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Failure of Ro 15-4513 to Antagonize Ethanol in Rat Lines Selected for Differential Sensitivity to Ethanol and in Wistar Rats

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HELLEVUO, K. AND E. R. KORPI. *Failure of Ro 15-4513 to antagonize ethanol in rat lines selected for differential sensitivity to ethanol and in Wistar rats.* PHARMACOL BIOCHEM BEHAV **30**(1) 183–188, 1988.—An imidazobenzodiazepine, Ro 15-4513, acting as a partial inverse agonist at the central benzodiazepine receptors has been recently reported to reverse efficiently the intoxicating effects of ethanol. In studies designed to delineate the role of benzodiazepine receptors in the ethanol-induced motor impairment difference between two rat lines selectively bred for high and low sensitivity to ethanol, however, we could not antagonize the effects of ethanol by Ro 15-4513 in the tilting plane and horizontal wire tests. Neither could we observe any consistent antagonism of ethanol actions in Han:Wistar rats, although we used a wide range of Ro 15-4513 doses, injected the drug intraperitoneally or intragastrically and before or after ethanol administration, and carried out the tests for motor impairment (rotarod, horizontal wire test and intoxication rating) at various times after the drug administration. The *ex vivo* assay of flunitrazepam binding in brain homogenates revealed the presence of compound(s) inhibiting the binding after administration of Ro 15-4513. Ro 15-4513 antagonized the motor impairing effects of lorazepam. In conclusion, Ro 15-4513 failed to function as a specific antagonist of moderate doses of ethanol in several tests for motor impairment in different rat lines.

Ethanol antagonism Motor impairment Ro 15-4513 Benzodiazepine receptors Selected rat lines

BENZODIAZEPINES, which are known to modify the GABAergic neurotransmission at the postsynaptic GABA/benzodiazepine receptor/chloride-ionophore level, resemble ethanol in many of their pharmacological effects [3]. The search for different benzodiazepine ligands has brought out a continuum of receptor agonists and antagonists which bind to the benzodiazepine site of the receptor complex (see [10,18]). One was the imidazobenzodiazepine Ro 15-4513 (ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a] [1,4] benzodiazepine-3-carboxylate). It is an azido analogue of the benzodiazepine receptor antagonist, Ro 15-1788, and was introduced as a photoaffinity label, binding to the same proteins as Ro 15-1788 [19]. There have been several recent reports that Ro 15-4513 is able to antagonize different actions of ethanol (see [13]). Ro 15-4513 was reported to be a partial inverse agonist at the benzodiazepine receptors [5] and an effective antagonist of behavioral actions of ethanol in mice and rats [6,21]. These findings were recently supported by Suzdak and coworkers [24] who reported that Ro 15-4513 could antagonize the intoxicating effects of ethanol *in vivo* and the ethanol-induced increase in chloride-ion flux into brain vesicles *in vitro* and by Mereu *et al.* [17] who found that Ro 15-4513 reversed the depressant action of ethanol on the firing rate of dopaminergic neurons in substantia nigra.

Previous work in our laboratory with the alcohol tolerant (AT) and alcohol non-tolerant (ANT) rat lines has also supported the involvement of GABAergic neurons in ethanol intoxication. The lines were developed by selective breeding for low (AT) and high (ANT) sensitivity to the motor impairment on the tilting plane from the same blood ethanol level [8]. It has been found that a barbiturate (barbital) and a benzodiazepine (lorazepam), induce a line difference in motor impairment similar to that with ethanol [11,23].

The initial aim of the present study was to use Ro 15-4513 to help in identifying the possible GABA/benzodiazepine receptor differences involved in the ethanol-induced motor impairment difference between the AT and ANT rat lines. When Ro 15-4513 failed to antagonize effects of ethanol in these lines, additional experiments were conducted to see if it would antagonize the intoxicating effects of ethanol on several behavioral tests in Wistar rats.

GENERAL METHOD

Subjects

Male, alcohol-insensitive AT (F_{29}–F_{30}) and alcohol-sensitive ANT (F_{29}–F_{30}) rats were bred at the Research Laboratories of the Finnish State Alcohol Company (Alko Ltd.). Male, Han:Wistar rats, were purchased from the University

of Helsinki, Department of Laboratory Animals, Helsinki. The animals were housed in group cages of 5–6 rats on a 12/12 hr light/dark cycle (lights on at 06:00 a.m.) with free access to food (standard rat chow R3, Ewos AB, Södertälje, Sweden) and water. The rats were transferred into individual cages 2 days before behavioral tests. The Wistar rats were 5–6 weeks of age and 163±2 g (mean±SEM, n=121) of body weight, and the AT and ANT rats 8 weeks of age and 277±5 g (n=37) and 283±3 g (n=36) of body weight, respectively, at the time of the testing. All the behavioral experiments were conducted between 8–12 a.m. with the person carrying out the tests not knowing the drug treatment of the animals.

Drugs

Ro 15-4513 was a gift from Hoffmann-La Roche & Co. Ltd., Basle, Switzerland. Lorazepam was purchased from Wyeth/Huhtamäki Pharmaceuticals, Turku, Finland and [N-methyl-^{3}H]flunitrazepam (specific radioactivity 3.15 TBq/mmol) from Amersham, Buckinghamshire, England. All other reagents were of analytical grade.

Ethanol was diluted with saline to 12% or 15% w/v. Lorazepam, dissolved in propylene glycol, was diluted 1:8 with saline and injected in a volume of 0.8 ml/100 g body weight. Preliminary experiments showed that at 20 min the accumulation of Ro 15-4513 (2.5 mg/kg, IP) (or [^{3}H]flunitrazepam binding inhibiting substances) into forebrain and cerebellar tissue was at least as great with a vehicle solution of 5% (w/v) gum arabic in water (0.5 ml/100 g body weight) as with other vehicles [4% Tween 80 in saline or 12% (v/v) propylene glycol in saline]. The flunitrazepam binding (% of vehicle-treated controls) were 72±10, 93±4 and 73±11 (mean±SEM, n=6) for gum arabic, Tween 80 and propylene glycol solutions of Ro 15-4513, respectively. We thus chose the gum arabic suspension as Ro 15-4513 vehicle.

Tilting Plane Test

In the tilting plane test [1,11], the animal was placed on a wire-cloth covered plane which was tilted at a constant speed from horizontal to vertical. The sliding angle of the rat was recorded. Each rat was given a pre-drug test, then injected with the drugs and tested again. The change in the sliding angle was used as the measure of motor impairment.

Rotarod Test

The "Treadmill for rats 7700" machine (Ugo Basile, Biological Research Apparatus, Milan, Italy) was used. Rats were placed on a rod, 6 cm in diameter, which rotated along its longitudinal axis at 20 rpm. The time spent on the rod before falling was recorded. Each rat was given training sessions on the day before the test until it performed at the criterion level of 120 sec on the rod. On the test day, the pre-drug performance of each rat was recorded, drug treatment given and the animal tested again. The change in performance was taken as the measure of motor impairment.

Horizontal Wire Test

The horizontal wire apparatus was kindly provided and the tests were performed as described by Bonetti and coworkers [4]. The rats were given two pre-drug tests which they all passed, i.e., each animal lifted at least one hindpaw onto the wire. Then the rats were injected with drugs, and subsequent tests were performed at various times after injection.

TABLE 1

THE EFFECT OF Ro 15-4513 ON ETHANOL-INDUCED MOTOR IMPAIRMENT IN THE ALCOHOL-INSENSITIVE AT AND ALCOHOL-SENSITIVE ANT RATS ON THE TILTING PLANE

	AT Rats		ANT Rats	
	Vehicle	Ro 15-4513	Vehicle	Ro 15-4513
Change in sliding angle (°)	23±4 (7)	19±3 (8)	26±3 (8)	27±2 (8)
Blood ethanol (mM)	59±3 (7)	54±2 (8)	51±3 (8)	53±5 (8)

Mean±SEM (n).

Ro 15-4513 (10 mg/kg, IP) was administered 15 min before ethanol (2.75 g/kg, IP) (45 min before the test). No significant differences between vehicle- and Ro 15-4513-treated groups within either rat line ($p > 0.05$, t-test).

Majchrowicz Scoring Test

The degree of ethanol intoxication was evaluated by the scoring test of Majchrowicz [15].

[^{3}H]Flunitrazepam Binding

Forebrain hemispheres were homogenized in 25 vol. of ice-cold Tris-HCl buffer (50 mM, pH 7.4, 100 mM NaCl) with Kinematica Polytron PT 10/35 for 20 sec. Aliquots of these suspensions (about 0.5 mg protein per sample measured by the fluorescamine method of Böhlen and coworkers [2]) were incubated in an ice-cold water bath for 60 min in the presence of [^{3}H]flunitrazepam (1 nmol/l) with or without added Ro 15-4513 (1 μmol/l). Incubations were terminated by addition of 4 ml of ice-cold assay buffer, quickly filtered through Whatman GF/B glass fiber filter paper using a Brandel cell harvester (model M-48R), and finally washed once with 4 ml of assay buffer. The filter discs were allowed to dry, after which 8 ml of Aquasol^R (New England Nuclear) scintillation cocktail was added, and the samples were counted for radioactivity with an LKB Wallac liquid scintillation counter (Ultrobeta 1210), using the external standard channels ratio method. Binding of [^{3}H]flunitrazepam with the above method in homogenates from naive animals was potently inhibited by Ro 15-4513 with an IC_{50} concentration of 9.6±0.4 nmol/l (mean±SEM, n=4).

Blood Ethanol Assay

Tail-tip or trunk blood samples (100 μl) were collected immediately after each experiment, except when the horizontal wire test was used (Experiments 2 and 6). Ethanol concentrations were determined by headspace gas chromatography [7]. There were no significant differences in blood ethanol concentrations between the vehicle-treated and the Ro 15-4513-treated groups in any of the Experiments 1, 3, 4 and 5 as shown in Tables 1, 3 and 4.

Statistical Analysis

Student's t-test and nonparametric Fisher exact probability test [22] were used to analyze statistical differences between different treatment groups.

TABLE 2

THE EFFECT OF Ro 15-4513 ON THE ETHANOL-INDUCED MOTOR IMPAIRMENT IN THE ALCOHOL-INSENSITIVE AT AND ALCOHOL-SENSITIVE ANT RATS ON THE HORIZONTAL WIRE

Time (min)	AT Rats		ANT Rats	
	Vehicle	Ro 15-4513	Vehicle	Ro 15-4513
10	4/9	2/7	1/7	2/6
20	5/9	4/7	2/7	2/6
30	6/9	4/7	3/7	2/6
40	6/9	4/7	3/7	1/6
50	6/9	5/7	2/7	1/6
60	6/9	4/7	2/7	1/6

Ro 15-4513 (30 mg/kg, IP) was injected 5 min after ethanol (1.6 g/kg, IP to the AT rats, 1.3 g/kg, IP to the ANT rats). The results are expressed as the number of animals per group being able to perform the task at various times after the administration of ethanol. No significant differences between vehicle- and Ro 15-4513-treated groups ($p>0.05$, Fisher test).

EXPERIMENT 1

The tilting plane test, which is being used in the selection program for the AT and ANT rat lines, was now used to observe a possible antagonistic effect of Ro 15-4513 on the motor impairment induced by ethanol in these rat lines.

Procedure

The dose of ethanol (2.75 g/kg, IP) was chosen so that both AT and ANT rats were intoxicated. It was injected 15 min after administration of Ro 15-4513 (10 mg/kg, IP). The rats were tested 30 min after administration of ethanol.

Results

There were no statistically significant ($p>0.05$, t-test) differences between the Ro 15-4513 or vehicle-treated groups within either the AT or ANT rat line (Table 1). The motor performances before drug administration were 71±1° (AT vehicle, mean±SEM) (n=7), 74±1° (AT Ro 15-4513) (n=8), 69±3° (ANT vehicle) (n=8) and 71±1° (ANT 15-4513) (n=8).

EXPERIMENT 2

Ethanol doses needed to see impairment in the tilting plane test are higher than those in the horizontal wire test. To see whether Ro 15-4513 was effective in counteracting the effects of lower doses of ethanol, we used the horizontal wire test and different ethanol doses for the AT and ANT rats.

Procedure

Ro 15-4513 (30 mg/kg, IP) or vehicle was injected 5 min after administration of ethanol. Subsequent tests were performed 10, 20, 30, 40, 50 and 60 min after administration of Ro 15-4513 or vehicle.

Results

In the pre-drug test, the rats of both rat lines were able to perform the task on the horizontal wire. There was a significant line difference ($p<0.05$, Fisher test) in the performance of the AT and ANT rats on the horizontal wire from 20 min onwards after the same dose of ethanol (1.4 g/kg, IP), the ANT rats being more affected than the AT rats (data not shown). In order to be able to test the effects of Ro 15-4513, different doses of ethanol were used for the two lines. The effects of ethanol (1.6 g/kg for the AT rats and 1.3 g/kg for the ANT rats) in the horizontal wire test could not be antagonized by Ro 15-4513 in either rat line at any time point (Table 2).

Since we did not see any effect of Ro 15-4513 in the AT and ANT rats, Wistar rats were used in subsequent experiments.

EXPERIMENT 3

In this experiment Han:Wistar rats received a high dose of Ro 15-4513 (100 mg/kg), and its effects on the rotarod performance as affected by ethanol was measured. In order to confirm that Ro 15-4513 was accumulating in the brain, the cerebral hemispheres were measured *ex vivo* for inhibition of [^{3}H]flunitrazepam binding.

Procedure

Ro 15-4513 (100 mg/kg, IP) or vehicle was administered to Wistar rats 20 min after administration of ethanol (1.5 g/kg, IP). The rats were then tested on the rotarod 10 min later and decapitated within 1 min after the test. Forebrain hemispheres were stored at −80°C for receptor binding studies that were carried out within two weeks.

Results

The high dose of Ro 15-4513 (100 mg/kg), injected intraperitoneally after ethanol, failed to antagonize the effects of ethanol on the rotarod [impairment 59±25 sec (mean±SEM) and 66±15 sec for the vehicle-treated (n=5) and Ro 15-4513-treated (n=7) groups, respectively]. There was a decrease of 68±6% in the [^{3}H]flunitrazepam binding in cerebral hemispheres of the Ro 15-4513-treated animals compared to the vehicle-treated controls suggesting that significant amounts of Ro 15-4513 were present in the brain immediately after the test.

EXPERIMENT 4

Since Bonetti and coworkers [6] used oral administration of Ro 15-4513 in their experiments, we gave various doses of Ro 15-4513 intragastrically after ethanol.

Procedure

Ro 15-4513 (3, 10 and 100 mg/kg) or vehicle was administered to Wistar rats by intragastric intubation 20 min after administration of ethanol (1.5 g/kg, IP). The rotarod test was performed 30 min after ethanol.

Results

As shown in Table 3, none of the three different doses of Ro 15-4513 significantly antagonized the effect of ethanol on the rotarod.

EXPERIMENT 5

In this experiment we followed the experimental procedure of Suzdak and coworkers [24], and gave Ro 15-4513 before ethanol administration.

Procedure

Ro 15-4513 (2.5 mg/kg, IP) or vehicle (IP) was injected to

TABLE 3

THE EFFECT OF INTRAGASTRIC ADMINISTRATION OF Ro 15-4513 ON THE ETHANOL-INDUCED MOTOR IMPAIRMENT ON THE ROTAROD IN WISTAR RATS

	Vehicle	Ro 15-4513 3 mg/kg	Ro 15-4513 10 mg/kg	Ro 15-4513 100 mg/kg
Impairment on rotarod (sec)	55±10 (19)	60±14 (7)	63±14 (7)	45±9 (20)
Blood ethanol (mM)	29±1 (19)	29±2 (7)	30±2 (7)	27±1 (20)

Mean±SEM (n).

Ro 15-4513 (IG) was administered 20 min after ethanol (1.5 g/kg, IP) (10 min before rotarod test). No significant differences between the vehicle-treated group and each of the Ro 15-4513-treated groups ($p>0.05$, t-test).

TABLE 4

THE EFFECT OF Ro 15-4513 ON ETHANOL INTOXICATION IN WISTAR RATS

	Vehicle	Ro 15-4513
Majchrowicz scores	1.5±0.2 (10)	1.6±0.2 (6)
Impairment on rotarod (sec)	84±13 (10)	70±23 (6)
Blood ethanol (mM)	32±3 (10)	38±6 (6)
Flunitrazepam binding (% of vehicle treated)	—	75±10 (6)

Mean±SEM (n).

Ethanol (2 g/kg, IP) was injected 5 min after Ro 15-4513 (2.5 mg/kg, IP), intoxication score was estimated at 11-15 min, which was promptly followed by rotarod test. No significant differences between vehicle-and Ro 15-4513-treated groups ($p>0.05$, t-test).

Wistar rats, and ethanol (2 g/kg, IP) was administered 5 min later. The degree of ethanol intoxication was evaluated by the Majchrowicz scoring test [15] at 11–15 min after administration of Ro 15-4513 or vehicle and by the rotarod test immediately thereafter. Rats were then decapitated, the forebrain hemispheres stored for receptor binding assays.

Results

Ro 15-4513 (2.5 mg/kg, IP) administered before ethanol did not reverse the effects of ethanol as measured by Majchrowicz scoring test nor did it have a significant effect on impairment on the rotarod test (Table 4). The decrease of [^{3}H]flunitrazepam binding in Ro 15-4513-treated rats suggested that it had accumulated into the brain.

EXPERIMENT 6

Finally, we compared efficacy of Ro 15-4513 to antagonize the effects of ethanol and lorazepam in the horizontal wire test with Han:Wistar rats.

Procedure

Ro 15-4513 (30 mg/kg, IP) or vehicle was injected 15 min after administration of ethanol (2 g/kg, IP) or 10 min after administration of lorazepam (4 mg/kg, IP). Subsequent tests were performed 5, 10 and 20 min after administration of Ro 15-4513 or vehicle.

Results

In the pre-drug test each animal was able to perform the task. As shown in Table 5, Ro 15-4513 could significantly antagonize the effects of lorazepam. In a preliminary experiment, also, the motor impairing effect of ethanol (1.5 g/kg, IP, 5 min before Ro 15-4513) could significantly be antagonized by Ro 15-4513 (30 mg/kg, IP) at 20 min (1 out of 5 vehicle-treated and 6 out of 6 Ro 15-4513-treated rats were able to perform the task; $p<0.05$, Fisher test). The latter finding could not, however, be confirmed in a subsequent experiment (Table 5).

GENERAL DISCUSSION

In our study, Ro 15-4513 failed to antagonize the motor impairing effects of ethanol both in the ANT and AT rat lines on the tilting plane or in the horizontal wire test. Similarly a wide dose range of Ro 15-4513, administered by different routes at various times before and after ethanol, failed to antagonize the effects of ethanol on the rotarod performance or the Majchrowicz intoxication scores in Han:Wistar albino rats. The only case in which a significant effect of Ro 15-4513 on ethanol intoxication was found was with the horizontal wire test at one time point in a preliminary study, but this finding could not be confirmed. Ro 15-4513 efficiently antagonized the effects of lorazepam in the horizontal wire test.

There are several reports of the ability of Ro 15-4513 to antagonize the intoxicating effects of ethanol. Ro 15-4513 has been reported to reverse the effects of ethanol in the horizontal wire test in rats and mice [6], in an anticonflict test in rats [24] and in locomotor activity test in rats [21]. Ethanol intoxication assessed by the Majchrowicz scoring procedure [24] and the mortality caused by ethanol [9] have also been reported to be reduced by Ro 15-4513. This imidazobenzodiazepine has been shown to have no effect on ^{35}S-TBPS binding, but it is known to antagonize the ethanol-induced decrease in cerebellar cGMP [6] and the ethanol-induced stimulation in $^{36}Cl^-$-uptake into brain vesicles [24]. There is evidence that Ro 15-4513 has partial benzodiazepine inverse

TABLE 5

THE EFFECT OF Ro 15-4513 ON THE ETHANOL- OR LORAZEPAM-INDUCED MOTOR IMPAIRMENT ON THE HORIZONTAL WIRE IN WISTAR RATS

Time (min) After Ro 15-4513 Administration	Ethanol		Lorazepam	
	Vehicle	Ro 15-4513	Vehicle	Ro 15-4513
−1	0/7	0/8	0/8	0/9
5	1/7	1/8	0/8	9/9*
10	1/7	1/8	0/8	8/9*
20	2/7	2/8	0/8	8/9*

Ro 15-4513 (30 mg/kg, IP) or vehicle was injected 15 min after ethanol (1.5 g/kg, IP) or 10 min after lorazepam (4 mg/kg, IP). The results are expressed as the number of animals per group being able to perform the task after ethanol or lorazepam injections. The significance of the differences between the vehicle- and Ro 15-4513-treated groups (Fisher test): $*p<0.01$.

agonist properties that can be blocked by Ro 15-1788 and certain other properties that cannot be blocked by it [5,17]. It has been suggested that some of these properties may explain the ability of the compound to antagonize at least partly the effects of ethanol (see [13]).

There may be several reasons for the apparent discrepancies between our results and published results of others. The poor solubility of Ro 15-4513, like benzodiazepine ligands in general, causes problems in the selection of vehicle. Tween 80-saline suspensions have been used in other studies [9,24], whereas we used 5% gum arabic. The rate of degradation of the drug in the suspension is not known, but it is unlikely that all of the drug is immediately degraded when fresh suspensions are administered. An interactive effect of the drug and vehicle might promote the effects of the drug on the receptor site. The selection of the vehicle also affects the bioavailability of drugs. At least the absorption of another benzodiazepine receptor ligand, a pyrazoloquinoline CGS 8216, has been shown to be greatly affected by the vehicle used following intraperitoneal injection [14]. The *ex vivo* inhibition of the flunitrazepam binding in our study showed, however, that Ro 15-4513 (or some other exogenous compound inhibiting the binding) remained in the brain after administration of Ro 15-4513 in gum arabic suspension, and we also found that 5% gum arabic was as good a vehicle for Ro 15-4513 as Tween 80 or propylene glycol solutions to produce inhibition of fluintrazepam binding (see the General Method section).

The route and time of administration of both ethanol and Ro 15-4513 may be critical. In many studies ethanol has been intubated intragastrically [6, 9, 21], whereas we have used intraperitoneal administration of ethanol. We did not find any difference between the intragastric or intraperitoneal administration of Ro 15-4513, nor did we see effects of Ro 15-4513 given prior or after ethanol. An additional difference to other studies is that we have used AT, ANT and Han:Wistar rats instead of Sprague-Dawley rats [9,24].

In another series of experiments the motor impairments from ethanol, barbital and lorazepam could be antagonized by picrotoxin in the AT and ANT rats in the tilting plane test [12]. These results support the hypothesis that the GABA/benzodiazepine receptor/chloride-ionophore complex is involved in the intoxicating mechanism of ethanol [16]. In the present study, however, the imidazobenzodiazepine Ro 15-4513 was not an effective ethanol antagonist even in the alcohol-sensitive ANT rats. Thus, while Ro 15-4513 is able to antagonize lorazepam-induced motor impairment, it is not an efficient and specific ethanol antagonist for use in the treatment of ethanol intoxication. This conclusion is in agreement with the results of Nutt and Lister [20] on the ability of Ro 15-4513 to fully antagonize the anticonvulsant effects of diazepam but only slightly those of ethanol.

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NOTE ADDED IN PROOF

The ability of our three different samples of Ro 15-4513 to photolabel cerebrocortical membrane benzodiazepine receptors has now been determined to estimate their integrity. Photoactivation (3 min, 366 nm) in the presence of all three samples (100 nM), followed by extensive washing, effectively decreased (30 to 50%) the specific binding of [^{3}H]Ro 15-4513 (5 nM).