vehicle, ■ + Ro 15-4513 (3.0 mg/kg).

Ro 15-4513 (3 mg/kg) and the rest received the water/Tween vehicle. Eight minutes after the second injection each animal was tested in the holeboard.

In all experiments data were analysed using analysis of variance. Where appropriate, simple main effects were evaluated as described in [33].

RESULTS

Exploratory Head-Dipping

For the sake of clarity, the results of experiment 1 have been presented in 3 separate figures (Figs. 1–3). It should be noted that the same controls are plotted in each figure.

In experiment 1 there were significant diazepam × Ro 15-4513 interactions in the analysis of the number of head-dips, $F(2,54)=6.2$, $p<0.005$, and the duration of head-dipping, $F(2,54)=9.4$, $p<0.001$. These interactions are most clearly interpreted by referring to Fig. 1. Ro 15-4513 alone significantly reduced both the number of head-dips, $F(1,54)=3.61$, $p<0.01$, and the duration of head-dipping, $F(1,54)=20.6$, $p<0.01$. The lowest dose of diazepam had no effect on exploration but significantly reversed the reduction in exploration caused by Ro 15-4513 ($p<0.05$). The highest dose of diazepam significantly reduced both measures of exploratory head-dipping ($p<0.05$) and this effect was completely reversed by Ro 15-4513 ($p<0.05$).

There were significant ethanol × Ro 15-4513 interactions in the analysis of both the number, $F(2,52)=7.28$, $p<0.002$, and duration, $F(2,52)=6.46$, $p<0.005$, of head-dips. These interactions are most easily discussed by referring to Fig. 2. The 1 g/kg dose significantly increased the number of head-dips ($p<0.05$). Ro 15-4513 reduced the number of head-dips made both by animals that received 1 g/kg ethanol, and by animals treated with the vehicle ($p<0.05$). In contrast to the effect of the lower dose, the higher dose of ethanol caused a significant reduction in the number of head-dips ($p<0.05$). This effect was completely reversed by Ro 15-4513 ($p<0.01$).

The lower dose of ethanol failed to alter the duration of head-dipping, but completely reversed the reduction in head-dipping caused by Ro 15-4513 ($p<0.01$). The higher dose of ethanol reduced the duration of head-dipping ($p<0.01$). A reversal of this effect by Ro 15-4513 just failed to reach significance.

Significant sodium pentobarbital × Ro 15-4513 interactions were found in the analysis of both the number of head-dips, $F(2,52)=4.3$, $p<0.02$, and the duration of head-dipping, $F(2,52)=5.7$, $p<0.01$. In Fig. 3 it can be seen that the lower dose of sodium pentobarbital increased the number of head-dips, and reversed the reduction in head-dipping caused by Ro 15-4513 ($p<0.05$). This dose did not significantly alter the duration of head-dipping, but did significantly reverse the effect of Ro 15-4513 ($p<0.05$). The higher dose of the barbiturate significantly decreased both the number of head-dips and the duration of head-dipping ($p<0.05$). Ro 15-4513 significantly reversed the effect of the barbiturate on the number of head-dips ($p<0.05$). The reversal of the reduction in the duration of head-dipping failed to reach significance (see Fig. 3).

Experiment 2 examined the effects of a higher dose of Ro 15-4513 (3 mg/kg) on the behavioral response to the high doses of ethanol and sodium pentobarbital.

Significant ethanol × Ro 15-4513 interactions were found both for the number of head-dips, $F(1,33)=19.9$, $p<0.0002$, and the duration of head-dipping, $F(1,33)=10.5$, $p<0.005$ (see Fig. 4). Ro 15-4513 reduced both measures of exploration ($p<0.01$). Ethanol alone did not significantly alter the number of head-dips, but significantly reversed the effects of Ro 15-4513 ($p<0.01$). While both ethanol and Ro 15-4513 reduced the duration of head-dipping ($p<0.05$) when administered alone, animals that received the drug combination did not differ from vehicle-treated controls.

Significant sodium pentobarbital × Ro 15-4513 interactions were again observed in the analysis of both the number of head-dips, $F(1,34)=16.1$, $p<0.0005$, and the duration of head-dipping, $F(1,34)=4.2$, $p=0.05$. Ro 15-4513 reduced the number of head-dips, and sodium pentobarbital alone failed to alter this measure. However, animals that received the combination of these two drugs made more head-dips than those that received either drug alone ($p<0.05$). A similar pattern was observed for the duration of head-dipping—animals that received the drug combination tended to spend longer head-dipping than animals that received either drug alone (see Fig. 4). It may be noted in Fig. 4 that the effects of ethanol and sodium pentobarbital alone on exploratory head-dipping were not as marked as in experiment 1. This was largely a result of a couple of animals in each group having high exploratory activities. This also led to an increased variance in these two groups.

Locomotor Activity

Table 1 shows the effects of the drugs on locomotor activity in experiment 1. Diazepam caused a significant increase in locomotor activity at both doses tested, $F(2,54)=5.04$, $p<0.01$. Ro 15-4513 completely reversed the effects of the lower dose of diazepam ($p<0.05$).

Ethanol significantly increased locomotor activity, $F(2,52)=33.9$, $p<0.0001$. There was no significant reversal of this effect by Ro 15-4513.

Sodium pentobarbital increased locomotor activity, $F(2,52)=16.1$, $p<0.0001$. Ro 15-4513 did not significantly alter this effect.

TABLE 1

LOCOMOTOR ACTIVITY SCORES OF ANIMALS 28 MIN AFTER TREATMENT WITH DIAZEPAM (1 OR 2 mg/kg), ETHANOL (1 OR 2 g/kg), SODIUM PENTOBARBITAL (PB 15 OR 30 mg/kg) OR VEHICLE, AND 8 MIN AFTER TREATMENT WITH Ro 15-4513 (1.5 mg/kg) OR ITS VEHICLE

Vehicle	Vehicle	206 ± 8
Vehicle	Ro 15-4513	203 ± 9
Diazepam 1	Vehicle	251 ± 13*
Diazepam 1	Ro 15-4513	215 ± 14†
Diazepam 2	Vehicle	252 ± 19*
Diazepam 2	Ro 15-4513	233 ± 9
Ethanol 1	Vehicle	248 ± 7*
Ethanol 1	Ro 15-4513	216 ± 10
Ethanol 2	Vehicle	331 ± 30*
Ethanol 2	Ro 15-4513	331 ± 17*
PB 15	Vehicle	243 ± 16
PB 15	Ro 15-4513	211 ± 14
PB 30	Vehicle	280 ± 19*
PB 30	Ro 15-4513	302 ± 24*

*Significantly different from mice that received both vehicles, $p<0.05$, and see text.

†Significantly different from animals that received diazepam alone, $p<0.05$.

Scores are means ± SEM, n=9 or 10 per group.

TABLE 2

LOCOMOTOR ACTIVITY SCORES OF ANIMALS 28 MIN AFTER TREATMENT WITH ETHANOL (2 g/kg), SODIUM PENTOBARBITAL (PB 30 mg/kg) OR VEHICLE, AND 8 MIN AFTER TREATMENT WITH Ro 15-4513 (3.0 mg/kg) OR ITS VEHICLE

Vehicle	Vehicle	206 ± 8
Vehicle	Ro 15-4513	182 ± 4
Ethanol 2	Vehicle	317 ± 21
Ethanol 2*	Ro 15-4513	305 ± 15
PB 30	Vehicle	329 ± 25
PB 30*	Ro 15-4513	312 ± 16

*Significant drug effect, $p<0.001$, see text.

Scores are means ± SEM, n=9 or 10 per group.

The data from experiment 2 are shown in Table 2. Ethanol again significantly increased locomotor activity, $F(1,33)=78.4$, $p<0.0001$. There was no indication that Ro 15-4513 reversed this effect. Sodium pentobarbital also significantly increased locomotor activity, $F(1,34)=65.4$, $p<0.0001$, and Ro 15-4513 failed to modify this effect.

DISCUSSION

Although considerable interest has focused on the ability of Ro 15-4513 to reverse the effects of ethanol, the intrinsic behavioral activity of this compound has received less attention. In both experiments of the present study Ro 15-4513 reduced exploratory head-dipping as we have previously reported [16]. This effect on exploration resembles that observed following treatment with other benzodiazepine receptor inverse agonists [4,17].

In experiment 1, the low doses of diazepam, ethanol and sodium pentobarbital were all capable of reversing the reductions in head-dipping caused by Ro 15-4513. These doses

did not significantly alter the duration of head-dipping when administered alone (although both ethanol and sodium pentobarbital significantly increased the number of head-dips).

The higher doses of all 3 drugs reduced exploratory head-dipping. Ro 15-4513 (1.5 mg/kg) completely reversed the effects of the higher dose of diazepam on both measures of exploration, animals receiving the drug combination behaving no differently from controls. This is consistent with other reports that Ro 15-4513 is a very potent antagonist of the behavioral effects of benzodiazepines [1,24]. It also completely reversed the reduction in the number of head-dips caused by the higher dose of ethanol, even though both drugs alone reduced this measure. A similar pattern of results was produced when Ro 15-4513 was combined with the higher dose of the barbiturate. A comparison of Figs. 2 and 3, and examination of Fig. 4 shows that the interactions of Ro 15-4513 with ethanol and with sodium pentobarbital are very similar. This contrasts with the data reported by Suzdak *et al.* [31] who found that Ro 15-4513 reversed the behavioral effects of ethanol but not sodium pentobarbital, but is in agreement with the observations of Bonetti *et al.* [2] and Nutt and Lister [24]. These investigators found that Ro 15-4513 altered the behavior of ethanol- and barbiturate-treated animals in a similar way.

Interestingly, the interactions between ethanol, sodium pentobarbital and Ro 15-4513 were most clearly seen on the measures of exploration. Ro 15-4513 failed to antagonise the locomotor stimulant effects of the high doses of these drugs. It may be noted from Table 1 that there was a tendency for Ro 15-4513 to reverse the stimulant effect of the low doses of sodium pentobarbital and ethanol. However, the reversals failed to reach statistical significance. We have found a slight, but significant reduction of the locomotor stimulant effect of ethanol by Ro 15-4513 in another experiment [17]. It might be argued that locomotion and head-dipping are incompatible behaviors and that a drug that increased locomotion would be likely to decrease exploratory head-dipping. In previous experiments using ethanol, we have found that low doses of ethanol can increase both locomotor activity and exploratory head-dipping [14,15]. This was also observed in the current experiment, where ethanol (1 g/kg) increased both the number of exploratory head-dips and locomotor activity. The observation that Ro 15-4513 partially reversed the decreases in exploration caused by the high doses of ethanol and sodium pentobarbital in experiment 1, without showing any indication of reversing the locomotor stimulant effects of these doses also suggests that reduced exploration and increased locomotion can occur independently.

There have been several reports that benzodiazepine receptor inverse agonists can reverse, at least in part, the behavioral effects of barbiturates [25,27]. This is not particularly surprising in view of the observations that inverse agonists decrease the effects of GABA, and barbiturates enhance GABAergic neurotransmission [6, 28, 30]. GABA antagonists such as pentylenetetrazole and picrotoxin also antagonise the effects of barbiturates [8]. The interactions between inverse agonists and ethanol have not been well documented. A number of studies implicate GABA in the behavioral effects of ethanol, and suggest that ethanol enhances GABAergic neurotransmission [6, 10, 12, 13, 20, 21, 23, 32]. Since several GABA antagonists antagonise the effects of ethanol [6, 8, 9, 12, 13, 20], it might be expected that inverse agonists would exert similar effects. In the holeboard test, both Ro 15-4513 and FG 7142 are capable of reversing, at least partially, the effects of ethanol on exploratory head-dipping [17]. Further, both Ro 15-4513 and FG 7142 are able to reverse the release in punished responding produced by ethanol in a conflict paradigm [3,11].

In conclusion, the results of the present experiment are in agreement with other data implicating GABA in the behavioral effects of Ro 15-4513 [2, 24, 26, 31]. Benzodiazepines, barbiturates and ethanol all potentiate the effects of GABA [7, 10, 21, 23, 30, 32], and the interactions of Ro 15-4513 with both the low and high doses of these agents are consistent with a pharmacological interaction. It is unclear to what extent Ro 15-4513's behavioral effects are related to its observed effects on chloride uptake into brain vesicles. Suzdak *et al.* [31] reported that Ro 15-4513 reversed ethanol- but not pentobarbital-stimulated chloride uptake and in the present study we found that Ro 15-4513 interacted similarly with ethanol and the barbiturate. Further, although Ro 15-4513 antagonised ethanol-stimulated chloride uptake, the inverse agonist FG 7142 did not. We have found that FG 7142 resembles Ro 15-4513 in its interaction with ethanol in the holeboard test [17].

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