Pharmacology Biochemistry & Behavior, Vol. 31, pp. 733–749. © Pergamon Press plc, 1989. Printed in the U.S.A. 0091-3057/88 $3.00 + .00

Ro 15-4513: Partial Inverse Agonism at the BZR and Interaction With Ethanol[1]

E. PIETRO BONETTI,[2] WILLY P. BURKARD, MARKUS GABL, WALTER HUNKELER, HANS-PETER LOREZ, JAMES R. MARTIN, HANNS MOEHLER, WOLFGANG OSTERRIEDER, LORENZO PIERI, PETAR POLC, J. GRAYSON RICHARDS, RUDOLF SCHAFFNER, RICHARD SCHERSCHLICHT, PETER SCHOCH AND WILLY E. HAEFELY

Pharmaceutical Research Department, F. Hoffmann-La Roche & Co., Ltd., CH-4002 Basel, Switzerland

BONETTI, E. P., W. P. BURKARD, M. GABL, W. HUNKELER, H.-P. LOREZ, J. R. MARTIN, H. MOEHLER, W. OSTERRIEDER, L. PIERI, P. POLC, J. G. RICHARDS, R. SCHAFFNER, R. SCHERSCHLICHT, P. SCHOCH AND W. E. HAEFELY. *Ro 15-4513: Partial inverse agonism at the BZR and interaction with ethanol.* PHARMACOL BIOCHEM BEHAV **31**(3) 733–749, 1988.—The imidazobenzodiazepinone derivative Ro 15-4513 has the activity profile of a partial inverse (low efficacy) agonist at the benzodiazepine receptor (BZR). It reverses central nervous depressant effects of diazepam, and, in part, of phenobarbitone and ethanol in mice, rats and cats in behavioural, electrophysiological, and neurochemical paradigms. The interaction of Ro 15-4513 with barbiturates and ethanol is due to its inverse agonistic (negative allosteric modulatory) property at the BZR, as it was reversed by the selective BZR blocker flumazenil (Ro 15-1788). In the present experiment situations, other BZR partial inverse agonists in subconvulsant or overt convulsant doses were less effective against ethanol effects than Ro 15-4513. Possible mechanisms for this differential activity of BZR inverse agonists are discussed.

Ethanol antagonism BZR ligands

Ro 15-4513, the azido analogue of the benzodiazepine receptor (BZR) blocker Ro 15-1788 (flumazenil, ANEXATE®) (Fig. 1), was designed as a photoaffinity label to the BZR. Upon photolysis Ro 15-4513 was indeed found to be covalently incorporated into proteins of the BZR (30). In a behavioural and electrophysiological characterization Ro 15-4513 was shown to antagonize not only benzodiazepine but also some barbiturate effects (3), thus behaving like a partial (low efficacy) inverse agonist at the BZR (34). Shortly thereafter, Ro 15-4513 was found to reverse acute depressant ethanol effects in behavioural, electrophysiological and neurochemical studies in mice, rats and cats (4,33). Reversal of acute ethanol effects by Ro 15-4513, both in vitro and in vivo (1, 5, 7, 14, 16, 18, 25, 27, 36, 38, 44, 45), has since been reported also from other laboratories. The mechanism of action that might underlie this effect is unclear (19, 42, 43), but has generally been interpreted in terms of intrinsic stimulant properties of Ro 15-4513 due to its inverse agonistic activity at the BZR (13, 16, 17, 20, 24, 31, 46). This paper summarizes the results of our studies performed on the intrinsic effect of Ro 15-4513 and on the interaction of Ro 15-4513 with benzodiazepine ligands, barbiturates and ethanol in a number of behavioural, electrophysiological and neurochemical experiments.

METHOD

Animals

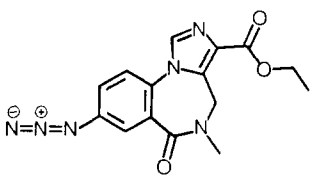
If not stated otherwise, mice and rats used in these studies were Specific Pathogens Free (SPF) albino animals of Füllinsdorf (Fü) strain. Room temperature was kept at 21°C, relative humidity was 55–65%. A 12-hr light-dark cycle was maintained in the rooms. Food and water were allowed ad lib.

Acute Toxicity

In a dose range finding study (N=10 per dose) mice were given Ro 15-4513 (suspended in acacia) up to 5000 mg/kg by gastric tube (PO) and observed for lethality at 24 hours, 3 and

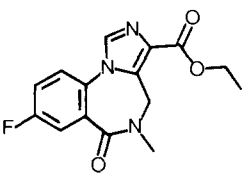
[1]Abstracts of part of this work have been presented at the Eighth European Neuroscience Congress, the Hague, The Netherlands, September 11th–15th, 1984, at the Summer Meeting of the British Pharmacological Society, Southampton, U.K., July 17th–19th, 1985, and at the 17th Annual Meeting of the Society for Neuroscience, New Orleans, LA, November 15th, 1987, at the Symposium "Antagonizing the behavioural effects of ethanol using drugs that act at the benzodiazepine/GABA receptor macromolecular complex."

[2]Requests for reprints should be addressed to E. Pietro Bonetti.



Ro 15-4513

Ethyl 8-azido-5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5-a] [1,4] benzodiazepine-3-carboxylate



Ro 15-1788, FLUMAZENIL

Ethyl 8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo [1,5-a] [1,4] benzodiazepine-3-carboxylate, **ANEXATE®**

FIG. 1. The structural diagrams of Ro 15-4513 and Ro 15-1788 (flumazenil).

8 days after administration under conventional light and, up to 14 days, under long-wave UV light irradiation.

Behavioural Studies

Observation of free behaviour. The free behaviour of mice and rats was observed by conventional techniques as described previously (2). Ro 15-4513 was tested up to 5000 mg/kg PO in mice and in rats.

Induction of loss of righting reflex (LRR). In mice, Ro 15-4513 (100 mg/kg PO) and, for comparison, the BZR partial inverse agonist Ro 15-3505 (9) (10 mg/kg PO), were administered 15 min prior to either ethanol (4700 mg/kg injected intraperitoneally, IP) or hexobarbitone (125 mg/kg IP). The animals were placed gently on their side after the ethanol or hexobarbitone injection and the time to the spontaneous reappearing of the righting reflex was measured.

Palatable food consumption. It has previously been demonstrated that inverse agonists at the BZR induce hypophagia in nondeprived rats presented with palatable food, whereas BZR full agonists (as well as some barbiturates) induce hyperphagia (6,23). In the present experiments, rats experienced in such a palatable food consumption paradigm and weighing approximately 200–300 g were used. A dose of Ro 15-4513 (1 mg/kg IP) which, when administered alone, failed to affect food intake, was given in an attempt to attenuate the hyperphagic effects of diazepam (5 mg/kg PO) and phenobarbitone (30 mg/kg PO). The treatment combinations in one experiment were: vehicle/vehicle, diazepam/vehicle, vehicle/Ro 15-4513, and diazepam/Ro 15-4513. In a second experiment, using another group of rats, the combinations were the same except that phenobarbitone was given in place of diazepam. Each treatment combination was administered to 8 rats. The rats first received oral administration of diazepam, phenobarbitone, or vehicle followed 15 min later by an injection of either Ro 15-4513 or vehicle. During this period the rats were kept in their home cages in the absence of both food and water. Thirty min after the initial administration, the rats were placed in a plastic cage (26×20×13 cm) with a water bottle attached and a preweighed piece of boiled potato. Consumption of this palatable food was measured for a 30-min session. The test compounds were prepared in 0.3% (v/v) Tween 80 and administered in a volume of 2 ml per kg body weight. Each rat received only a single treatment combination: the effects of these combinations were analyzed with the Mann-Whitney U-test.

Spontaneous locomotor activity. Male rats (180–200 g) were placed in groups of 3 into round covered activity cages (2,32). After 30 min of adaptation during which the animals explored, ethanol (3000 mg/kg) or saline was administered PO at 9.30 a.m. At 9.45 a.m. Ro 15-4513 (3–30 mg/kg) or the BZR partial inverse agonist FG 7142 (11) (10–100 mg/kg), both compounds being suspended in water with 0.3% Tween 80, were given PO.

Spontaneous movements were counted during 2 hr after drugs or vehicle and recorded automatically. Statistical evaluation was performed as reported (2,32).

Rotating rod. Female albino mice (Charles River, 19–21 g) were used. The test (32) consisted of placing the mice on a horizontal metal rod rotating twice per min, 30 min after PO administration of the test substance (suspended in 0.3% Tween 80), and measuring the time the mice remained on it.

Chimney test. Female albino Charles River mice (19–21 g) were used. Thirty min after PO administration of the test substance (suspended in 0.3% Tween 80), each mouse was introduced into one end of a glass tube (32) and gently pushed from behind with a cotton padded rod towards the opposite end whilst turning vertically the tube on the bench; in this way the mouse was placed head down at the bottom end of the bench. The number of animals unable to reach the 20 cm mark within 30 sec was noted.

Horizontal wire test (HWT). Male mice (18–20 g) and rats (70 to 80 g) were lifted by the tail and allowed to grasp a horizontally strung wire with their forepaws (2). The number of animals on a total of 10 per treatment group was then assessed which were unable to grasp, within 3 seconds, the wire with at least one hindpaw as well. In control groups administered the vehicle (acacia) this number was constantly found to be zero. Both impairment and reversal of impairment of performance was studied. ED values were computed by probit analysis by taking the number of animals out of 10 showing the maximum impairment or impairment reversal at the different doses.

Induction of seizures by chemical and physical convulsants. Pentylenetetrazol (PTZ, 120 mg/kg IP, dissolved in saline) or 3-mercaptopropionic acid (3-MPA, 48.8 mg/kg IP, dissolved in distilled water) elicited emprosthotonus and tonic extension of fore- and hindlimbs in all control mice within 4 min after administration. Protection by Ro 15-4513 (1–100 mg/kg PO, suspended in 5% acacia), was studied by administering it 1 hr before PTZ or 3-MPA, respectively. Ten female mice were used per dose. The number of animals protected from the tonic episode was noted.

Prevention and reversal by Ro 15-4513 of the anticonvulsant activity of diazepam (5 mg/kg IP), meprobamate (200 mg/kg IP) or phenobarbitone (60 mg/kg IP) was studied by giving the azide PO 15 min prior or 45 min after diazepam, meprobamate or phenobarbitone, and 15 min before PTZ.

Facilitation of seizures by Ro 15-4513 was studied by administering it PO to female mice (19 to 21 g) and female rats (120–140 g) 15 min prior to a liminal dose of PTZ (60 mg/kg IP in mice, 70 mg/kg IP in rats) which produced tonic convulsions in only a few animals out of groups of 20 evaluated within 30 min after the convulsant was given. Reversal by flumazenil of this facilitation was tested by administering flumazenil (10 mg/kg IP, 10 and 20 mg/kg PO) to the mice either 10 min after Ro 15-4513 (10 mg/kg PO) and 5 min before PTZ, or 15 min before Ro 15-4513 (same dose) or 25 min before PTZ, given 10 min after Ro 15-4513.

Audiogenic convulsions in mice. Male DBA/2J (inbred

strain) mice, 21 days old, received auditory stumulation (14 kHz, sound level around 83 dB) which typically produced tonic convulsions in only about 3–4% of vehicle-treated animals during the 60 sec period of exposure. Facilitation by Ro 15-4513 was studied by administering the compound PO in 5% acacia 30 min before stimulation. Reversal of this facilitation was tested by administering flumazenil 5 min after or before Ro 15-4513. Reversal of the anticonvulsant effect of diazepam (auditory stimulation: 110 dB) by Ro 15-4513 was also studied.

Electrophysiological Studies

Electroencephalographic (EEG) studies. Experimental animals were male rats (ca. 400 g body weight) with chronic electrodes bilaterally on the fronto-parietal cortex and in the dorsal hippocampus. For recording the rats were placed on a moving tread mill (3.75 cm/sec) which was automatically switched on and off every 10 min in order to keep the animals awake in the on-phases. The EEG signals were transmitted from the animal to a polygraph by cables. Bipolar EEG recordings were written on paper (5 mm/sec) and examined visually. In the cortical leads 8/sec spike/wave groups of about 5 sec duration were counted. Each single experiment consisted of a predrug and a drug session of 3 hr duration on consecutive days. The doses of Ro 15-4513 (suspended in 0.3% Tween 80) were 3, 6 and 12 mg/kg IP (5 animals per dose). The ED_{50} for number of responders was calculated using probit analysis. In interaction experiments 4 rats were injected flumazenil (12 mg/kg IP) 30 min after Ro 15-4513 (also 12 mg/kg IP).

Cat spinal cord. The lumbosacral spinal cord of artificially ventilated unanaesthetized spinal cats was exposed. Spontaneous activity of γ-motoneurones was recorded by means of platinum electrodes attached to a ventral rootlet L_7 (34). The spontaneous impulse rate of γ-motoneurones was determined by counting the impulses during intervals at 200 msec, every 2 sec, for a 1-min period. Ro 15-4513, FG 7142 and 40% w/v ethanol were slowly injected into the right femoral vein. Stable controls were taken as 100%. Either Ro 15-4513 (0.3 mg/kg IV) or FG 7142 (10 mg/kg IV) were administered after the control period in 8 experiments and the effects measured 5 min after injection. Ethanol (500 mg/kg IV) was injected 10 min after Ro 15-4513 and FG 7142 and its effect evaluated 10 min later. In a separate group of experiments (4 cats), ethanol was injected first, followed 30 min later by Ro 15-4513. Finally, flumazenil (0.3 mg/kg IV) was injected 10 min after Ro 15-4513. Drug effects were expressed in percent deviation from control values. The paired *t*-test was used for statistical analysis (35).

Myocardial calcium inward current. For determination of the effect of Ro 15-4513 on the myocardial calcium inward current, myocytes were isolated from guinea-pig myocardium by a treatment with collagenase (15). For voltage-clamp recordings, myocytes were allowed to settle to the bottom of a recording chamber which was perfused with Krebs-Henseleit solution (composition in mM: NaCl 120, KCl 5.9, $MgCl_2$ 1.2, $NaHCO_3$ 15, glucose 11 and $CaCl_2$ 1.8; pH 7.3, temperature 37°C). Measurement of the whole-cell calcium inward current was made using the one-electrode voltage-clamp technique (12). Patch-clamp pipettes (resistance 2–3 MΩ) were filled with (in mM): KCl 30, K-aspartate 100, Na_2ATP 3, $MgCl_2$ 1, KH_2PO_4 10, glucose 5.5 and HEPES 5 (pH 7.3). The electrode was connected to the head stage of a potential follower with current injection, the output of which was connected to a voltage-clamp amplifier for recording of membrane currents. The transmembrane calcium inward current was elicited by transiently (200 msec) depolarizing the cell membrane to +10 mV from a −50 mV holding potential (at a frequency of 0.33 Hz). For original recordings see, e.g., (15). The drugs to be tested were added to the perfusion solution to the final concentration indicated: Ethanol (3% w/v), Ro 15-4513 alone (10^{-6}–10^{-4} M) and Ethanol + Ro 15-4513 (3%w/v + 1 mM).

Neurochemical Studies

[^{3}H]Diazepam binding. [^{3}H]Diazepam binding was performed according to (28).

[^{35}S]TBPS binding. Synaptic membranes were prepared from bovine cerebral cortex as follows. The tissue was homogenized in 0.32 M sucrose, diluted to a final volume of 20 times the original wet weight and centrifuged for 10 min at 1000×g. The supernatant was recentrifuged for 20 min at 17,000×g. The membrane fraction was subjected to a hypotonic shock by suspending the pellet in cold distilled water (10 volumes of original wet weight) for 20 min. The suspension was again centrifuged for 20 min at 17,000×g after which the hypotonic shock was repeated with the upper layer of the pellet. The membranes were collected by centrifugation at 43,000×g for 10 min and washed another 5 times by suspending them in assay buffer followed by centrifugation. They were kept frozen at −20°C for at least 2 days before use. Before measuring [^{35}S]t-butylbicyclophosphonothionate ([^{35}S]TBPS) binding, performed according to (41), the membranes were thawed and washed once in assay buffer. Membranes corresponding to an original wet weight of bovine cortex of about 70 mg were incubated with 10 nM [^{35}S]TBPS (New England Nuclear, >60 Ci/mMol) for 90 min at room temperature. Assay buffer was either PBS (phosphate-buffered saline) (pH 7.4) or 50 mM Tris/HCl, pH 7.8, 200 mM NaBr, 1 mM EDTA as indicated in the legend of Fig. 15. Both buffers contained additionally 1 ml aprotinin (Sigma, A6279) per liter. Nonspecific binding was determined in the presence of 0.1 mM picrotoxin (Fluka AG, Ch-Buchs). Incubation was terminated by filtration on Whatman GF/B filters using a BRANDEL cell harvester. The filters were washed twice with about 3 ml of cold assay buffer before counting the retained radioactivity.

Cerebellar cGMP level. Determination of cerebellar cGMP level was performed with male rats (140–170 g) or mice (38–44 g) (29). In short, the animals were killed by exposure of the heads to a microwave beam for 1.3 and 1.8 sec, respectively. The cerebellum was homogenized in 10 volumes of 1% perchloric acid. The homogenate was heated in boiling water for 2 min, centrifuged at 9,000×g for 10 min and the cGMP measured by using a radioimmunoassay kit (New England Nuclear).

Cerebral glucose utilization. As an index of the cerebral glucose utilization, the rate of the cerebral uptake of [^{3}H]2-deoxyglucose was measured in male rats (190–210 g) (21,22, 40). Ethanol (6000 mg/kg, 40% w/v) or water was administered PO. After 15 min, Ro 15-4513 (100 mg/kg) or vehicle (acacia 5% in water, 4 ml/kg) was given PO. Ten min later, 2-deoxy-D-[1,2^{3}H (N)]glucose (27 Ci/mM, 70 μCi/kg in saline, 3 ml/kg) was injected in a jugular vein via a chronic indwelling catheter. Thirty-five min after [^{3}H]2-deoxyglucose, sodium pentobarbitone (50 mg/kg in saline)

TABLE 1

Ro 15-4513: INTRINSIC EFFECTS

Experiment*	Animal Species	Dose (mg/kg) or Concentration Active	Inactive
Induction of convulsions	Mouse		up to 5000 PO†
	Rat		up to 5000 PO†
Locomotor activity	Rat		up to 30 PO†
Rotating rod	Mouse		up to 100 PO†
Chimney	Mouse		up to 100 PO†
Prevention of convulsions induced by:			
Pentylenetetrazol (PTZ)	Mouse, Rat		up to 100 PO†
3-Mercaptopropionic acid (3-MPA)	Mouse		up to 100 PO†
Facilitation of convulsions induced by:			
PTZ	Mouse, Rat	1‡–100 PO§	
Auditory stimulation	Mouse	ED_{50} 0.86 PO§	
Induction of spike/wave paroxysms	Rat	ED_{50} 7.3 IP§	
Enhancement of γ-motoneurone activity	Cat	0.3‡–3 IV¶	
[^{3}H]Diazepam binding in vitro	Rat	IC_{50} 4.6 nmol/l	
[^{3}H]Flumazenil binding in vitro	Rat	IC_{50} 4.5 nmol/l#	
Increase of cerebellar cGMP	Mouse		0.3–30 PO
	Rat	ED_{50} 0.04 PO	
Brain glucose utilization	Rat		100 PO

*See the Method section.
†Highest dose tested.
‡Lowest effective dose.
§Antagonized by flumazenil.
¶Not antagonized by flumazenil.
#A significant negative GABA-shift (see the Method section and Fig. 20) was seen in the presence of 10 μM GABA in frontal cortex, olfactory bulb and inferior colliculus.

was injected via the jugular catheter and the rats were decapitated. Trunk blood was collected in heparinized tubes for the determination of plasma glucose by the hexokinase method. The brains were removed and dissected into cerebellum, myelencephalon, pons plus midbrain, hippocampus, septum, neostriatum, thalamus plus hypothalamus, cerebral cortex and olfactory bulbs. The tissues were weighed, digested with Protosol® and total radioactivity (dpm/10 mg wet weight) measured by scintillation counting and taken as an index of the glucose utilization rate. The experiments were performed twice with 7 to 9 rats per group. Values are given as means±SEM of the pooled experiments.

Cytopharmacological Studies

Receptor autoradiography. Parasagittal cryostat sections of mouse and rat brain were incubated with [^{3}H]Ro 15-4513 (23 Ci/mmol, New England Nuclear) (5 nM in 15 mmol Krebs-Tris buffer, pH 7.4) for 15 min at 4°C in subdued light. In competition binding experiments the sections were coincubated with the radioligand in the presence of either diazepam, clonazepam, the triazolopyridazine BZR agonist Cl 218872 (11), the BZR inverse agonist β-CCE (11), the BZR partial inverse agonists CGS 8216 (11), flumazenil, the BZR partial inverse agonist Ro 15-3505, the BZR antagonist Ro 14-7437 (11) or the convulsant benzodiazepine Ro 05-3663 (11) at a concentration of 50 nM and 1 μM. All sections were rinsed with buffer (2×30 sec + 1 min), dipped in distilled water and rapidly dried under a stream of ice-cold nitrogen gas. Sections were coexposed with tritium brain paste standards to LKB Ultrofilm® for 3 weeks at 4°C. Autoradiograms were evaluated quantitatively by image analysis (Sequier *et al.*, in preparation). The regional IC_{50} values for Ro 15-4513, in [^{3}H]flumazenil binding in vitro, were determined in the absence and presence of 10 μM GABA (GABA-shift).

RESULTS

Acute Toxicity

In the *acute toxicity studies* in mice, no lethality was observed with doses up to 5000 mg/kg Ro 15-4513 PO during the 8-day period under conventional light and during the 14-day period under UV light irradiation following administration of Ro 15-4513.

Behavioural Effects

In *observation of free behaviour* no convulsions were induced by Ro 15-4513 in mice and rats up to high doses (Table 1). In mice, mild signs of stimulation (increased motility) were seen during 15 min after 30 and 100 mg/kg Ro 15-4513 PO. Ro 15-4513 and Ro 15-3505, administered at doses of 100 mg/kg and 10 mg/kg PO, respectively, did not significantly shorten the *duration of LRR* induced by ethanol or by hexobarbitone (Table 5). Administration of Ro 15-4513 at a single dose which alone failed to appreciably affect palatable food consumption (present experiments) was effective in significantly reducing hyperphagic effect of both oral di-

TABLE 2

REVERSAL OF DIAZEPAM EFFECTS BY Ro 15-4513

Diazepam* Effect	Animal Species	ED_{50} in mg/kg PO (95% confidence limits)
Impairment of performance, horizontal wire test	Mouse	0.33 (0.17–0.62)
	Rat	0.45 (0.22–0.90)
Prevention of pentylenetetrazol (PTZ)-induced convulsions	Mouse	3.5 (2.9–4.3)
	Rat	1.9 (1.6–2.4)
Prevention of audiogenic convulsions	Mouse	1.2 (1.0–1.5)
Hyperphagia	Rat	1.0†

*Doses in the Method section.
†IP, single dose evaluated reduced diazepam-induced hyperphagia by 36%.

TABLE 3

REVERSAL OF PHENOBARBITONE EFFECTS BY Ro 15-4513

Phenobarbitone* Effect	Animal Species	Dose (mg/kg PO) Active	Inactive
Depression of locomotor activity	Rat		up to 30†
Depression of performance in the horizontal wire test	Mouse	ED_{50} 0.89‡	up to 100† (no dose-dependent effect)
	Rat		
Prevention of convulsions induced by pentylenetetrazol: clonic	Mouse	3§–100	
tonic			up to 100†
Hyperphagia	Rat	1.0¶	

*Doses in the Method section.
†Highest dose tested.
‡Antagonized by flumazenil.
§Lowest effective dose.
¶IP, single dose tested reduced phenobarbitone-induced hyperphagia by 38%.

azepam (−36%) and oral phenobarbitone (−38%) in the *palatable food consumption* paradigm (see Tables 2 and 3). Ro 15-4513 and FG 7142 were devoid of intrinsic effects on the spontaneous *locomotor activity* in rats (Table 1 and Fig. 2). However, Ro 15-4513, but not FG 7142 reversed the reduction of rat locomotor activity caused by ethanol (Table 4 and Fig. 2). In this experimental situation, Ro 15-4513 was inactive against phenobarbitone (Table 3). No effect was observed by Ro 15-4513 per se on mice performance on the *rotating rod* and *chimney* paradigms (Table 1), and no reversal of the ethanol effect in the rotating rod (Table 4). In the *mouse HWT*, the potency of Ro 15-4513 in reversing the central nervous depressant effect of diazepam was of the same order of magnitude as that against phenobarbitone and against ethanol (Figs. 3, 4 and 5). In this test, Ro 15-4513 was inactive against meprobamate (not shown). In rats, there was a dose-dependent reversal by Ro 15-4513 of the diazepam effect with a potency comparable to that in mice (Fig. 3), and a dose-dependent reversal of the ethanol effect with a 10 to 20 times lower potency (Fig. 5). No dose-dependent effect was seen in rats by Ro 15-4513 against phenobarbitone (Table 3 and Fig. 4). In mice HWT, the reversal of the phenobarbitone as well as of the ethanol-induced motor impairment by Ro 15-4513 was abolished by flumazenil (Figs. 6 and 7), which by itself was not active against ethanol (Fig. 7) or against phenobarbitone (2). The ethanol-induced impairment in the HWT was not affected by the BZR partial inverse agonists Ro 15-3505 or CGS 8216, which, unlike the BZR blocker flumazenil, did not abolish the reversal by Ro 15-4513 of the ethanol effect (Fig. 8A and B). In mice HWT, the partial BZR inverse agonist β-CCM, active against phenobarbitone in doses as low as 0.003 mg/kg IV, did show an effect against ethanol in doses more than 1000 times higher, reaching the convulsant range (Fig. 9).

Seizures Induced by Chemical and Physical Convulsants

No protection by Ro 15-4513 was found against *PTZ or*

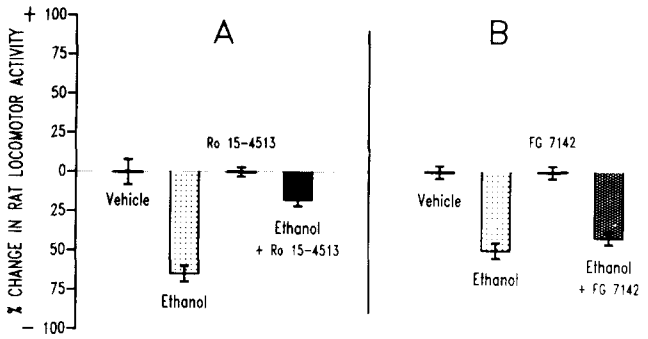


FIG. 2. Effects of ethanol (3000 mg/ PO), Ro 15-4513 (10 mg/kg PO), FG 7142 (100 mg/kg PO) and the combinations thereof on the spontaneous locomotion in rats. Columns are means of 3 animals ±S.E.M. The sequence of drug administrations in interaction experiments was: Ethanol 15 min before Ro 15-4513 in A, or FG 7142 in B.

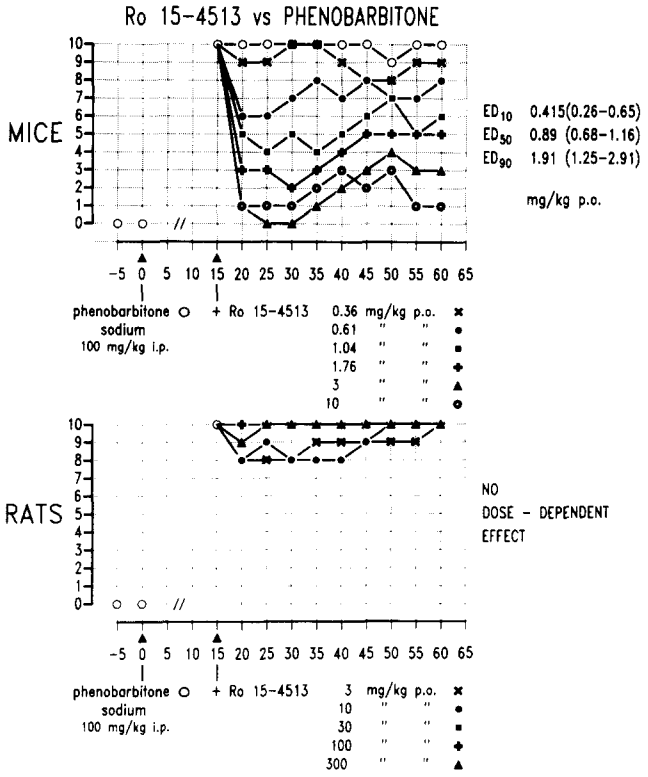


FIG. 4. Reversal by Ro 15-4513 in mice, but not in rats, of the phenobarbitone-induced impairment of motor performance in the HWT. Details as in Fig. 3.

3-MPA-induced tonic convulsions (Table 1). Ro 15-4513 facilitated tonic convulsions produced by a liminal dose of PTZ in mice and rats (Table 7). When mice and rats were administered 100 mg/kg Ro 15-4513 PO alone, no convulsions were observed during the subsequent 60 min period. The facilitation by Ro 15-4513 of PTZ activity in mice was both prevented and reversed by flumazenil (Fig. 10a and b). Ro 15-4513 was active in reversing the anticonvulsant activity of diazepam, meprobamate or phenobarbitone (Tables 2, 3 and 6). In DBA/2J mice receiving *auditory stimulation*, Ro 15-4513 significantly increased the occurrence of tonic convulsions (see Table 1). This facilitation of audiogenic seizures was dose-dependently both prevented and reversed by flumazenil (Fig. 11A and B). The ED_{50} value for the reversal of the anticonvulsant effect of diazepam by Ro 15-4513 in mice audiogenic convulsions was 1.2 mg/kg PO (Table 2).

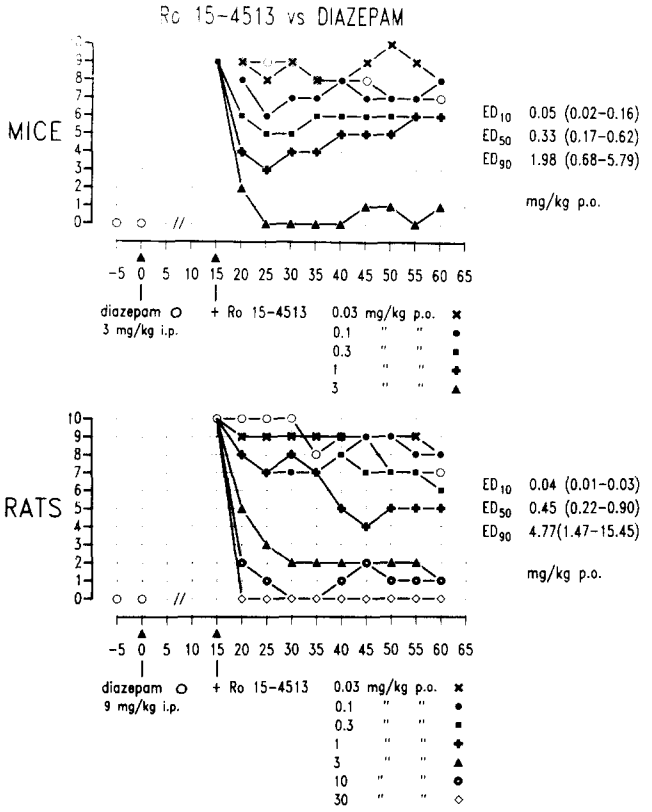


FIG. 3. Dose-dependent reversal by Ro 15-4513 of the depressant effect of diazepam on the performance of mice and rats in the HWT. Ro 15-4513 was administered orally, 15 min after diazepam, at the doses indicated. Abscissa: time in min. Ordinate: number of animals showing performance impairment out of a total of 10 animals per treatment group. Potency determinations ($ED_{10,50,90}$) were performed by taking the maximum effects observed within 45 min of Ro 15-4513 administration.

Electrophysiological Studies

Ro 15-4513 induced spike/wave paroxysms in the *cortical EEG* of rats at doses higher than 3 mg/kg IP (Fig. 12). This intrinsic activity of Ro 15-4513 was antagonized by flumazenil (Fig. 13). Ro 15-4513 (0.3 mg/kg IV) and FG 7142 (10 mg/kg IV) enhanced the spontaneous activity of *γ-motoneurones* in all experiments within 5 min after injection. The ethanol- (500 mg/kg IV) induced depression of γ-motoneuronal activity was antagonized by a subsequent injection of Ro 15-4513 (Fig. 14), but not by FG 7142 (results not shown). Flumazenil (0.3 mg/kg IV), administered after Ro 15-4513, reversed the effect of Ro 15-4513 (Fig. 14). The effects of Ro 15-4513 and ethanol on the *slow inward* (transmembrane) Ca^{2+} *current* were investigated in isolated

TABLE 4

Ro 15-4513 AND ACUTE ETHANOL EFFECTS

Ethanol Effect	Dose (g/kg)	Animal Species	Dose (mg/kg) Active	Inactive
Depression of locomotor activity	3 PO	Rat	3§–30 PO†	
Impairment of performance on the rotating rod	2 IP	Mouse		10–30 PO* 10–30 IP† 10–30 PO†
Impairment of performance on the horizontal wire	3 PO	Mouse	ED_{50} 0.84 PO†¶	
	6 PO	Rat	ED_{50} 8.6 PO†¶ 3.0 IP†	
Depression of γ-motoneurone activity	0.5 IV	Cat	0.3 IV†¶	
	0.5 IV		0.3–3 IV*¶	
Decrease of cerebellar cGMP	2 PO	Mouse		0.3–30 PO‡
	2 PO	Rat	1§–100 PO‡¶	
Depression of brain glucose utilization	6 PO	Rat	100 PO†	

*Given before ethanol.
†Given after ethanol.
‡Given at the same time as ethanol.
§Lowest effective dose.
¶Antagonized by flumazenil.

TABLE 5

LACK OF PREVENTION BY THE BZR PARTIAL INVERSE AGONISTS Ro 15-4513* AND Ro 15-3505† OF THE LOSS OF RIGHTING REFLEX (LRR) INDUCED IN (N) MICE BY ETHANOL‡ OR HEXOBARBITONE§

Duration of LRR (mean min ± S.E.M.)			
Ethanol	Ro 15-4513 + Ethanol	Ethanol	Ro 15-3505 + Ethanol
51 ± 8 (N=50)	45 ± 12 (N=50)	33 ± 9 (N=10)	24 ± 6 (N=10)
Hexobarbitone	Ro 15-4513 + Hexobarbitone	Hexobarbitone	Ro 15-3505 + Hexobarbitone
29 ± 4 (N=30)	25 ± 3 (N=30)	34 ± 4 (N=20)	30 ± 3 (N=20)

*One hundred mg/kg PO, given 15 min before either ethanol or hexobarbitone.
†Ten mg/kg PO, as above.
‡Forty-seven hundred mg/kg IP.
§One hundred twenty-five mg/kg IP.

myocardial cells from guinea-pig heart. Ro 15-4513 alone caused only a slight reduction in Ca^{2+} inward current (29±8% decrease at 10^{-4} M, N=4). In another series of experiments (N=4), ethanol alone and in combination with Ro 15-4513 was tested. In the ethanol solution used (3% w/v) it was possible to dissolve 1 mM of the test compound. Ethanol (3% w/v) reduced inward Ca^{2+} current to 62±1% of the control value. Addition of Ro 15-4513 did not reverse the reduction but caused a further current decrease to 39±1%.

Neurochemical Studies

The results of competitive binding studies with *[^{3}H]diazepam* and *[^{35}S]TBPS* with several compounds using rat cortical membranes are given in Table 8. Ro 15-4513 is a high-affinity ligand of the BZR. It clearly does not competitively interfere with [^{35}S]TBPS binding sites, nor do the β-carbolines and flumazenil. Agonists and inverse agonists at the BZR, however, are known to allosterically modulate [^{35}S]TBPS binding (41). This is demonstrated in Fig. 15. Diazepam enhances [^{35}S]TBPS binding, whereas DMCM reduces it. Ro 15-4513 has no effect and behaves in this experiment like the antagonist flumazenil. No effect of Ro 15-4513 on [^{35}S]TBPS binding was detected in the presence of GABA either. The inhibitory action of GABA was not affected; the effect of diazepam on the enhancement of GABA, however,

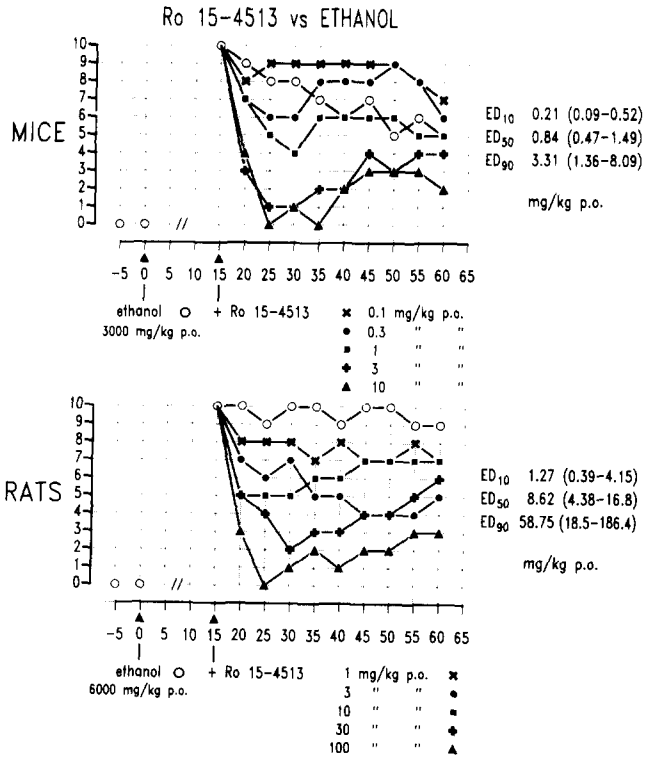


FIG. 5. Reversal by Ro 15-4513 of the ethanol-induced impairment of motor performance of mice and rats in the HWT. Details as in Fig. 3.

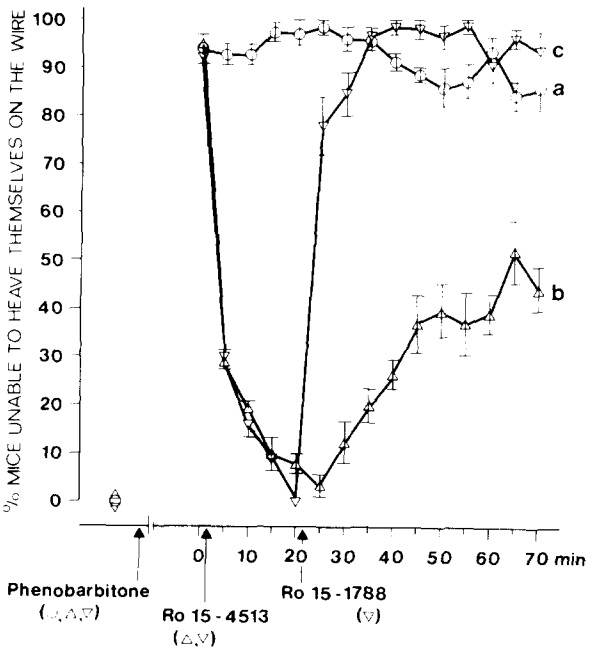


FIG. 6. Abolition by flumazenil (Ro 15-1788, 3 mg/kg PO) of the reversal by Ro 15-4513 (3 mg/kg PO) of the phenobarbitone- (100 mg/kg IP) induced impairment of mice motor performance in the HWT. Phenobarbitone was injected to 6 groups of 10 mice and depressed performance in all animals (a). In the 6 groups labelled b, Ro 15-4513 was administered as soon as the full phenobarbitone effect was reached. In the 6 groups labelled c, the administration of Ro 15-4513 was followed 15 min later by flumazenil. The symbols are the means±S.E.M.

was blocked by Ro 15-4513. Most important in the present context, Ro 15-4513 was unable to counteract the inhibitory action of ethanol on [^{35}S]TBPS binding to the presumed chloride channel (Fig. 15).

Ro 15-4513 dose-dependently increased the *cerebellar cGMP* level in rats, maximally to almost the double of control levels but had virtually no effects in mice (Fig. 16A). In both species, ethanol (2 g/kg PO) reduced cGMP level by more than 50%. Concomitant administration of increasing doses of Ro 15-4513 with 2 g/kg ethanol PO led to a reduction of the ethanol effect back to control levels in rats but not in mice (Fig. 16B). The effect of 10 mg/kg Ro 15-4513 PO on the ethanol-induced decrease of cGMP in rats was antagonized by 100 mg/kg flumazenil PO (Fig. 17). In contrast to Ro 15-4513, β-CCM at the low dose of 0.3 mg/kg IV did not antagonize the effect of ethanol on cGMP in rats (Fig. 17). As shown in Fig. 18, ethanol (6000 mg/kg PO) reduced the *regional uptake of [^{3}H]2-deoxyglucose* in rat brain by approximately 20%. This reduction was counteracted by Ro 15-4513. Ro 15-4513 by itself induced only a small increase in the [^{3}H]2-deoxyglucose uptake in some brain regions, e.g., in the cerebellum. Drug-induced changes in cerebral deoxyglucose uptake (tissue radioactivity) are proportional to changes in glucose utilization rate if the drug did not change the plasma glucose level (40). As compared with controls, plasma glucose in trunk blood was not altered by Ro 15-4513 but was similarly increased after administration of both ethanol and ethanol plus Ro 15-4513 (controls: 109±4 mg/100 ml=100±3.3%; ethanol: 113±2.7%; Ro 15-4513: 101±2.7%; ethanol + 15-4513:114±6.9%).

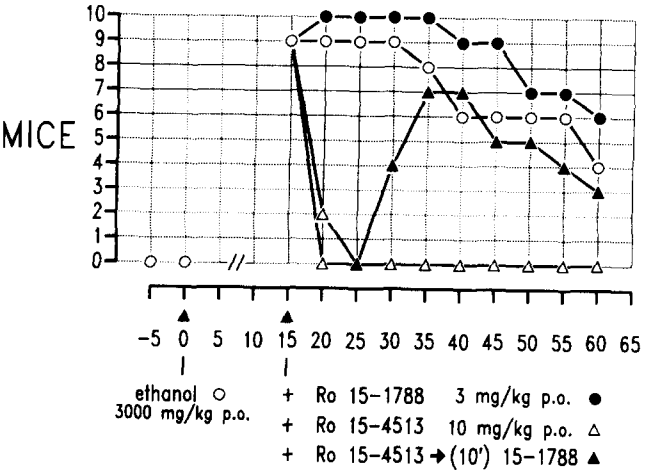


FIG. 7. Abolition by flumazenil (Ro 15-1788), by itself not active against ethanol, of the reversal by Ro 15-4513 of the ethanol-induced impairment of mice motor performance in the HWT. Details as in Fig. 3.

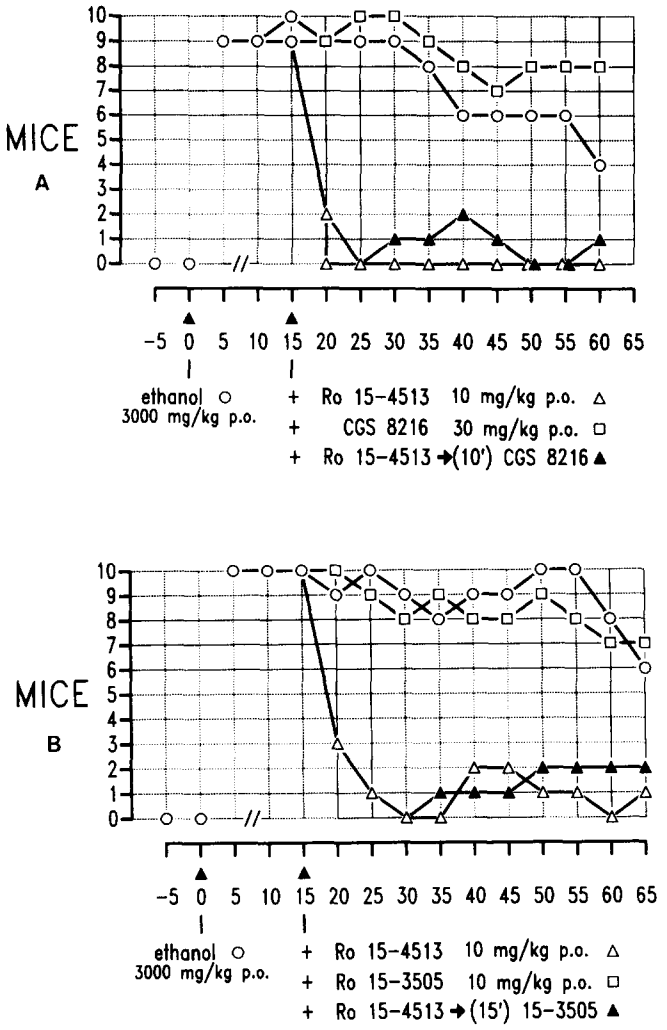


FIG. 8. Lack of activity of the BZR partial inverse agonist CGS 8216 (A) and Ro 15-3505 (B) against ethanol and against the reversal by Ro 15-4513 of the ethanol-induced impairment of mice motor performance in the HWT. Details as in Fig. 3.

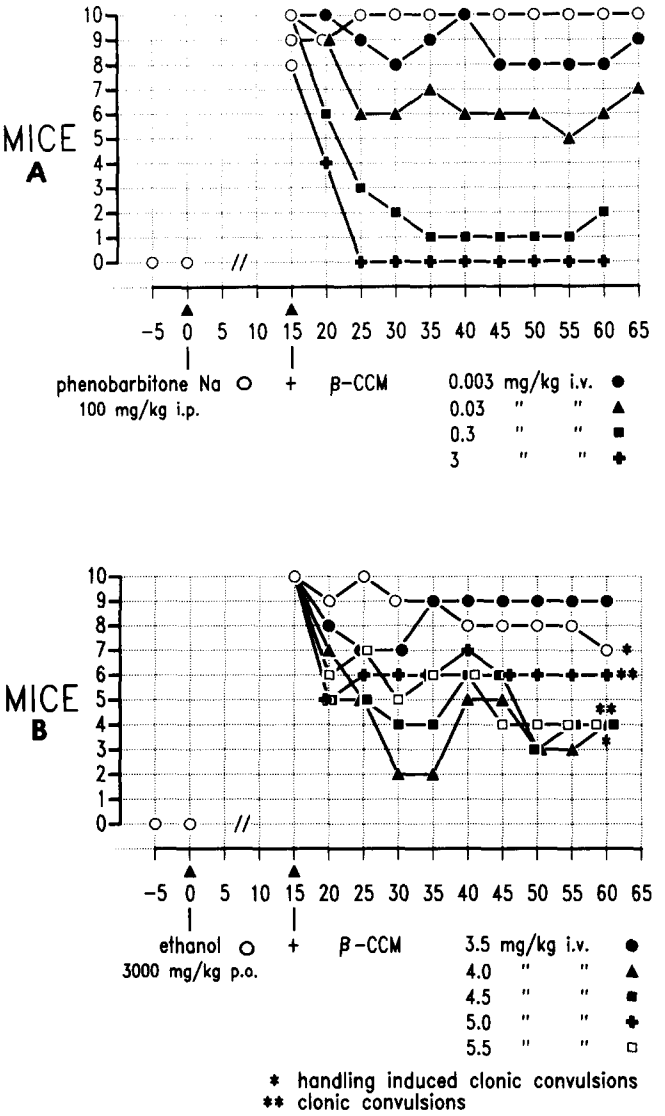


FIG. 9. Interaction of the BZR inverse agonist β-CCM with phenobarbitone and ethanol in the mice HWT. Dose-dependent reversal of the phenobarbitone- but not of the ethanol-induced impairment of motor performance. Details as in Fig. 3. The computed ED_{50} value for the effect against phenobarbitone was 0.05 mg/kg IV (about 1/1000 of the convulsant dose active against ethanol).

Cytopharmacological Studies

The distribution and density of [^{3}H]Ro 15-4513 binding sites in rat and mouse brain sections (Fig. 19A) were similar, but not identical, to those previously described for other benzodiazepine receptor ligands (37, 39, 47). The granular layer of the cerebellum contained an unusually high density of binding sites, only 50% of which could be blocked by the receptor ligands tested at 50 nM. Several compounds (diazepam, clonazepam, Cl 218872, β-CCE or Ro 14-7437) failed to competitively inhibit this binding even at a concentration of 1 μM (Fig. 19B). The IC_{50} values for Ro 15-4513 in [^{3}H]flumazenil binding in vitro (Fig. 20) showed little regional variation. Similar results have been obtained with Ro 15-3505 and flumazenil (not shown). The GABA-shift profile (Fig. 20) was similar to that obtained for other partial inverse agonists such as Ro 15-3505. In other words, GABA reduced the affinity of Ro 15-4513 for [^{3}H]flumazenil binding in some but not all regions investigated.

DISCUSSION

An overview of the pharmacological effects of Ro 15-4513 observed in the present experiments is given in Tables 1 to 3 (intrinsic effects, reversal of diazepam and phenobarbitone effects) and 4 (interactions with acute effects of ethanol). Ro 15-4513 is relatively untoxic in acute single doses and did not produce manifest behavioural signs of either excitation or depression.

Ro 15-4513 as a Partial Inverse Agonist at BZR

The term partial inverse BZR agonist has been proposed by Polc *et al.* (34) for ligands of the BZR with submaximal

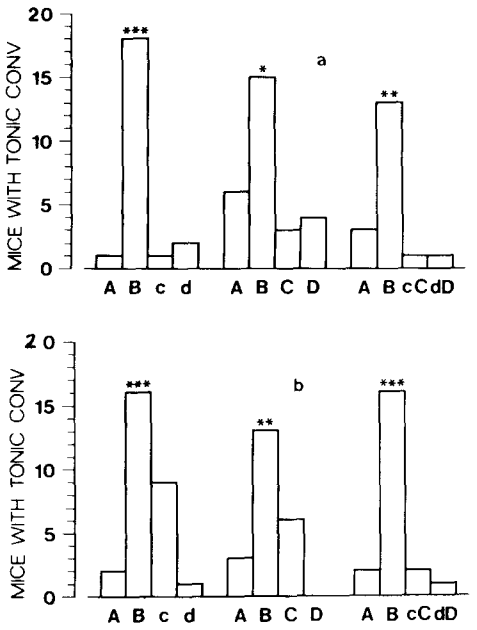


FIG. 10 (a) Antagonism by Ro 15-1788 (10 mg/kg IP, 10 and 20 mg/kg PO) of the PTZ-facilitating effect of Ro 15-4513 when given 10 min after Ro 15-4513 (10 mg/kg PO) and 5 min before PTZ (60 mg/kg IP). (b) Antagonism by Ro 15-1788 (10 mg/kg IP, 10 and 20 mg/kg PO) of the PTZ-facilitating effect of Ro 15-4513 when given 10 min before Ro 15-4513 (10 mg/kg PO) and 15 min before PTZ (60 mg/kg IP). A=PTZ, B=PTZ + Ro 15-4513, c=PTZ + Ro 15-4513 + Ro 15-1788 10 mg/kg IP, d=PTZ + Ro 15-1788 10 mg/kg IP, C=PTZ + Ro 15-4513 + Ro 15-1788 10 mg/kg PO, D=PTZ + Ro 15-1788 10 mg/kg PO, cC=PTZ + Ro 15-4513 + Ro 15-1788 20 mg/kg PO, dD=PTZ + Ro 15-1788 20 mg/kg PO. *<0.05, **<0.01, ***<0.001 (vs. PTZ controls, Chi-squared test).

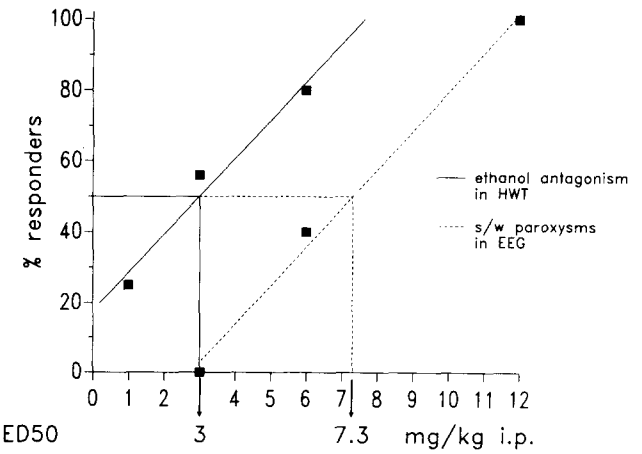


FIG. 12. Ro 15-4513: dose-response curve for the induction of spike/wave paroxysms in the cortical EEG of rats (dotted line, 5 animals per dose) compared to that for the reversal of the depressant effect of ethanol in the rat HWT (solid line, 10 animals per dose). Probit analysis.

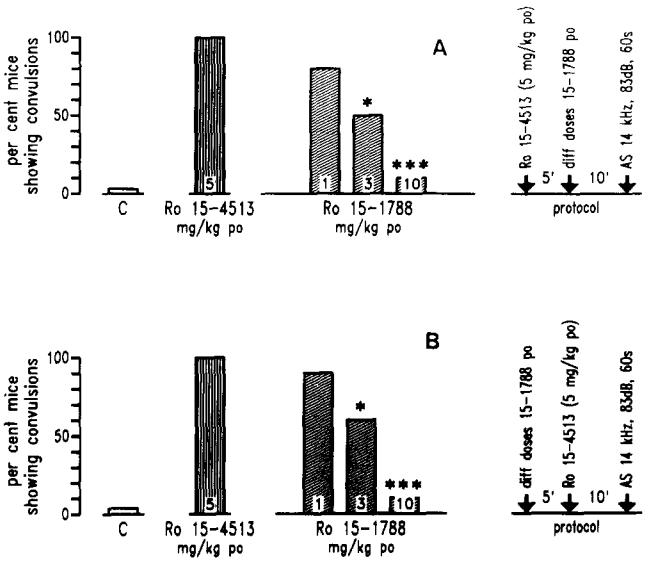


FIG. 11. Audiogenic convulsions induced by a subliminal sound level (83 dB): dose-dependent reversal (A) and prevention (B) by flumazenil PO of the facilitating effect of a supramaximal dose of Ro 15-4513 (5 mg/kg PO). AS=Auditory stimulation.

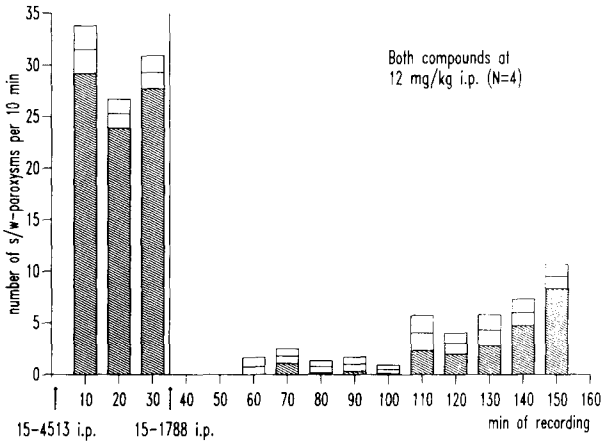


FIG. 13. Reversal by flumazenil (Ro 15-1788, 12 mg/kg IP) of the spike/wave paroxysms induced in the cortical EEG of 4 rats by Ro 15-4513 (12 mg/kg IP). White areas on top of columns: means±S.E.M.

negative modulatory activity (low negative efficacy agonists) on the function of the $GABA_A$ receptor complex. Ro 15-4513 fulfills all of the criteria for partial inverse agonism at the BZR.

It binds with high affinity to central but not to peripheral binding sites. Its potency as inhibitor of [^{3}H]diazepam binding in vitro is about half that of its congener flumazenil and similar to that of β-CCM. Ro 15-4513, in contrast to the full inverse agonist DMCM, does not interact with another allosteric site of the $GABA_A$ receptor on which numerous convulsants and sedatives act and which can be identified by the cage convulsant ligand TBPS. However, as a BZR ligand it

TABLE 6

ATTENUATION BY Ro 15-4513 OF THE PROTECTIVE EFFECT OF MEPROBAMATE (200 mg/kg) AND PHENOBARBITONE (60 mg/kg) IN MICE AGAINST CONVULSIONS INDUCED WITH A SUPRAMAXIMAL DOSE OF PENTYLENETETRAZOL (PTZ)

Treatment 60 min Prior to PTZ	Treatment 15 min Prior to PTZ	Number of Mice out of 20 Which Exhibited Clonic Convulsions	Tonic Convulsions
I. Meprobamate	Vehicle	6	0
Meprobamate	Ro 15-4513 3 mg/kg, PO	12	9†
Meprobamate	Ro 15-4513 10 mg/kg, PO	12	9†
Meprobamate	Ro 15-4513 30 mg/kg, PO	17†	13†
Meprobamate	Ro 15-4513 100 mg/kg, PO	18†	13†
II. Phenobarbitone	Vehicle	3	0
Phenobarbitone	Ro 15-4513 3 mg/kg, PO	10*	1
Phenobarbitone	Ro 15-4513 10 mg/kg, PO	17†	0
Phenobarbitone	Ro 15-4513 30 mg/kg, PO	11*	2
Phenobarbitone	Ro 15-4513 100 mg/kg, PO	11*	2

*<0.05, †<0.01, in comparison with the meprobamate-vehicle condition. Chi-squared test.

TABLE 7

FACILITATION OF PTZ-INDUCED LIMINAL TONIC CONVULSIONS IN MICE AND RATS BY ORAL PRETREATMENT WITH Ro 15-4513

Species	Dose (mg/kg)	Number of Animals out of 20 Which Exhibited Convulsions After Ro 15-4513	Vehicle
Rat	0.1	7	
	1	15*	6
	10	15*	
	100	20†	
Mouse	1	7	
	3	9*	
	10	18†	2
	30	18†	
	100	18†	

*<0.05, †<0.01 compared with the vehicle-treated group, Chi-squared test.

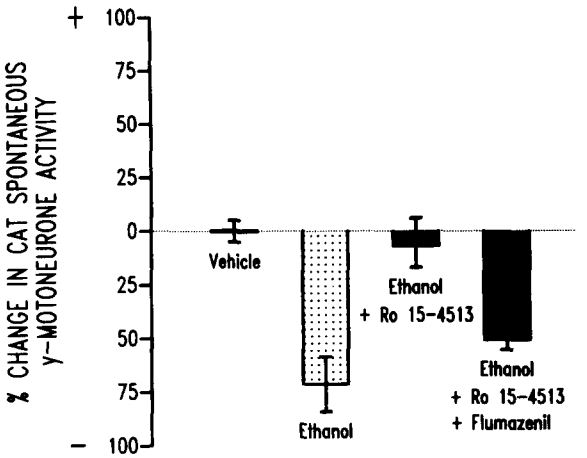


FIG. 14. Effects of ethanol (500 mg/kg IV), the combination of ethanol and Ro 15-4513 (0.3 mg/kg IV) as well as with the benzodiazepine receptor antagonist flumazenil (0.3 mg/kg IV), on the ongoing γ-motoneurone activity in spinal cats. Columns are the means of 4 cats ±S.E.M. The sequence of drug injections was: 30 min after ethanol Ro 15-4513 was injected, followed 10 min later by flumazenil.

TABLE 8

DISPLACEMENT OF [^{3}H]DIAZEPAM AND [^{35}S]TBPS BY Ro 15-4513 AND OTHER BZR LIGANDS (IC_{50} VALUES) IN RAT CEREBRAL CORTICAL MEMBRANES

Radioligands	Flumazenil	Ro 15-4513	β-CCE	β-CCM
[^{3}H]Diazepam	2.3 nmol/l	4.6 nmol/l	0.9 nmol/l	3.9 nmol/l
[^{35}S]TBPS	>10 μmol/l	>10 μmol/l	>10 μmol/l	>10 μmol/l

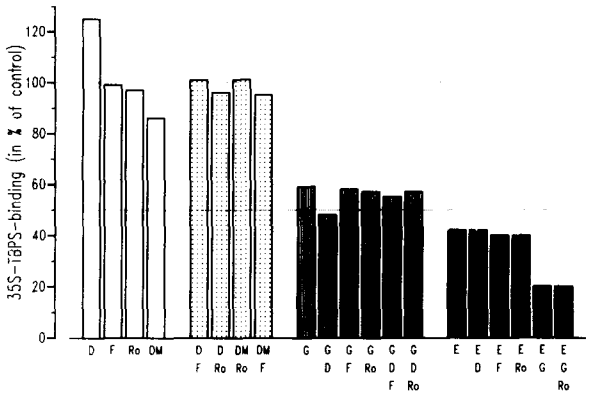


FIG. 15. [^{35}S]TBPS binding to synaptic membranes from bovine cerebral cortex in the presence of various agents affecting the GABA/BZR. Indicated is the specifically bound [^{35}S]TBPS in % of control binding. The first two groups of experiments (white and dotted columns) were performed in PBS, the last two groups (hatched and black columns) in 50 mM Tris/HCl buffer, pH 7.8, containing 200 mM NaBr. Control binding in these two buffers corresponded to about 34,000 and 74,000 dpm, respectively. S.E.M. values were consistently lower than 2%. Abbreviations: D: 0.1 μM diazepam; DM: 0.1 μM DMCM (methyl 6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate); E: 3% ethanol; F: 1.0 μM flumazenil; G: 1.0 μM GABA; Ro: 1.0 μM Ro 15-4513.

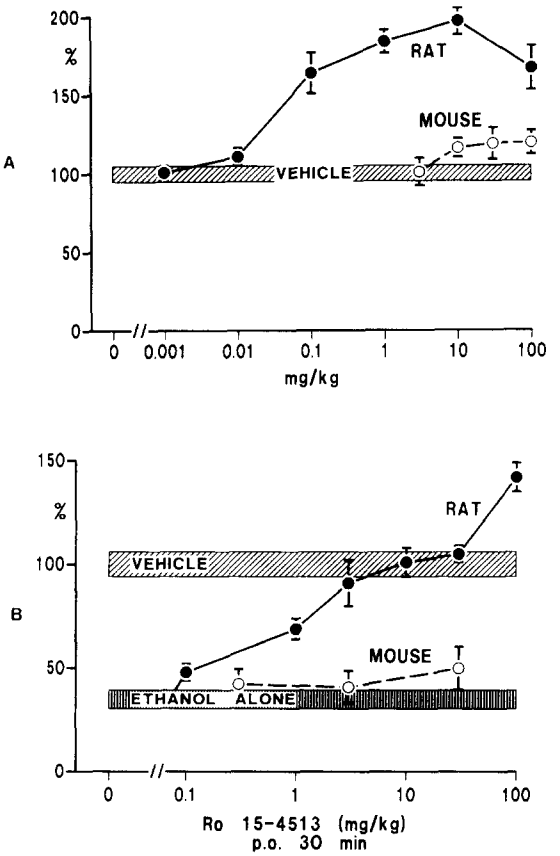


FIG. 16. Dose-dependent effects of Ro 15-4513 on the level of cerebellar cGMP 30 min after oral administration. (A) Effect of increasing doses of Ro 15-4513 on the ethanol-induced decrease of cerebellar cGMP level. Ethanol (2 g/kg) was administered either alone or together with Ro 15-4513 PO, 30 min before sacrifice. (B) the cGMP level of control animals (vehicle) was 1.2±0.04 nmol/g for rats and 0.47±0.05 nmol/g for mice. Each point represents the mean±S.E.M. of at least 12 animals.

blocks the allosteric interaction of diazepam with [^{35}S]TBPS binding. The autoradiographic distribution of radiolabelled Ro 15-4513 in rodent brain sections in vitro was found to be very similar to that of other specific BZR ligands, the only major exception being observed in the cerebellar cortex. There is a high density of binding sites for [^{3}H]Ro 15-4513 in the cerebellar granular layer; only about 50% of these are blocked by diazepam, while 1 μM flumazenil completely inhibited binding in this area. Although the function of these binding sites is not clear, a corresponding 53 kD protein in the cerebellum has been identified by photoaffinity labelling. The affinity of Ro 15-4513 for BZR was very similar in all rat CNS regions studied. GABA reduced the binding of Ro 15-4513 in some regions (frontal cortex, olfactory bulb and inferior colliculi) but was without effect in most other areas (weak negative GABA shift).

A characteristic property of inverse and partial inverse agonists is their convulsive or proconvulsive effect. In the present study no dose of Ro 15-4513 was found to produce overt seizure activity in rodents. In monkeys, the induction of convulsions has been reported (26). However, Ro 15-4513 induced flumazenil-sensitive electrocortical spike/wave activity with an Ed_{50} of 7.3 mg/kg IP in the rat. This nonconvulsive cortical paroxysmal activity occurred at doses of Ro 15-4513 slightly higher than those reversing the effect of ethanol in the HWT. Ro 15-4513 sensitized both mice and rats to the convulsive effect of PTZ, an effect which was antagonized by flumazenil. Essentially the same was found with audiogenic seizures in DBA/2J mice.

Like other inverse and partial inverse agonists, Ro 15-4513 increased the spontaneous firing rate of spinal

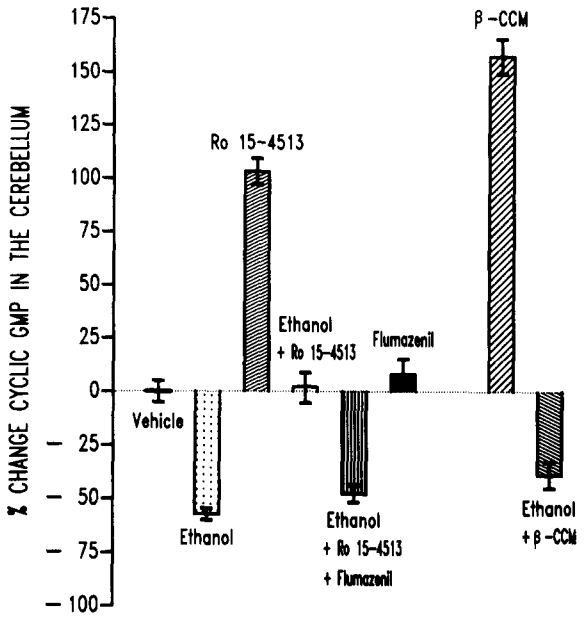


FIG. 17. Effect of Ro 15-4513 and Ro 15-4513 + flumazenil on the ethanol-induced decrease in cerebellar cGMP level of the rat. Ethanol (2 g/kg PO) or Ro 15-4513 (10 mg/kg PO) was administered either alone, together or in combination with flumazenil (100 mg/kg) PO, 30 min before sacrifice of the animal. The cGMP level of control rats was 1.31±0.05 nmol/g. β-CCM 0.3 mg/kg was injected IV and the animal sacrificed 10 min later. In addition, β-CCM was injected to rats pretreated with ethanol (2 g/kg PO) 20 min before β-CCM. The values represent means±S.E.M. of at least 12 animals.

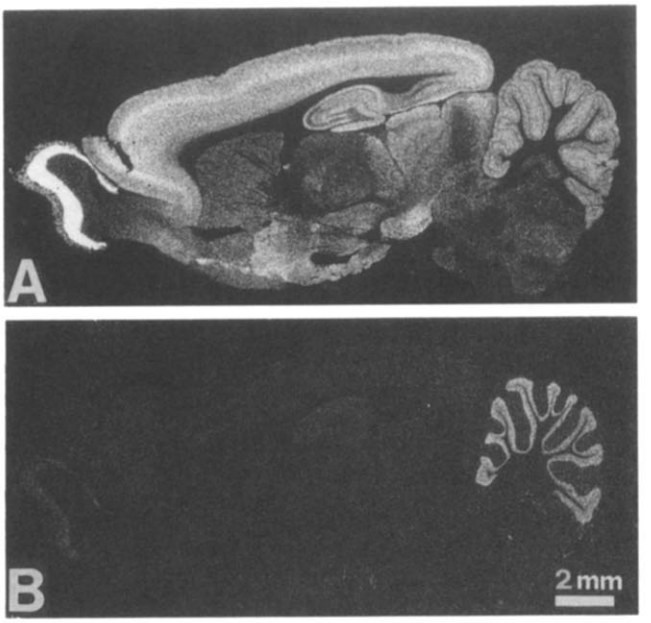


FIG. 19. Autoradiographic distribution of [^{3}H]Ro 15-4513 binding sites in parasagittal sections of rat brain in vitro. (A) Total binding at 5 nm. (B) Competitive inhibition by 1 μM diazepam.

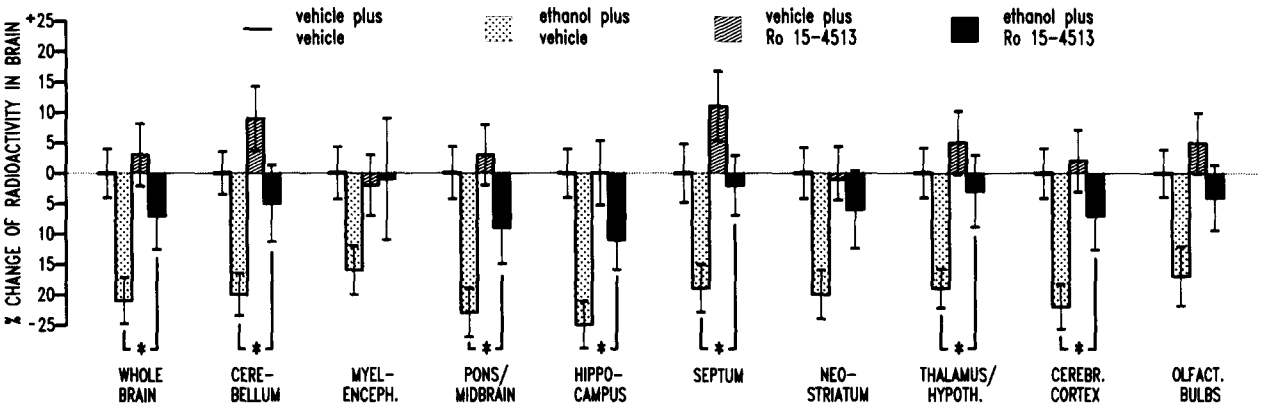


FIG. 18. Effect of ethanol, Ro 15-4513 and ethanol plus Ro 15-4513 on the regional [^{3}H]2-deoxyglucose uptake in rat brain. Rats (N=12–16) were treated with ethanol (6000 mg/kg PO) 60 min prior to sacrifice, with Ro 15-4513 (100 mg/kg PO) 45 min prior to sacrifice or ethanol plus Ro 15-4513. The animals were injected with [^{3}H]2-deoxyglucose (70 μCi/kg IV) 35 min prior to sacrifice. Tissue radioactivity (dpm/10 mg wet weight) was determined by scintillation counting in various brain regions and the % differences from controls given on the ordinate. The values represent means±S.E.M. of two pooled experiments. The difference ethanol vs. controls is significant in each region (<0.01, *t*-test). Significant differences between ethanol and ethanol plus Ro 15-4513 are indicated with an *(<0.05).

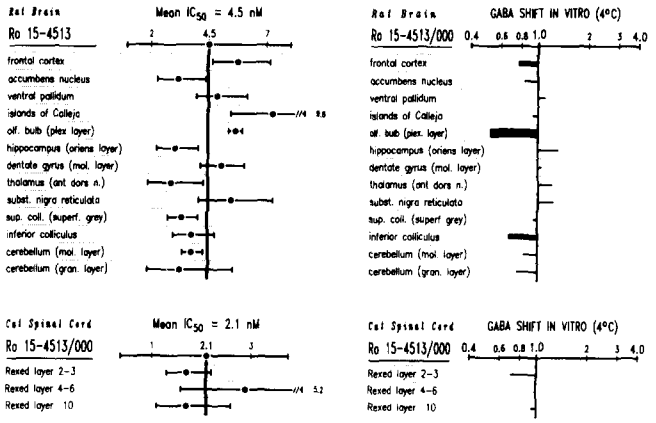


FIG. 20. IC_{50} and GABA-shift profiles for Ro 15-4513 in competition binding experiments with [^{3}H]flumazenil in rat brain and lumbosacral cat spinal cord in vitro. For the IC_{50} profile, the regional means (with 95% confidence limits) are compared with the mean IC_{50} (4.5 nM) for all 13 brain regions and spinal cord regions investigated. For the GABA-shift, the ratio

$$\frac{IC_{50}}{IC_{50} + GABA}$$

was determined for each region. Values significantly less than 1.0 (thick bars) indicate a negative GABA-shift, characteristic of a partial inverse agonist.

γ-motoneurones in high spinalized cats and reduced dorsal root potentials (3) which are the electrophysiological signs of GABA-mediated presynaptic depolarization at primary afferent endings. As expected, flumazenil reversed the latter effect of Ro 15-4513 but, suprisingly, not its stimulant effect on γ-motoneurones (3) (see below).

The cGMP level of the cerebellar cortex is a rather reliable index of the neuronal activity in this region, although it does not itself distinguish between the mechanisms determining this activity, such as excitation, disinhibition or inhibition. Ro 15-4513 dose dependently elevated the cGMP content of the rat cerebellum. A doubling of the normal value was obtained with 10 mg/kg PO; the dose of 100 mg/kg tended to be less effective. Surprisingly, Ro 15-4513 had only a borderline effect on the cerebellar cGMP level in the mouse. In line with its lack of a clearcut stimulant effect on behaviour, Ro 15-4513 produced only a borderline increase of central 2-deoxyglucose uptake in the cerebellum and septum at the high dose of 100 mg/kg PO and failed to alter the plasma glucose level.

For reasons discussed earlier (34) inverse agonists of the BZR reduce some effects of barbiturates; the ability to reverse barbiturate effects is generally a function of the negative intrinsic efficacy of inverse and partial inverse agonists. Ro 15-4513 was found to be very potent in reversing or preventing the effects of barbiturates in some tests (anti-PTZ and HWT in the mouse, hyperphagia in the rat) but was ineffective in other conditions (hexobarbitone-induced LRR in mice, phenobarbitone-depressed locomotor activity in rats). Systematic comparisons with other inverse and partial inverse agonists are lacking. The interaction of Ro 15-4513 with barbiturates was blocked by the BZR antagonist flumazenil.

Partial inverse agonists of BZR should of course antagonize the effects of agonists. Ro 15-4513 showed this effect in all those experiments performed to test it. In our routine test to assess diazepam-antagonistic activity, the HWT, Ro 15-4513 was a very potent diazepam antagonist (Ed_{50}=0.33 and 0.45 mg/kg PO in mice and rats, respectively).

In summary then, Ro 15-4513 is clearly characterized, by the present results and those reported by other authors, as a highly specific partial inverse agonist of the BZR. Except for the increase of spinal γ-motoneurone activity, all effects of Ro 15-4513 described here were antagonized by the BZR antagonist flumazenil. What is, unfortunately, lacking is the exact assessment of the negative intrinsic efficacy of Ro 15-4513 in comparison to other partial inverse agonists, e.g., by measuring its negative regulation of $GABA_A$ receptor function in single neurones. This greatly hampers the interpretation of its interaction with ethanol, as will be discussed below. Preliminary results obtained with chloride uptake measurements suggest that Ro 15-4513 is a low efficacy inverse agonist, its efficacy ranging between β-CCM and Ro 15-3505 (P. Schoch, unpublished).

There is no positive evidence so far that Ro 15-4513, in spite of being a suitable photoaffinity labelling ligand of the BZR in vitro, interacts covalently with the BZR under the usual conditions of in vivo animal experimentation.

Interaction of Ro 15-4513 With Ethanol

Our original finding of a partial reversal of some ethanol effects by Ro 15-4513 (4,33) was considered interesting enough to be communicated but we did not believe that this particular drug was sufficiently effective, safe, and long acting to be useful as an antidote to ethanol. The reports of Suzdak *et al.* (42–44) of an antagonistic interaction between ethanol and Ro 15-4513 on the level of behaviour and membrane chloride flux raised considerable scientific and mass media interest. Conflicting results on this interaction have been published in recent years. Our own results can be summarized and commented as follows.

Ro 15-4513 does not alter the lethal effect of ethanol (10), in contradiction to another study (8), but consistent with information from several investigators. In the present study, failures to find an antagonistic interaction between these two agents were also noted for the loss of righting reflex in mice, depression of rotarod performance in mice, decrease of cerebellar cGMP in mice, and inhibition of [^{35}S]TBPS binding in vitro. In contrast, Ro 15-4513 prevented the ethanol-induced decrease of locomotor activity in rats and the ethanol-induced impairment in the HWT was dose-dependently reversed by Ro 15-4513; in mice the compound was very potent (Ed_{50}=0.84 mg/kg PO) however, in rats it had only $^1/_{10}$ this potency. The antagonistic effect of Ro 15-4513 in the HWT was abolished by flumazenil. The decrease by ethanol of the cerebellar cGMP level was dose-dependently prevented by Ro 15-4513 in the rat, however, in the mouse the ethanol action was resistant to Ro 15-4513. It is worth noting that Ro 15-4513 alone produced a clear dose-dependent increase of cGMP in the rat, but was virtually ineffective in the mouse. The interaction between ethanol and Ro 15-4513 in the rat cerebellum was blocked by flumazenil. The rather uniform decrease by ethanol of [^{3}H]2-deoxyglucose uptake in various rat brain regions was virtually abolished by Ro 15-4513 which, by itself, produced a small increase in two brain regions. In the cat spinal cord, Ro 15-4513 reversed the ethanol-induced decrease of spontaneous activity of γ-motoneurones in a flumazenil-sensitive way.

Possible Mechanisms of Ro 15-4513-Ethanol Interaction

Theoretically, the interaction between ethanol and Ro 15-4513 could be accounted for by two major mechanisms. a) The first possibility is that Ro 15-4513 acts by virtue of its partial inverse agonistic activity, i.e., by its negative allosteric modulation of the $GABA_A$ receptor function. As a partial inverse BZR agonist, Ro 15-4513 could reduce some effects of ethanol if the latter acted at the BZR or at some other site on the $GABA_A$ receptor chloride channel complex to produce a positive allosteric modulation of the $GABA_A$ receptor function. The interaction could also be simply functional, the negative modulatory effect of Ro 15-4513 on the GABA receptor function producing (mildly stimulant) changes in neuronal activity that neutralize the CNS depressant alterations induced by ethanol through whatever mechanisms. b) Alternatively, Ro 15-4513 might affect neuronal activity by a yet unknown mechanism unrelated to the BZR; again this hypothetical mechanism could oppose that of ethanol at a common target or it could be a functional antagonism.

If Ro 15-4513 were to produce its interaction with ethanol through the BZR, the ethanol antagonism by Ro 15-4513 should, on the one hand, be blocked by a BZR antagonist, like flumazenil, and, on the other hand, other partial inverse agonists as well as full inverse agonists should be expected to mimic the ethanol-Ro 15-4513 interaction.

Before discussing these two important issues we should like to make some general comments pertinent to drug interaction studies. Pharmacological investigations of drug interactions and, in particular, their interpretations, are most frequently biased due to the lack of appropriate dose-response curves and the lack of appropriate replacement of the two interacting substances with other similarly acting agents. In the present context this means that both ethanol and Ro 15-4513 ought to be tested over a wide dose-range and that comparative studies ought to be performed with other agents acting similarly to ethanol and with other agents acting as full or partial BZR inverse agonists, if possible from diverse chemical classes. In most studies reported in the literature, as well as in the present one, a single dose of ethanol was administered in a given test paradigm. This dose varied between 2 and 6 g/kg IP and PO in our experiments which, in man, would produce marked drunkenness to severe intoxication. The reason for choosing one dose per paradigm is the necessity of obtaining consistent and quantifiable effects. In most of our tests we have used several doses of Ro 15-4513. Neither we nor other investigators have systematically studied the effect of Ro 15-4513 against other central depressant agents, e.g., barbiturates, meprobamate, other alcohols, general anesthetics, etc. We have conducted a few comparative studies with other negative modulators of the BZR but they are not sufficient for a final evaluation of the issue.

In cases where flumazenil was tested, the ethanol-Ro 15-4513 interaction was blocked by this specific BZR antagonist (HWT, cGMP, γ-motoneurones). Therefore, it is very likely that the interaction of Ro 15-4513 with ethanol was due to its action at the BZR. It is important that flumazenil itself did not interact with ethanol in our experiments. However, not enough data with other BZR antagonists are available to substantiate the conclusion that Ro 15-4513 interacts with ethanol through BZR. If negative modulation of the $GABA_A$ receptor function by Ro 15-4513 were indeed the mechanism of its ethanol reversal, one would expect that other partial inverse agonists of the BZR and, even more so, full inverse agonists, would be as effective antagonists of ethanol as Ro 15-4513 or even more powerful. We have only performed a few nonsystematic studies with Ro 15-3505, a benzodiazepinone derivative with very weak negative intrinsic efficacy, with the phenylquinoline CGS 8216, and with the β-carbolines FG 7142, β-CCE and β-CCM. The only interactions with ethanol were found with β-CCM for performance in the HWT and for cerebellar cGMP in the mouse (4). These predominantly negative results cannot, however, be considered to be conclusive before systemic dose-response relationships have been explored which take into account the duration of action and the variable bioavailability of these agents.

As a working hypothesis it is proposed that ethanol reversal by negative modulators of the BZR is not a linear function of their negative intrinsic efficacy, meaning that the most pronounced interaction with ethanol does not occur with full inverse agonists but with agents possessing a critical submaximal negative intrinsic efficacy. The reason could be that a critical moderate reduction of the tonic GABAergic inhibitory action in some distinct neuronal circuits may counteract some effects of ethanol more efficiently than a more generalized and more marked depression of $GABA_A$ receptor function.

The present and other published data overall favour the view that the interaction of Ro 15-4513 with some of the ethanol effects in some but not all test situations is due to its negative intrinsic efficacy at the BZR. However, our results do not help to decide whether this interaction is at the level of the GABA-gated chloride channel or whether the two agents act at different sites in a functionally antagonistic manner. Chloride flux experiments (42) support the former possibility. Unfortunately, the interaction of ethanol with $GABA_A$ receptor-mediated chloride conductance is not unambiguously demonstrated in electrophysiological studies.

Practical Relevance of the Ethanol-Ro 15-4513 Interaction

In view of the considerable impact of ethanol abuse on the affected individuals and the community, it would be of utmost importance to develop drugs that efficiently interact with the various aspects of ethanol's actions. a) One possible application of antiethanol drugs could be the reversal of acute drunkenness to reduce, e.g., traffic accidents. Such a "sober-up pill" would have to abolish reliably and completely the acute CNS effects of ethanol in the lower to medium dose range. Unpredictable ethanol antagonism would, of course, even increase the dangers of drunkenness. Whether a fully effective sober-up pill would be advantageous at all is subject to considerations of psychological, ethical and legal aspects. Clearly, Ro 15-4513 is very far away from an active and safe sober-up pill. b) Life-threatening severe intoxications with ethanol cannot realistically be expected to respond to agents that block specific or nonspecific effects of ethanol. Indeed, Ro 15-4513 is not capable of reducing lethality due to acute ethanol (10). What would be required for these situations are means to rapidly eliminate ethanol from the body or to accelerate its transformation into a nontoxic metabolite. c) A means of helping ethanol addicts to avoid resuming drinking would be a major breakthrough. Ro 15-4513 was suggested to be able to reduce the craving for ethanol or its reinforcing property (38). Only very indirect evidence for such an action can be expected on the basis of animal experiments. It is unlikely that reduc-

ing the acute CNS effects of ethanol would be the desired mechanism.

Hence, Ro 15-4513 and similarly acting agents should for the time being be considered as scientific tools for exploring certain aspects of the mechanisms of action of ethanol on the CNS.

CONCLUDING REMARKS

Ro 15-4513 is a benzodiazepine derivative with high affinity for the central BZR and a weak to moderate negative intrinsic efficacy reflected, e.g., by a proconvulsive activity and a reduction of dorsal root potentials. It was found to reverse and/or to prevent some, but not all, effects of ethanol and this interaction was blocked by the BZR antagonist flumazenil. In some tests active doses of Ro 15-4513 for ethanol interaction were lower than proconvulsive doses, in others they were in the same range. Some dramatic species differences in this ethanol interaction cannot yet be explained. Overall, the evidence suggests that Ro 15-4513 interacts with ethanol through its negative modulatory action of the $GABA_A$ receptor function but whether the interaction occurs at this receptor or whether it is a functional antagonism is not elucidated by our study. Out of a few other partial BZR inverse agonists tested in a nonsystematic manner only β-CCM (injected intravenously) was found to mimic Ro 15-4513 in its reversal of the ethanol induced performance deficit in the HWT. Some odd results call for systematic extension of experiments, in particular, for more dose-response studies. Ro 15-4513 is not neutralizing the effect of ethanol in a consistent manner and, therefore, a clinical evaluation of its therapeutic potential in man is not warranted.

ACKNOWLEDGEMENTS

The graphical assistance of Mrs. Brigitte Fritz is greatly appreciated. We thank also Mrs. Brigitte Schlienger for all secretarial work and for the untiring typing of the manuscript.

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