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Benzodiazepine Receptor Affinities, Behavioral, and Anticonvulsant Activity of 2-Aryl-2,5-dihydropyridazino[4,3-b]indol-3(3H)-ones in Mice

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DE SARRO, G., A. CAROTTI, F. CAMPAGNA, R. MCKERNAN, M. RIZZO, U. FALCONI, F. PALLUOTTO, P. GIUSTI, C. RETTORE AND A. DE SARRO. *Benzodiazepine receptor affinities, behavioral, and anticonvulsant activity of 2-aryl-2,5-dihydropyridazino[4,3-b]indol-3(3H)-ones in mice.* PHARMACOL BIOCHEM BEHAV **65**(3) 475–487, 2000.—The anticonvulsant properties of 1,4-benzodiazepines (BDZs), pyrazoloquinolones (CGS), 2-aryl-2,5-dihydropyridazino[4,3-b]indol-3(3H)-ones (PIs) **1**, and abecarnil were studied after intraperitoneal (IP) administration in mice. The anticonvulsant effects were evaluated on seizures evoked by means of auditory stimulation in DBA/2 mice or on seizures induced by administration of pentylenetetrazole (PTZ) in Swiss mice. In DBA/2 mice abecarnil was the most potent compound studied. The rank order of potency for anticonvulsant activity was abecarnil > flunitrazepam > **1i** > diazepam > pinazepam > **1d** > quazepam > prazepam > halazepam > **1f** > **1e** > **1b** > CGS 9896 > **1c** > **1h**, and **1a**, the latter being inactive against audiogenic seizures. Some PIs **1** and abecarnil showed anticonvulsant properties against seizures induced by PTZ with a potency lower than that observed in audiogenic seizures. The pharmacological actions of **1d**, **1f**, and **1i** were significantly reduced by a treatment with flumazenil (8.24 μmol/kg IP), suggesting a clear involvement of benzodiazepine mechanisms in the anticonvulsant activity of these compounds or their metabolites. The anticonvulsant activity of **1d**, **1f**, and **1i** was also evaluated against seizures induced by two β-carbolines namely methyl-β-carboline-3-carboxylate (β-CCM) and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM), in DBA/2 mice: they gave better protection against seizures induced by β-CCM than the ones by DMCM. The potency of various BDZs and PIs as inhibitors of specific [^{3}H]flumazenil binding to neuronal membranes, was also evaluated. The radioligand binding study, carried out on stable cell lines expressing definite combinations of benzodiazepine receptor subunits, demonstrated that **1b**, **1e**, **1d**, and **1h** have preferential interaction with α_1, β_3, γ_2, receptor subtypes. © 2000 Elsevier Science Inc.

1,4-Benzodiazepines 2-Aryl-2,5-dihydropiridazino[4,3-b]indol-3(3H)ones DBA/2 mice Epilepsy β-Carbolines Pentylenetetrazole Flumazenil Rotarod Audiogenic seizures

THE benzodiazepine (BDZ) binding site has been identified as part of the GABA receptor–Cl^- ionophore molecular complex (44,46). Although initial studies indicated that BDZs bind to the α-subunit and that GABA binds to the β-subunit of the receptor complex (5), it was later found that the γ_2-subunit contributes to the formation of a functional BDZ site (41,42). A total of at least 13 subunits (α_1 to α_6, β_1 to β_3, γ_1 to γ_3, and δ) of the $GABA_A$ receptor have been identified by

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molecular cloning, and these subunits are thought to assemble into a pentameric structure to form a Cl^- channel (36,46). Most functional subtypes of $GABA_A$ receptors contain α, β, and γ subunits, in different combinations and show high sensitivity to different benzodiazepine receptor ligands (16,31,33).

Numerous attemps to correlate benzodiazepine receptor (BDZR) occupancy with functional effects have been done by several authors (10,19,32,33,39). Besides BDZs, β-carbolines, pyrazoloquinolones (CGS_1), and several other classes of chemicals bind to BDZR. Previous reports demonstrating the different binding properties of some BDZs have permitted the identification of multiple subtypes of BDZRs (17,27,46). Unwanted effects (i.e., sedation and muscle relaxation) have been observed with several BDZR ligands (29). Thus, there has been increasing effort devoted to the development of BDZR ligands with highly selective in vivo activities. A major strategy used towards this end by various investigators has been based on the hypothesis of "partial agonism." Mennini and Garattini (35) and Petersen et al. (39) have showed that diazepam required higher fractional receptor occupancy to produce motor-impairing effects than to induce anticonflict activity. Furthermore, partial agonists such as bretazenil, CL 218872, Ro 17-1812, RU 33368, and FG 8205, differ from diazepam, requiring higher BDZR occupancy to elicit equivalent effects (3,21–24,48). The diverse in vivo activities observed for BDZR ligands range from full agonists (anxiolytic, hypnotic, and anticonvulsant agents), through antagonists (nil efficacy), to inverse agonists (proconvulsant and anxiogenic agents) (9,48).

As a follow-up of our investigation on binding and anticonvulsant activity of several classes of BDZR ligands (4,14–16,38), aimed at a better definition of the pharmacophore for BDZR ligands, in the present report we describe, compare, and discuss the BDZR affinity, the behavioral and antiseizure effects of some classic BDZs, CGS, and 2-aryl-2,5-dihydropyridazino[4,3-b]indol-3(3H)-ones (PIs, **1**) (Fig. 1). The latter have been previously synthesized in our laboratory (4,38). Molecular modeling and theoretical studies have shown that the relatively close heterocyclic nuclei of **1** and CGS are indeed diverse in terms of chemical and structural properties. In particular, a slightly different location of putative pharmacophore points as well as relatively different physicochemical properties might justify the diverse binding and pharmacological properties of these ligands. Preliminary studies have shown indeed that some PIs **1** inhibited [^{3}H]flunitrazepam binding (38) but to a lower extent than corresponding CGS compounds.

However, according to some previously proposed pharmacophore models (1,17,24,49), all the investigated compounds **1** should present the essential structural features for a relatively high BDZR affinity (4,38). The BDZR affinity of BDZs, CGS1, and PIs was evaluated both in the cerebellum and in the cortex which, present primarily α**1**-containing receptor subtypes (33,36), and in cell lines expressing recombinant receptors of structure $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, $\alpha_5\beta_3\gamma_2$, and $\alpha_6\beta_3\gamma_2$ (36).

The anticonvulsant and proconvulsant properties of the examined BDZR ligands against pentylenetetrazole (PTZ)-induced and audiogenic seizures, were assayed in Swiss and DBA/2 mice respectively. The latter strain of mice is genetically susceptible to sound-induced seizures, and has been considered an animal model for testing new anticonvulsant drugs (7,45). In addition, the modification of the observed pharmacological effects by the "neutral" benzodiazepine antagonist flumazenil and the ability to antagonize the seizures of two β-carbolines (β-CCM and DMCM) were studied for selected compounds **1**, diazepam, and quazepam.

METHOD

Testing of Anticonvulsant Activity in DBA/2 Mice

DBA/2 mice (6–12 g, 22–26 days old) were purchased from Charles River (Calco, Como, Italy), and after 5 days of habituation they were exposed to auditory stimulation, 45 min following intraperitoneal (IP) administration of vehicle or drugs. The experimental protocol was approved by University of Catanzaro Ethical Committee, and procedures are in compliance with the Nationals Institutes of Health Guide for Care and Use of Laboratory Animals (Pubblications No. 85-23, revised 1985) and European Communities Council Directive of 24 November 1986 (86/609 EEC).

For systemic injections from four to six doses of all compounds were given intraperitoneally (0.1 ml/10 g of body weight of the mouse) as a freshly prepared solution in 30% dimethylsulfoxide (DMSO) and 70% sterile saline (0.9% NaCl). Control animals received a vehicle solution containing 30% DMSO and 70% sterile saline. Flumazenil was prepared and used at a dose level that did not affect audiogenic seizure response in DBA/2 mice (13). Individual mice were placed under a hemispheric perspex dome (diameter 58 cm) and 60 s allowed for habituation and assessment of locomotor activity. Auditory stimulation (12–16 kHz, 109 dB) was applied for 60 s or until tonic extension occurred. Seizure response (S.R.) as previously reported (12) was assessed on the following scale: 0 = no response, 1 = wild running, 2 = clonus, 3 = tonus, 4 = respiratory arrest. The maximum response was recorded for each animal. Rectal temperature was recorded immediately prior to auditory testing using an Elektrolaboratoriet thermometer type T.E.3. Behavioral changes were observed during the period between drug administration and auditory testing. Ten mice were administered for each dose level of each compound studied.

Antagonism of BDZ Anticonvulsant Activity

The method used to evaluate antagonism of anticonvulsant activity of benzodiazepine was that previously described by Chapman et al. (8). Briefly, DBA/2 mice were administered IP with diazepam (0.1–3.3 mg/kg corresponding to 0.35–11.59 (μmol/kg) at a standard volume of 0.1 ml/10 g. Fifteen minutes later, a test substance (flumazenil, CGS 8216, 1a) was injected IP and followed (30 min later) by the auditory test. The total number of mice that developed seizures was tallied at each dose level.

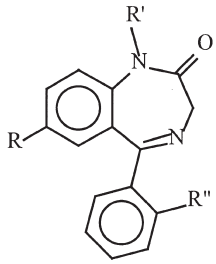
Anticonvulsant Properties Against Pentylenetetrazole-Induced Seizures in Swiss Mice

Male Swiss mice (20–26 g, 48–56 days old) were purchased from Charles River (Calco, Como, Italy), and were pretreated with vehicle or drugs 45 min before the subcutaneous (SC) administration of PTZ. The convulsive dose 97 (CD_{97}) of PTZ (85 mg/kg) or the subconvulsive dose (40 mg/kg) were injected in a volume of 0.1 ml/10 g of body weight of the mouse. The animals were then placed in isolated cages and observed for 30 min. A threshold convulsion is an episode of clonic spasms lasting for at least 5 s. Absence of this threshold convulsion over 30 min indicates that the test substance has the ability to elevate PTZ seizure threshold (47).

β-Carboline-Induced Seizures in DBA/2 Mice

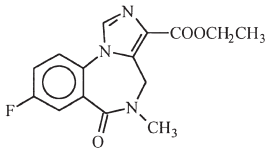
DMCM and β-CCM seizures were induced in adult DBA/2 mice (6–8 weeks old) by the IP injection of DMCM (3.18–6.36 (μmol/kg) or β-CCM (2.2–8.4 (μmol/kg) dissolved in a minimal amount (<5% of final volume) of glacial acetic acid, and

1,4-Benzodiazepines

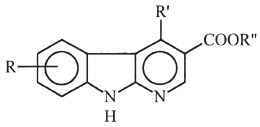


Common name	R	R'	R''
Diazepam	Cl	CH_3	H
Halazepam	Cl	CH_2CF_3	H
Quazepam	Cl	CH_2CF_3	F
Pinazepam	Cl	$-CH_2C{\equiv}CH$	H
Flunitrazepam	NO_2	CH_3	F

Flumazenil

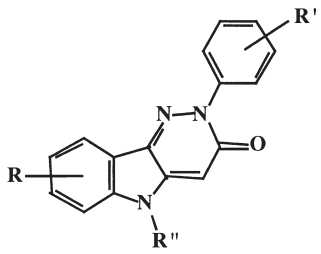


β-Carbolines

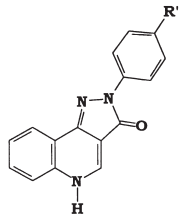


Common name and codes	R	R'	R''
Abecarnil	$OCH_2C_6H_5$	CH_2OCH_3	$CH(CH_3)_2$
β-CCM	---	---	CH_3
DMCM	$6,7(OCH_3)_2$	CH_2CH_3	CH_3

Pyridazinoindoles (PI) 1 and Pyrazoloquinolines (CGS)



1a-i



CGS

Compound	R	R'	R''
1a	H	H	H
1b	H	*p*-Cl	H
1c	H	*m*-Cl	H
1d	H	*p*-Br	H
1e	H	*p*-OCH_3	H
1f	H	*p*-CH_3	H
1g	H	*p*-NO_2	H
1h	8-Br	H	H
1i	H	*p*-Cl	CH_3
CGS 8216	-	H	-
CGS 9895	-	OCH_3	-
CGS 9896	-	Cl	-

FIG. 1. Chemical structures of compounds used in this study.

brought to volume with saline (n = 20–30 per group of pooled controls). The final solution showed a pH of 5–5.5. At least 10 mice were used for each dose of each compound studied. The mice were observed for 30 min for the incidence of clonic seizures. The animals were pretreated with diazepam (3.51 μmol/kg), quazepam (3.49 μmol/kg), **1d** (2.44 μmol /kg), **1f** (1.08 μmol /kg), and **1i** (0.97 μmol /kg) 15 min before the administration of DMCM or β-CCM. The doses of diazepam, quazepam, **1d**, **1f**, and **1i** used were calculated according to the method of Litchfield and Wilcoxon (30), and correspond to the doses that were able to antagonize the clonic phase of the audiogenic seizures in 95% of the DBA/2 mice.

Anxyolysis/Anxiogenesis

A computer-controlled elevated plus-maze test system was adapted to study the anxiolytic and anxiogenic properties of the test compounds. The apparatus was mounted on a 50 cm-high plastic base, and consisted of two open arms (50 × 10 cm) and two enclosed arms (50 × 40 × 10 cm) made from dark Plexiglas, connected by a central platform (10 × 10 cm). The apparatus was equipped with 12 pairs of infrared photocell units and connected to an IBM computer. Thirty minutes following drug or vehicle administration, the animal was placed in the center of the plus-maze, facing a closed arm. The number of entries and the time spent in the open, closed, and center arms were recorded over a 5-min period.

Sedation

The testing was done immediately after the anxiety test, for a duration of 10 min. The locomotor activity monitor was an enclosed soundproof stainless steel cubicle with a white Plexiglas bottom, 23 cm in diameter and 34 cm high, equipped with six pairs of photocell detectors. Interruptions of the photocell beams were recorded automatically by digital counter.

Effects on Motor Movements

Groups of 10 male Swiss mice (20–26 g 48–54 days old) were purchased from Charles River (Calco, Como, Italy), and were trained to do coordinated motor movements continously for 2 min on a rotarod 3 cm diameter 8 r.p.m. (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as the inability of the mice to remain on the rotarod for a 2-min test period (20). The ability of the mice to remain on the rotarod was tested 45 min after administration of various BDZR ligands and PIs **1**.

Membrane Preparation and [^{3}H]Flumazenil Binding Studies

Male SD/Rij rats (FRAR, S. Pietro al Natisone, UD, Italy), weighing 200–250 g, were decapitated, and different brain areas and spinal cord were rapidly dissected on ice. The brain region and spinal cord tissues were homogenized in 20 ml of ice-cold 0.32 M sucrose pH 7.4 by using a glass homogenizer with a Teflon pestle (10 up-and-down strokes). The homogenate was centrifuged at 1000 × *g* at 4°C for 10 min, the P1 pellet was discarded, and the supernatant was collected and recentrifuged at 20,000 × *g* at 4°C for 20 min. The resulting crude mitochondrial pellet (P2) was resuspended in 20 ml of ice-cold distilled water, and homogenized. The homogenate was centrifuged at 8000 × *g* at 4°C for 20 min, the supernatant was collected and recentrifuged at 48,000 × *g* at 4°C for 20 min, and the final crude microsomal pellet (P3) was frozen for at least 24 h. The pellet was resuspended in 10 ml of 50 mM Tris-HCl pH 7.4, centrifuged at 48,000 × *g* at 4°C for 20 min, and then resuspended in 10 vol of the same buffer for standard binding assay. Aliquots of membrane suspensions (100 μl, or 0.15 mg of protein) were added to incubation medium containing 1 nM of [^{3}H]flumazenil (specific activity 72.4 Ci/mmol) in a final volume of 1 ml of Tris-HCl 50 mM, NaCl 120 mM, and KCl 5 mM, pH 7.4 (16). All compounds were dissolved in DMSO at the final concentration of 1%. Incubations were carried out for 60 min at 4°C in triplicate and nonspecific binding measured in the presence of 10 μM of diazepam. Reactions were stopped by the addition of 5 ml ice-cold Tris-HCl followed by rapid filtration through Whatman GF/C glass fiber filters (Whatman Inc., Clifton, NJ) and two additional washes. The radioactivity trapped on the filters was counted after the addition of 8 ml of Filter Count (Packard), by liquid scintillation spectrometry. The experiments were run in triplicate with eight different concentrations of competing ligand leading to the determination of $IC_{50} \pm$ SD values.

Effects on Radioligand Binding of Stable Cell Lines

Stable cell lines were generated and radioligand binding studies carried out essentially as described by Hadingham et al. (28). Briefly, stable cell line expressing $\alpha_1\beta_3\gamma_2$, $\alpha_2\beta_3\gamma_2$, $\alpha_3\beta_3\gamma_2$, $\alpha_5\beta_3\gamma_2$, and $\alpha_6\beta_3\gamma_2$ were grown as previously described (25,33). Cells were harvested by scraping, and were washed twice by centrifugation at 1000 × *g* and resuspension in 50 mM phosphate, 120 mM NaCl, pH 7.5. Cells were either frozen as pellets or used immediately by resuspension in 10 ml of 50 mM phosphate buffer pH 7.5. Membranes (25–75 mg of protein) were incubated with [^{3}H]flumazenil in a total volume of 0.5 ml for 1 h at room temperature in the presence of varying concentrations of the test compound. Nonspecific binding was defined with 10 μM flunitrazepam. Incubations were terminated by filtration followed by three washes (5 ml) with ice cold buffer.

Statistical Analysis

Statistical comparisons between groups of control and drug-treated animals were made using Fisher's exact probability test (incidence of the seizure phases) or ANOVA followed by post hoc Dunnett's *t*-test (rectal temperatures). The percentage incidence of each phase of the audiogenic seizure was determined for each dose of compound administered, and dose–response curves were fitted using linear regression analysis of percentage response. ED_{50} values (±95% confidence limits) for each compound and each phase of seizure response were estimated using the method of Litchfield and Wilcoxon (30); the relative anticonvulsant activities were determined by comparison of respective ED_{50} values. The dose that induced 50% of mice to fall from the rotarod TD_{50} values (±95% confidence limits) for each compound was estimated using the method of Litchfield and Wilcoxon (30). The relative activities were determined by comparison of respective TD_{50} values. For the binding experiments ID_{50} values for the statistical package for the [^{3}H]flumazenil displacement were determined by the nonlinear curve-fitting program based on McPherson's ligand (34).

Drugs

The chemical structures, molecular weight (MW), and lipophilicity parameters of tested drugs are listed in Fig. 1 and Table 7. The sources of the drugs used were: diazepam was purchased from Sigma (St. Louis, MO), pinazepam, prazepam, and flunitrazepam were extracted with chloroform from the corresponding drug formulation and purified by crystallization; halazepam and quazepam were obtained from Schering-Plough (Milano, Italy), DMCM (methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, MW = 314.34), and β-CCM (methyl-β-carboline-3-carboxylate, MW = 226.26) were obtained from Schering (Berlin, Germany), PIs **1a–i** were synthesized as previously described (4,38). Flumazenil (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate, MW = 206) was obtained from Hoffmann–LaRoche (Basel, Switzerland). Abecarnil was obtained from Schering AG (Berlin, Germany). CGS 8216, CGS 9895, and CGS 9896 were obtained from Ciba-Geigy (Summit, NJ). [^{3}H]Flumazenil was obtained from New England Nuclear (Boston, MA).

RESULTS

Anticonvulsant Activity of Classical 1,4-Benzodiazepines (BDZ) Against Audiogenic Seizures in DBA/2 Mice

As shown in Table 1, classical BDZs were able to protect DBA/2 mice against the clonic and tonic phases of the audiogenic seizure response. In particular, the audiogenic seizures were significantly reduced 45 min after IP administration of diazepam (0.33, 0.66, and 1.0 μmol/kg), halazepam (3.3 and 10 μmol/kg), pinazepam (1.0 μmol/kg), prazepam (3.3 and 10 μmol/kg), flunitrazepam (0.33, 0.66, and 1.0 μmol/kg), and quazepam (0.33, 0.66, and 1.0 μmol/kg). The relative ED_{50} values (±95% confidence limits) are reported in Table 1. The wild running phase was significantly reduced after IP administration of all BDZs at the largest doses tested (Table 1). A significant fall in body temperature and sedative effects were observed after the highest doses of all BDZs studied (see below).

TABLE 1
ANTICONVULSANT ACTIVITY OF 1,4-BENZODIAZEPINES AND 2-ARYL-2,5-DIHYDROPYRIDAZINO[4,3-b]INDOL-3(3H)-ONES **1** AGAINST THE CLONIC AND TONIC PHASES OF THE AUDIOGENIC SEIZURES

Drug	Range of Doses (μmol/kg)	Clonic Phase (ED_{50}, μmol/kg)	Tonic Phase (ED_{50}, μmol/kg)
Diazepam	(0.1–1)	0.28 (0.20–0.39)	0.24 (0.15–0.39)
Halazepam	(0.33–10)	1.82 (1.23–2.68)	1.36 (0.74–2.52)
Pinazepam	(0.033–1)	0.38 (0.17–0.83)	0.18 (0.09–0.38)
Prazepam	(0.33–10)	1.45 (0.87–2.44)	1.01 (0.49–2.09)
Flunitrazepam	(0.033–1)	0.22 (0.11–0.41)	0.18 (0.09–0.36)
Quazepam	(0.1–1.0)	0.48 (0.20–0.90)	0.26 (0.10–0.65)
1a	(0.33–33.3)	NA	NA
1b	(0.33–10)	3.62 (1.76–7.44)	1.79 (0.79–4.06)
1c	(3.3–100)	48.42 (20.63–113.64)	43.18 (21.64–86.16)
1d	(0.1–1)	0.68 (0.59–0.80)	0.26 (0.11–0.59)
1e	(0.66–10)	3.12 (1.72–5.66)	2.26 (1.66–3.09)
1f	(0.1–10)	30.1 (2.27–3.99)	0.80 (0.29–2.18)
1g	(1–10)	4.73 (2.86–7.83)	4.15 (2.29–7.51)
1h	(0.33–33.3)	NA	NA
1i	(0.1–3.3)	0.26 (0.16–0.42)	0.19 (0.13–0.28)
Abecarnil	(0.001–0.1)	0.062 (0.041–0.092)	0.0084 (0.0037–0.0197)
CGS 8216	(0.33–33.3)	NA	NA
CGS 9895	(0.33–33.3)	NA	NA
CGS 9896	(0.33–33.3)	3.82 (2.16–6.76)	2.81 (2.03–3.89)
flumazenil + **1d**		2.53 (1.82–3.52)*	0.73 (0.45–1.17)*
flumazenil + **1f**		8.10 (7.59–8.64)*	5.81 (4.39–7.69)*
flumazenil + **1i**		0.83 (0.55–1.25)*	0.71 (0.48–1.05)*

ED_{50} values (with 95% confidence limits) were calculated according to the method of Litchfield and Wilcoxon (30). *Significant differences between ED_{50} values for group treated with flumazenil (8.24 μmol/kg + **1d**, flumazenil (8.24 μmol/kg) + **1f** or flumazenil (8.24 μmol/kg) + **1h** and group treated with **1d**, **1f**, and **1i** alone, $p < 0.01$. NA = not active.

*Anticonvulsant Activity of 2-Aryl-2,5-dihydropyridazino [4,3-b]indol-3(3H)-ones **1a–i** and CGS Derivatives Against Audiogenic Seizures in DBA/2 Mice*

As shown in Table 1, some PIs were able to protect DBA/2 mice against the clonic and tonic phases of the audiogenic seizure response. In particular, the audiogenic seizures were significantly reduced 45 min after IP administration of **1b** (3.3, 6.6, and 10 μmol/kg), **1c** (66 and 100 μmol/kg), **1d** (0.66 and 1.0 μmol/kg), **1e** (3.3, 6.6, and 10 μmol/kg), **1f** (3.3, 6.6, and 10 μmol/kg), **1g** (6.6 and 10 μmol/kg), and **1i** (0.33, 0.66, 1, and 3.3 μmol/kg). The wild running phase was significantly reduced after IP administration of compounds **1b–i**. The phases of the audiogenic seizures were not significantly affected by **1a** and **1h**. The CGS 9896 (6.6, 10, and 33.3 μmol/kg) administered 45 min before the test was able to significantly reduced the clonic and tonic phase of seizures, while CGS 8216 and CGS 9895 did not affect audiogenic seizures (Table 1). A significant fall in body temperature was observed after the highest doses of some derivatives (see below). The relative ED_{50} values (±95% confidence limits) for the anticonvulsant activity of PIs **1** and CGS_1 are shown in Table 1.

Hypothermia

As shown in Fig. 2, two partial agonists, abecarnil, $F(4, 21) = 9.78$, $p = 0.0002$, and CGS 9896, $F(4, 47) = 11.05$, $p = 0.0001$, **1i**, $F(5, 42) = 3.158$, $p = 0.01$, diazepam, $F(1, 10) = 12.22$, $p = 0.006$, **1a** $F(7, 4) = 11.3$, $p = 0.017$, **1d**, $F(4, 47) = 11.05$, $p = 0.0001$, and CGS 8216, $F(7, 42) = 10.96$, $p = 0.0001$, decreased rectal temperature 40 min after administration of these ligands, whereas **1e**, $F(5, 42) = 0.424$, $p = 0.83$, **1g**, $F(5, 42) = 2.35$, $p = 0.058$, and **1h**, $F(4, 45) = 0.676$, $p = 0.61$, had no effect (data not shown).

Treatment With Flumazenil

To ascertain the possible involvement of benzodiazepines receptors in the antiseizure activity of PIs **1**, the most active compounds (i.e., **1d**, **1f**, and **1i**) were administered concomitantly with flumazenil. In particular, the anticonvulsant effects of **1d**, **1f**, or **1i** derivative (0.1, 0.33, 1.0, 3.3, 6.6, and 10.0 μmol/kg) was reduced by a treatment with flumazenil (8.24 μmol/kg IP) 15 min after the administration of these compounds. In fact, a significant increase in the incidence of all phases of the audiogenic seizure response was seen in the groups treated with flumazenil compared to the corresponding groups receiving **1d**, **1f**, or **1i** derivative alone. The ED_{50} values for **1d**, **1f**, or **1i** derivative against the different phases of audiogenic seizures significantly increased (from 2.69- to 7.26-fold, Table 1). Flumazenil (8.24 μmol/kg, IP) did not modify the body temperature in DBA/2 mice. As for their anticonvulsant effects, flumazenil was able to antagonize the hypothermic actions of PIs in DBA/2 mice.

*Treatment With **1a***

To ascertain the possible involvement of benzodiazepine receptors in the activity of PIs, the derivative **1a**, which showed no anticonvulsant activity, was administered concom-

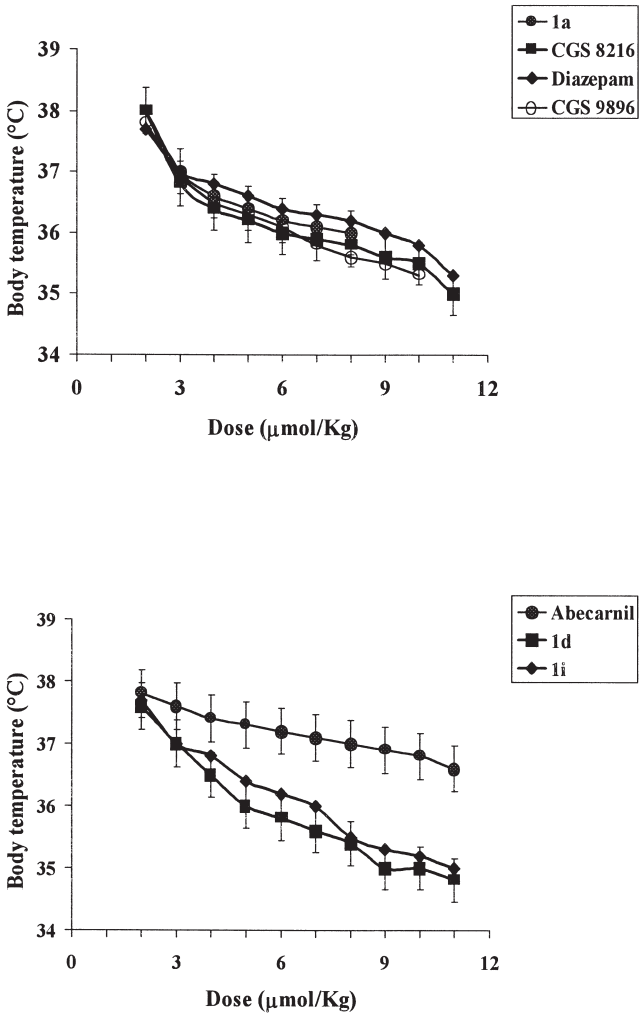


FIG. 2. The hypothermic effect of six benzodiazepine receptor ligands as measured by the change in rectal temperature following the increasing dose of each compound. Ordinate shows body temperature (°C), abscissa shows the dose expressed as μmol/kg. The parameter presented here is the mean ± SEM (n = 6–10).

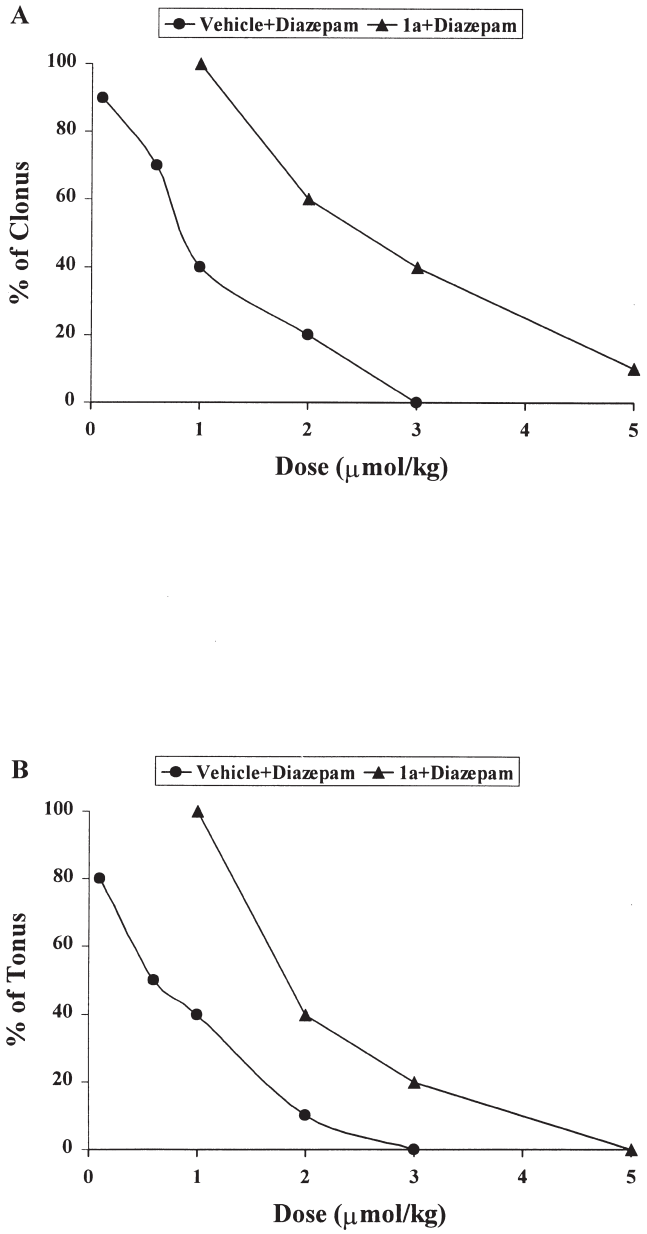


FIG. 3. Dose–response curves obtained following diazepam IP injection in DBA/2 mice pretreated with **1a** or vehicle. (A) % clonus; (B) % tonus. Ordinate shows percentage of clonus (A) or tonus (B); abscissa shows the dose of diazepam expressed as μmol/kg. For the determination of each points, 10 mice were used.

itantly with diazepam. **1a** (3.3 μmol/kg), administered IP, was able to reduce the anticonvulsant properties of diazepam (0.1–3.3 μmol/kg IP) in DBA/2 mice (Fig. 3). In particular, following concomitant treatment of **1a** + diazepam, the dose–response curve shifts to the right, and the ED_{50} values (±95% confidence limits) in DBA/2 mice were 0.74 (0.49–1.11) μmol/kg for clonus and 0.46 (0.33–0.64) μmol/kg for tonus. **1a** increased by 1.9–2.6-fold the ED_{50} values of diazepam. CGS 8216 was able to shift the dose–response curve of diazepam to the right, similarly to **1a** (data not shown).

Anticonvulsant Properties Against Pentylenetetrazol-Induced Seizures

Table 2 shows the ED_{50} values (±95% confidence limits) of those compounds that were active against clonic seizures induced by SC administration of PTZ in Swiss mice. In particular, the clonic seizures induced by PTZ were significantly reduced 45 min after IP injection of diazepam (0.66 and 1.0 μmol/kg), halazepam (3.3, 6.6, and 10 μmol/kg), pinazepam (1.0 μmol/kg), prazepam (3.3 and 10 μmol/kg), flunitrazepam (0.33, 0.66, and 1.0 μmol/kg), and quazepam (1, 2.1, and 3.3 μmol /kg). In addition, a pretreatment 45 min before with **1d** (21, 33, and 66 μmol/kg), **1e** (66 and 100 μmol/kg), **1f** (10 and 33 μmol/kg), **lg** (10, 21, and 33 μmol/kg), and **1i** (3.3, 6.6, and 10 μmol/kg) was able to significantly reduce the incidence of clonic seizures induced by PTZ. The CGS derivatives were not active or very weakly active (CGS 9896) to protect against PTZ-induced seizures. Diazepam and flunitrazepam were more potent (8.3- and 12.4-fold, respectively) than **1i**, which

TABLE 2

ANTICONVULSANT ACTIVITY AGAINST PENTYLENETETRAZOLE-INDUCED SEIZURES AND EFFECTS ON MOTOR MOVEMENTS OF 1,4-BENZODIAZEPINES AND 2-ARYL-2,5-DIHYDROPYRIDAZINO[4,3-b]INDOL-3(3H)-ONES **1**

Drug	ED_{50} Clonus (μmol/kg)	TD_{50} Locomotor Deficit (μmol/kg)	TD_{50}/ED_{50}
Diazepam	0.43 (0.27–0.68)	11.6 (9.2–14.6)	26.9
Halazepam	2.46 (1.78–3.40)	19.5 (11.3–33.7)	7.9
Pinazepam	0.61 (0.34–1.09)	6.9 (4.7–10.2)	11.3
Prazepam	2.55 (1.81–3.59)	18.1 (12.7–25.8)	7.1
Flunitrazepam	0.29 (0.18–0.47)	8.7 (6.1–12.4)	29.5
Quazepam	0.82 (0.62–1.19)	16.2 (12.1–21.7)	19.7
1d	17.62 (7.05–44.04)	>250	>14
1e	43.60 (18.2–104.5)	>250	>5.7
1f	8.03 (4.94–13.0)	156.5 (79.5–307.9)	19.5
1g	11.17 (5.91–21.1)	241.3 (192.9–301.7)	21.6
1i	3.6 (2.7–4.8)	>250	>69
Abecarnil	0.22 (0.15–0.32)	>250	>1136
CGS 8216	NA	ND	ND
CGS 9895	NA	ND	ND
CGS 9896	190.6 (87.9–413.1)	>250	>1.31

ED_{50} and TD_{50} values (with 95% confidence limits) were calculated according to the method of Litchfield and Wilcoxon (30). TD_{50}/ED_{50}, the therapeutic index represents the ratio between TD_{50} and ED_{50}.

NA = not active; ND = not determined.

was the most active anticonvulsant PI derivative in the PTZ protocol.

Anticonvulsant Activity Against β-Carboline-Induced Seizures

Following vehicle pretreatment (30 min before) β-CCM (2.2, 4.3, 6.5, and 8.4 μmol/kg IP) induced clonic seizures in DBA/2 mice in a dose-dependent manner. In particular, the lower dose of β-CCM (2.2 μmol/kg IP) elicited clonic convulsions in 30% of the mice. At the dose of 4.3 μmol/kg IP β-CCM elicited clonic convulsions in 58% of the mice, at 6.5 μmol/kg IP it induced clonic seizures in 80% of the mice, while at 8.4 μmol/kg it produced clonus in 93% of the animals. The ED_{50} value for β-CCM–induced clonic convulsions was 3.36 μmol/kg IP (Table 3). After vehicle pretreatment (30 min before) DMCM (3.18, 3.82, 4.77, and 6.36 μmol/kg IP) produced clonic seizures in DBA/2 mice in a dose-dependent manner. In particular, at the lower dose of 3.18 μmol/kg IP DMCM induced clonus in 10% of the mice, at 3.82 μmol/kg IP it elicited clonic seizures in 33% of the mice, and at 4.77 μmol/kg IP it caused clonic convulsions in 73% of the mice. At the highest dose tested (6.36 μmol/kg IP) DMCM produced clonus in 100% of animals. The ED_{50} value for DMCM-induced clonic convulsions was 4.17 μmol/kg IP (Table 3). A pretreatment 30 min before with diazepam (3.51 μmol/kg IP), quazepam (3.49 μmol/kg IP), **1d** (2.44 μmol/kg IP), **1f** (1.08 μmol/kg IP), and **1i** (0.97 μmol/kg IP) provided some anticonvulsant protection against β-carboline–induced seizures. In particular, diazepam increased by 2.1-fold the ED_{50} values for both β-CCM and DMCM, while quazepam, **1d**, **1f**, and **1i** provided better anticonvulsant protection against β-CCM seizures than against DMCM induced seizures (Table 3). Diazepam was the most potent in the protection against DMCM-induced seizures, whereas for the seizures induced by β-CCM the three

TABLE 3

ANTAGONISM OF THE CLONIC CONVULSANT ACTION OF THE β-CARBOLINES β-CCM AND DMCM IN ADULT DBA/2 MICE BY DIAZEPAM, QUAZEPAM AND SOME 2-ARYL-2,5-DIHYDROPYRIDAZINO[4,3-b]INDOL-3(3H)-ONES **1**

Pretreatment (30 Min Before)	ED_{50} μmol/kg		Potency Ratio	
	β-CCM	DMCM	β-CCM	DMCM
Vehicle	3.36 (2.08–4.99)	4.17 (3.75–4.61)	—	—
Diazepam (3.51 μmol/kg)	6.89 (5.66–8.40)	8.62 (8.05–9.26)	2.1*	2.1*
Quazepam (3.49 μmol/kg)	13.39 (10.92–16.44)	7.09 (6.65–7.54)	4.0*	1.7
1d (2.44 μmol/kg)	15.38 (10.74–22.01)	6.74 (5.41–8.43)	4.6*	1.6
1f (1.08 μmol/kg)	15.91 (11.93–21.21)	7.35 (4.74–11.32)	4.7*	1.8
1i (0.97 μmol/kg)	15.07 (9.41–24.13)	7.28 (5.57–9.48)	4.5*	1.7

ED_{50} values (with 95% confidence limits) were calculated according to Litchfield and Wilcoxon (30). Potency ratios are the ratios of ED_{50} values of drug over vehicle.

*Significant differences vs. respective control groups, $p < 0.01$.

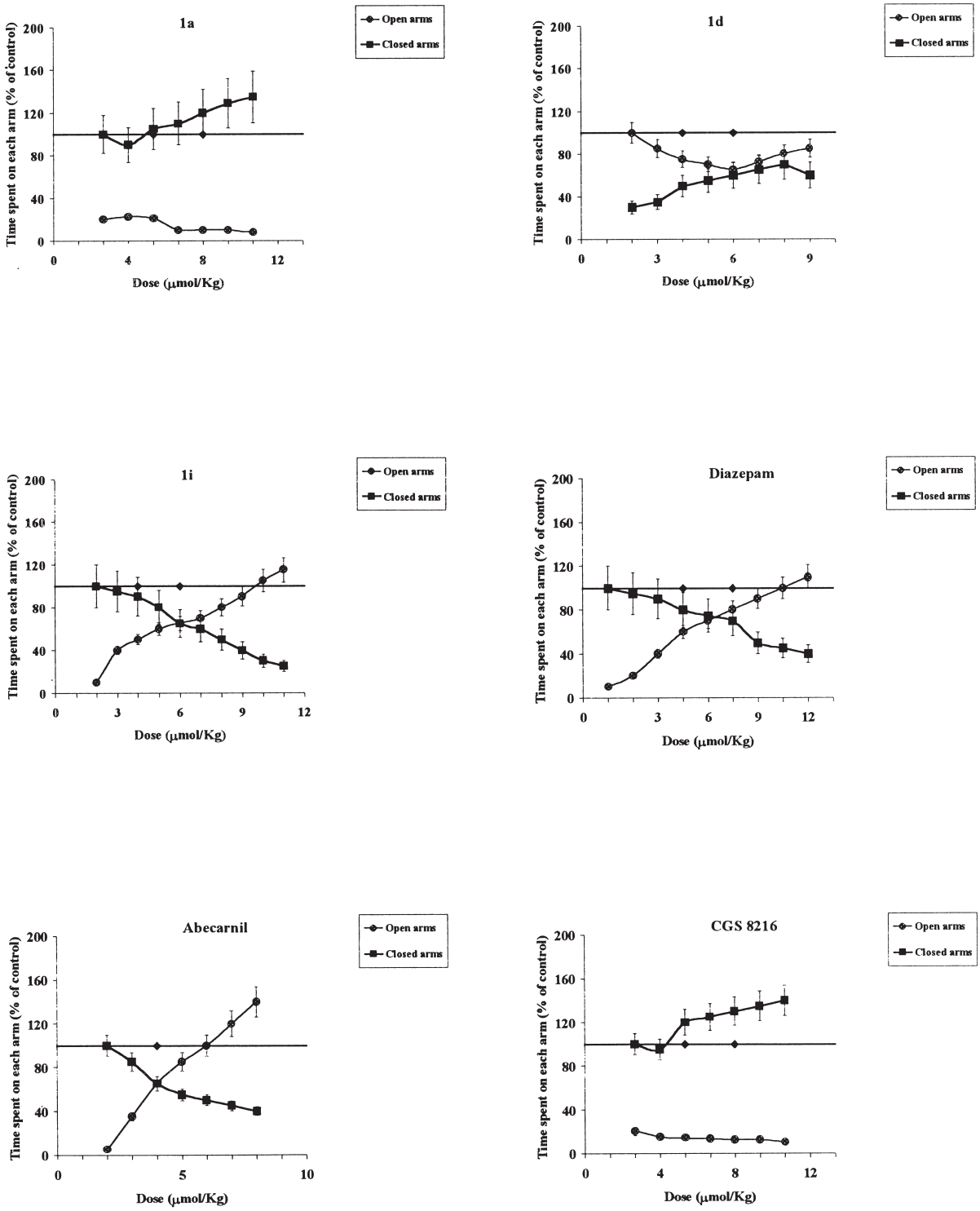


FIG. 4. The effect of six benzodiazepine receptor ligands on anxiolysis as tested in the plus-maze apparatus. For comparison among six compounds, data are presented as a percentage of control. See the Method section for protocol. The parameter presented here is the mean (n = 6–10).

tested PIs were more active than diazepam and quazepam. In particular, taking into account the low dose used, PI **1i** elicited an outstanding protective activity against both β-CCM- and DMCM-induced seizures.

Anxiolysis

As shown in Fig. 4, two of the three PI derivatives, with the exception of **1a**, were active at the anxiolytic end point in the elevated plus-maze test. CGS 8216, $F(7, 42) = 6.799$, $p = 0.0001$, and **1a**, $F(4, 25) = 3.649$, $p = 0.0179$, clearly displayed an anxiogenic profile. These results reinforce the ability of the plus-maze test to distinguish anxiogenic from anxiolytic activity, as very recently suggested by Chen et al. (11). Abecarnil, $F(4, 21) = 6.57$, $p = 0.001$, **1i**, $F(4, 45) = 2.593$, $p = 0.0001$, diazepam, $F(4, 47) = 11.05$, $p = 0.0001$, and **1d**, $F(4, 25) = 3.649$, $p = 0.0179$, significantly increased the time spent in the open arms. All of the anxiolytic ligands also decreased the time spent in the closed arms. No effect of any of the six ligands studied was detected on the number of entries into the open or closed arms or the central platform (data not shown).

Sedation

When monitored in the locomotor activity apparatus, mice receiving BDZR ligands were not completely immobile. The "partial agonist," abecarnil, $F(4, 21) = 9.178$, $p = 0.0002$, diazepam, $F(4, 45) = 2.91$, $p = 0.03$, and the PI derivative **1i**, $F(5, 42) = 5.542$, $p = 0.03$, as well as CGS 8216, $F(7, 42) = 2.513$, $p = 0.03$, significantly reduced locomotor activity. **1d**, $F(4, 45) = 1.118$, $p = 0.14$; 10.29 μmol/kg, $t = 2.78$, $p < 0.05$, and **1f**, $F(5, 42) = 1.78$, $p = 0.14$; 12.71 μmol/kg, $t = 2.716$, $p < 0.05$; 4 mg/kg, $t = 2.765$, $p < 0.05$, displayed weak agonist activity in this test, whereas **1a** had an antisedative effect, $F(4, 45) = 1.35$, $p = 0.27$. These results are shown in Fig. 5.

Effects on Motor Movements

Table 2 shows the TD_{50} values (with 95% confidence limits) obtained 45 min following the IP administration of various BDZs and PIs **1**. In general terms, BDZs appeared more potent than the related PIs to affect the rotarod test with the following rank order of potency: pinazepam > flunitrazepam > diazepam > quazepam > prazepam > halazepam > **1f** > **1g**. Abecarnil, **1d**, **1e**, and **1i** seem to affect the rotarod test at a dose level higher than 250 μmol/kg, and this led to particularly favorable therapeutic indices, especially for abecarnil and **1i**.

BDZR Binding Studies: [^{3}H]Flumazenil Displacement From Cerebellum, Cortex, or Spinal Cord Synaptosomal Membranes

The IC_{50} of abecarnil, various BDZR ligands, and PIs are reported in Table 4. The order of binding affinity was as follows: cerebellum, CGS 9895 > CGS 8216 > CGS 9896 > abecarnil > flunitrazepam > **1e** > **1b** > diazepam > pinazepam > quazepam > halazepam > prazepam > **1d** > **1c** > **1g** > **1h**; **1i** and **1f** were inactive (IC_{50} > 10,000); cortex, **1e** > CGS 8216 > CGS 9895 > CGS 9896 > abecarnil > **1b** > flunitrazepam > diazepam > **1d** > **1f** > **1c** > **1a** > **1h** > halazepam > pinazepam > prazepam > **1g**; **1i** was inactive; spinal cord, CGS 8216 > CGS 9895 > CGS 9896 > **1e** > flunitrazepam > diazepam > **1d** > **1a** > **1b** > quazepam, **1g** and **1i** were inactive. In general terms, BDZs and/or CGS derivatives were more potent than the related PIs in the cerebellum and spinal cord but not in the cortex.

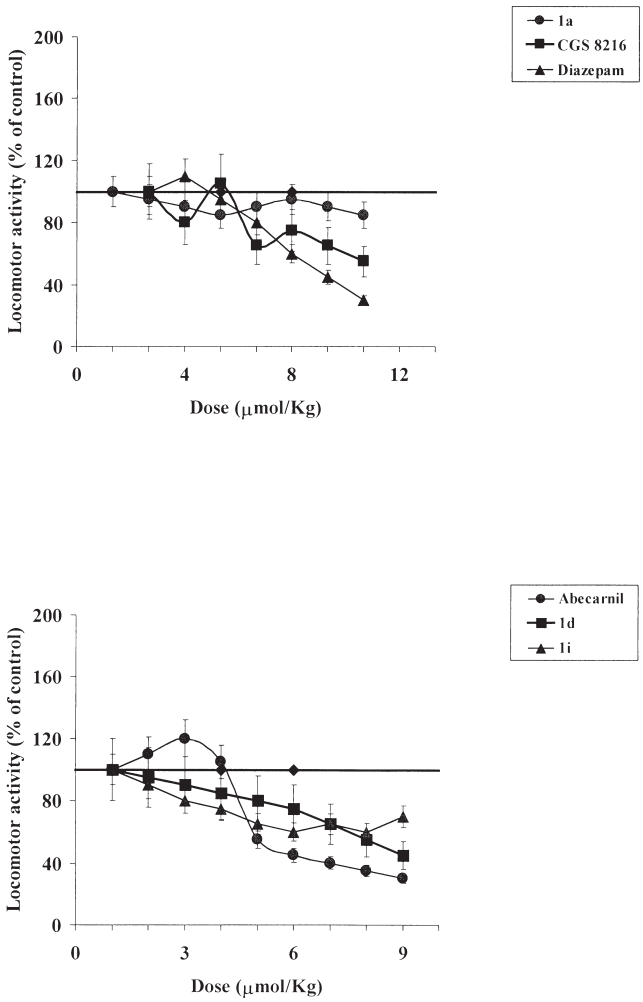


FIG. 5. The effect of six benzodiazepine receptor ligands on locomotor activity. Ordinate shows percentage of control in locomotor activity, abscissa shows the dose expressed as μmol/kg. For comparison among six compounds, data are presented as a percentage of vehicle control group. See the Method section for protocol. The parameter presented here is the mean (n = 6–10).

Radioligand Binding of Various 1,4-Benzodiazepines and 2-Aryl-2,5-dihydropyridazino[4,3-b]indol-3(3H)-ones 1 on Stable Cell Lines

Compounds **1a**, **1b**, **1d**, **1e**, and **1h** displayed a degree of subtype specificity resulting from a higher affinity, toward the $\alpha_1\beta_3\gamma_2$ subunit combination. This behavior was not observerd in CGS derivatives.

DISCUSSION

Previous studies (2,6,7,14–16) have indicated that the systemic administration of benzodiazepines and compounds binding BDZRs may produce anticonvulsant activity in experimental animals, and our present results confirm those effects. Most of the compounds studied demonstrated marked anticonvulsant activity in DBA/2 mice assay (Table 1); compounds **1e**, **1f**, **1g**, and **1b** were generally less potent as anticonvulsants than BDZs, and only derivatives **1d** and **1i**, the

TABLE 4

BINDING AFFINITY OF 1,4-BENZODIAZEPINES AND 2-ARYL-2,5-DIHYDROPYRIDAZINO[4,3-b]INDOL3(3H)-ONES **1** FROM [^{3}H]FLUMAZENIL DISPLACEMENT IN SYNAPTOSOMAL MEMBRANES FROM CEREBELLUM, CORTEX OR SPINAL CORD

Drug	IC_{50} (nM)		
	Cerebellum	Cortex	Spinal Cord
Diazepam	20 ± 3	42 ± 5	ND
Halazepam	62 ± 5	919 ± 34	ND
Pinazepam	28 ± 4	1550 ± 45	ND
Prazepam	80 ± 6	2520 ± 38	ND
Flunitrazepam	5 ± 2	7 ± 2	ND
Quazepam	55 ± 5	96 ± 10	ND
1a	280 ± 39	330 ± 40	230 ± 19
1b	12 ± 0.8	2.5 ± 1.3	320 ± 85
1c	312 ± 26	281 ± 18	84 ± 12.3
1d	140 ± 19	105 ± 14	41 ± 4.2
1e	5.4 ± 2.6	0.15 ± 0.0097	2.1 ± 0.082
1f	>10,000	121 ± 7	78,7 ± 23.1
1g	376 ± 24	2556 ± 42	>10,000
1h	284 ± 19	521 ± 20	1560 + 126
1i	>10,000	>10,000	>10,000
Abecarnil	1.55 ± 0.21	0.95 ± 0.023	ND
CGS 8216	0.21 ± 0.13	0.21 ± 0.018	0.04 ± 0.0034
CGS 9895	0.17 ± 0.15	0.38 ± 0.025	0.069 ± 0.0034
CGS 9896	0.41 ± 0.05	0.81 ± 0.045	0.24 ± 0.028

The IC_{50} ± SD values were estimated with a nonlinear curve program based on Ligand (34). ND = not determined.

most active compounds of our new series, showed an anticonvulsant activity higher than or comparable to that of the BDZs studied. BDZs were instead always more potent than PIs **1** in the prevention of seizures induced by PTZ (Table 2). However, it is worth noting that PIs **1** always gave better results than their CGS analogs. The binding studies indicate that, in all tissues studied, the inhibition of [^{3}H]flumazenil binding occurred from a low to high nanomolar range. In the cortex, BDZ and CGS derivatives exhibited IC_{50} significantly higher than in the cerebellum. A less clear picture comes from PI ligands **1**: **1a**, **1g**, and **1h** behaved as BDZ and CGS derivatives, whereas **1b**, **1c**, **1d**, **1e**, and **1f** showed an opposite effect. These results suggest different ligand binding with different benzodiazepine receptor subtypes, and could be interpreted in terms of a certain selectivity either for BDZ1 or BDZ2 receptors that are prevalent in the cortex and cerebellum, respectively (14,15,18,25,37,48). In addition, the mice pretreatment with some PIs provides better anticonvulsant protection

TABLE 5

BINDING AFFINITIES (K_i) OF VARIOUS 1,4-BENZODIAZEPINE AND 2-ARYL-2,5-DIHYDROPYRIDAZINO[4,3-b]INDOL-3(3H)-ONES **1** AS INHIBITORS OF SPECIFIC [^{3}H]FLUMAZENIL BINDING IN STABLE CELL LINES

Compound	α1β3γ2	α2β3γ2	α3β3γ2	α5β3γ2	α6β3γ2
Diazepam	14.08 ± 2.14	12.77 ± 4.16	15.89 ± 3.65	8.18 ± 0.52	>10,000
Pinazepam	0.33 ± 0.03	0.27 ± 0.09	0.65 ± 0.44	0.22 ± 0.06	>10,000
Prazepam	0.29 ± 0.01	0.46 ± 0.11	0.88 ± 0.32	0.35 ± 0.01	>10,000
Flunitrazepam	3.00 ± 0.03	1.46 ± 0.56	1.66 ± 0.78	1.51 ± 0.30	>10,000
Quazepam	53 ± 3.62	1408 ± 104.8	619 ± 45	777 ± 75	>10,000
1a	32.7 ± 8.05	43.0 ± 7.71	145.4 ± 26.0	126.1 ± 15.4	>10,000
1b	22 ± 6.24	150 ± 38.15	255 ± 17.43	101 ± 13.67	>10,000
1e	7.71 ± 1.62	20.42 ± 4.63	66.51 ± 6.70	84.46 ± 19.01	>10,000
1d	15.76 ± 4.70	37.0 ± 14.92	140.44 ± 0.56	75.29 ± 13.48	>10,000
1h	55 ± 7.77	875 ± 106	2219 ± 435	56 ± 10.4	>10,000
CGS 8216	40.2 ± 9.1	41 ± 8.2	174 ± 34	146 ± 18.2	>10,000
CGS 9895	65.6 ± 6.7	57.0 ± 19.2	144 ± 10.7	125.9 ± 13.8	>10,000
CGS 9896	241 ± 55	341 ± 64	224 ± 42.8	121 ± 22.6	>10,000

Binding were determined by displacement of [^{3}H] flumazenil from $GABA_A$ subtype of stable cell lines. The K_i values obtained are expressed as μM and represent means ± SEM.

TABLE 6

FUNCTIONAL EFFECTS OF BENZODIAZEPINE RECEPTOR LIGANDS AT FOUR BEHAVIORAL TESTS

Ligands	Anxiolysis	Sedation	Hypothermia	Anticonvulsant Activity
Diazepam	Agonist	Agonist	Agonist	Agonist
1a	Inverse agonist	Antagonist	Agonist	Inverse agonist
1d	No effect	Agonist	Agonist	Agonist
1i	Agonist	Agonist	Agonist	Agonist
Abecarnil	Agonist	Agonist	Agonist	Agonist
CGS 8216	Inverse agonist	Agonist	Agonist	Inverse agonist

against β-CCM seizures than against DMCM seizures, further supporting a preferential action at the BDZ1 receptor subtype, analogously to what has been found for quazepam (8).

The pharmacological characterization of the present compounds showed that PIs **1** had a mixed activity profile, different to that elicited by diazepam and abecarnil. Moreover, the examined series of PIs **1** elicited agonist, antagonist, partial agonist, and, sometimes, no activity at some end point. The diverse functional responses coming from four different behavioral tests of PIs **1** provide robust evidence for interactions with different BDZR subtypes and/or with benzodiazepine receptors with multiple functionalities (18,22,23). Examining each column of Table 6, corresponding to the behavior of all compounds at a given end point, it appears that PI **1a** and CGS 8216 have a similar type of functional activity, whereas agonist activity was always detected for diazepam and abecarnil. The present results suggest that pharmacological properties of PIs **1** cannot be explained by an interaction with a single, structurally homogeneous, benzodiazepine receptor. For example, comparing anxiolysis and sedation, two end points frequently used to determine "partial agonist" behavior (9,28), not only was the rank order of agonist activity not preserved, but two of the seven compounds tested had qualitatively different activity at the two end points. **1d** had no effect on the anxyolitic end point, but was an agonist in sedation, whereas **1f** was an agonist at the anxiolytic end point and an antagonist in sedation (data not shown).

Despite some decrease in body temperature, **1a** was an antagonist of both the sedative and anticonvulsant effects of diazepam. In addition, the unsubstituted, closely related congeners **1a** and CGS 8216, displayed similar effects on all end points, except sedation for which an antagonist and agonist activity was found, respectively.

The most plausible explanation of these behavioral profiles is that different in vivo end points may be initiated by ligand binding to different benzodiazepine receptor subtypes that may have different requirements for activation (24). On the other hand, some effects may be due to possible metabolites with different activities. The lack of any type of activity (agonist, antagonist, or inverse agonist) at a given end point might be explained by the low binding affinity of some PIs for the benzodiazepine receptor subtype that mediated the end point (9,48). Agonist activity of a given compound at one end point and antagonist or inverse agonist activity at another one may be explained by a high affinity of the ligand for the BDZR subtypes responsible for eliciting the two activities, but different ability to activate each receptor subtypes. Implicit in this hypothesis is the prediction that a compound, such as **1d**, that has no activity at some end points, would be more subtype selective than the other compounds that are either agonists, antagonists, or inverse agonists at all the end points, and hence, would bind with high affinity to all the BDZR subtypes initiating those end points (9). The consistency of our hypothesis on the observed different pharmacological effects was, at least in part, proved by radioligand binding studies carried out on stable cell lines (Table 5) expressing diverse receptor subunit combinations. Indeed, binding studies involving recombinant $GABA_A$ receptors with varying subunits and the same β and γ subunit combinations, showed that changing one subunit modulated the relative efficacy of benzodiazepine receptor ligands (28,33,36,43). For example, in the subunit combination $\alpha_3\beta_2\gamma_2$ (28) abecarnil produce a response similar to that of flunitrazepam on GABA-evoked chloride ion flux, but in the $\alpha_5\beta_2\gamma_2$ subunits combination, abecarnil potentiated the GABA response to a lesser extent. These and other similar findings demonstrate that the BDZR ligands may act as partial agonists at certain receptor subtypes, but can be full agonists at other receptor subtypes. It is interesting to note that **1f** and **1g**, which were active as anticonvulsants and impaired motor activity, were able to displace [^{3}H]flumazenil from its binding sites. Some PIs **1**, although apparently show lower affinity for BDZR than diazepam [see Tables 4, 5, and (4,38)] could be metabolized to more active BDZR ligands. This hypothesis was confirmed, at least in part, by the ability of flumazenil to increase the ED_{50}

TABLE 7

MOLECULAR WEIGHT AND RELATIVE LIPOPHILICITY OF 1,4-BENZODIAZEPINES, 2-ARYL-2,5-DIHYDROPIRIDAZINO (4,3-b)INDOLONES **1** AND PQ (CGS)

Drugs	Molecular Weight	ClogP*	CLIP*	RM*
Diazepam	284.76	3.08 (2.99)†	2.46	2.91
Halazepam	351.74	4.37	3.62	—
Pinazepam	308.77	3.18	2.65	—
Prazepam	324.81	4.06	3.22	3.45
Flunitrazepam	313.31	2.06	1.46	2.71
Quazepam				
1a	261.28	3.35	1.10	
1b	295.73	4.06	1.70	
1c	295.73	4.06	1.70	
1d	340.18	4.21	1.85	
1e	291.31	3.275	1.29	
1f	275.31	3.85	1.65	
1g	306.28	3.09	0.55	
1h	340.18	4.225	1.83	
1i	309.76	4.10	1.76	
Abecarnil	404.47			
CGS 8216	261.27	3.35	1.05	2.41
CGS 9895	291.31	3.275	1.22	—
CGS 9896	295.70	4.06	1.61	3.47

*ClogP and CLIP are estimated logP values according to the method of Leo (26) and Gaillard (21), respectively. Rm are measured retention parameters from reversed phase thin-layer chromatography (Stationary phase: Whatman K C18 F plates; Mobile phase: CH_3OH/Phosphate buffer or, in patenthesis CH_3LN/phosphate buffer). Rm is defined as: $Rm = \log (1/Rf - 1)$ (CLogP and CLIP programs are available at Biobyte Corp. (Claremont, USA) and Inst. de Chim. Therap. Univ. of Lousanne–Lousanne–SW).

†Measured logP value (octanol/water): by method previously described by Pietrogrande et al. (40).

values of **1d**, **1f**, and **1i** (Table 1) vs. audiogenic seizures under identical experimental conditions. The derivative **1a** was also able to increase the ED_{50} values of diazepam (see Fig. 3). The derivative **1i** was also unable to displace [^{3}H]flumazenil from its binding sites even if it showed pharmacological effects similar to BDZs; most likely, a metabolization reaction leading to a ligand with much higher affinity takes place in vivo. Overall, our results suggest that the action of PIs **1**, or their metabolites, are principally mediated by benzodiazepine receptor sites. As expected, although most PIs **1** are more lipophilic than BDZs, and this property was reported to induce a greater anticonvulsant activity and produce a lower impairment of locomotor performance (21,40), this was not confirmed in our study (Table 2). In addition, compounds possessing very similar ED_{50} values in DBA/2 mice, such as flunitrazepam, diazepam, and **1i** have a different degree of lipophilicity, suggesting the importance of other parameters for BDZR activation. The lower affinity for benzodiazepine receptor of PIs **1** with respect to BDZ derivatives could be due to a different spatial positions of the putative pharmacophoric points in the two different tricyclic systems, and in addition, it cannot be ruled out that the different pharmacological activities of BDZs and PIs could be attributable to a different diffusion into the CNS through the blood–brain barrier (1,4,11, 17,24,26). Therefore, we must consider that the different pharmacological potency of BDZs and PIs might arise from a more or less easy crossing of the ligand, or possible metabolites, through the blood–brain barrier. It is worth stressing that the anticonvulsant effects of active compounds was evident at dose levels that did not affect sedation, locomotor activity, and in some cases, the body temperature. Further experiments concerning efficacy, absorption, and metabolism of PIs are warranted to better clarify the in vivo pharmacological effects of this new interesting class of BDZR ligands.

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