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Anticonvulsant Activity of Azirino[1,2-*d*][1,4]Benzodiazepines and Related 1,4-Benzodiazepines in Mice

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DE SARRO, G. B., A. CHIMIRRI, R. MCKERNAN, K. QUIRK, P. GIUSTI AND A. DE SARRO. *Anticonvulsant activity of azirino[1,2-*d*][1,4]benzodiazepines and related 1,4-benzodiazepines in mice.* PHARMACOL BIOCHEM BEHAV **58**(1) 281–289, 1997.—The anticonvulsant properties of several 1,4-benzodiazepine and azirino[1,2-*d*][1,4]benzodiazepine (ABDZ) derivatives were studied after intraperitoneal (IP) administration in DBA/2 mice (a strain genetically susceptible to sound-induced seizures) and in Swiss mice. The anticonvulsant effects were evaluated on seizures evoked by means of auditory stimulation (109 dB, 12–16 kHz) in animals placed singly under a hemispheric Perspex dome or on seizures induced by administration of pentylenetetrazole. The 1,4-benzodiazepines were generally more potent than the related ABDZ derivatives. The rank order of potency for anticonvulsant activity was flunitrazepam > diazepam > pinazepam > ABDZ5 > ABDZ4 > prazepam > halazepam > ABDZ1 > ABDZ3 > camazepam > ABDZ6 > ABDZ2. The impairment of locomotor performance following IP administration of these derivatives was also evaluated by means of the rotarod test. The rank order of potency for impairment of coordinated motor movements was pinazepam > flunitrazepam > diazepam > ABDZ5 > prazepam > halazepam > ABDZ4 > ABDZ3 > ABDZ1 > camazepam > ABDZ2 = ABDZ6. The potency of various 1,4-benzodiazepines and ABDZs as inhibitors of specific [^{3}H]flumazenil binding to membranes from cerebellum or cortex was evaluated. In general, ABDZs were active as anticonvulsants and inhibited [^{3}H]flumazenil binding in the micromolar range. Radioligand binding studies carried out in stable cell lines demonstrated that none of the ABDZs tested showed a particular subtype specificity. The pharmacological actions of ABDZ4 and ABDZ5, which appeared to be the most potent ABDZs as anticonvulsants, were significantly reduced by 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 ABDZ4 and ABDZ5 was also evaluated against seizures induced in DBA/2 mice by two β-carbolines: methyl-β-carboline-3-carboxylate (β-CCM) and methyl-6,6-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM). Both ABDZ4 and ABDZ5 give better protection against seizures induced by *β*-CCM than DMCM, suggesting a preferential action on the benzodiazepine receptor subtype BDZ1. © 1997 Elsevier Science Inc.

1,4-Benzodiazepines Azirino[1,2-*d*][1,4] benzodiazepines Epilepsy β-Carbolines Pentylenetetrazole Flumazenil Rotarod and audiogenic seizures

BENZODIAZEPINES are widely used as therapeutic agents active in a variety of neurological and psychiatric disorders such as epilepsy, anxiety, insomnia, spasticity, and depression (26). The benzodiazepine (BDZ) binding site has been identified as part of the γ-aminobutyric acid (GABA) receptor–Cl ionophore supercomplex (42,47), although other BDZ effects that are unrelated to GABA have been demonstrated (7,31,34). Although initial studies indicated that BDZs bind to

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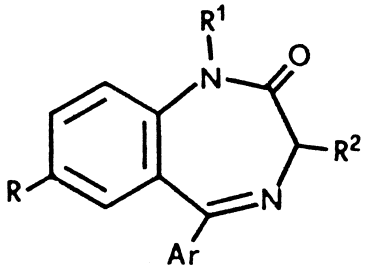
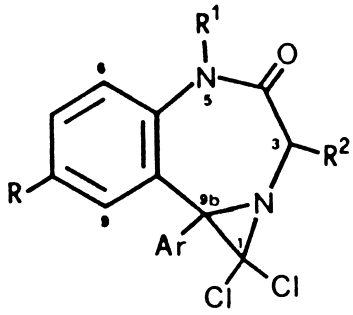
the α-subunit and that GABA binds to the β-subunit of the GABA–BDZ receptor complex (8), it was later found that the γ2-subunit contributes to the formation of a functional BDZ site (40,41). Early attempts to correlate BDZ receptor occupancy with functional effects were made by several authors (4,5,13,20,36). β-Carbolines and other substances binding BDZ receptors and some BDZs permitted identification of multiple subtypes of BDZ receptors (2,19,28,44–46). Mennini and Garattini (35) and Petersen et al. (37) 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 in requiring higher BDZ receptor occupancy to elicit equivalent effects (6,22,24,39,49). Based on different reported studies of in vivo activities, BDZ ligands have come to be labeled as agonist, antagonist, or inverse agonist (38), and the number of molecules being placed into this classification is increasing. As a follow-up to our investigations on anticonvulsant activity of annelated BDZ derivatives (16,17) and a contribution to the identification of a pharmacophore for BDZ receptors, in the present report we describe, compare, and discuss the BDZ receptor affinity and antiseizure effects of several 1,4-benzodiazepine and azirino[1,2-*d*][1,4]benzodiazepine (ABDZ) derivatives (Fig. 1) previously synthesized in our laboratory (12). The ABDZs are characterized by introduction of the dichloro three-membered ring into the BDZ system; this introduction was used both to increase the lipophilic properties (30) of BDZ precursors and to verify the influence of the conformational mobility of the heptatomic diazepine nucleus (12) and the phenyl substituent at C-S on the anticonvulsant activity of BDZs. It has recently been reported (23) that the reduced freedom of the molecules and, in particular, the spatial position of the phenyl group could influence the activity of BDZ receptor ligands; the optimum structure appears to be that with the phenyl ring almost perpendicular to the main plane of the molecule. According to recently proposed pharmacophore models (1,18,23,50), all of the investigated compounds display the structural features essential for both BDZ receptor affinity and efficacy: i.e., a planar aromatic system, two lipophilic moieties (i.e., the fused benzene and the aromatic substituent at the C-N group), and two proton-accepting atoms (nitrogen and carbonyl oxygen) at particular spatial orientations and distances. However, the conformational characteristics of the two groups of substances (BDZs and ABDZs) differ because of the presence of the azirine nucleus, which reduces both the heptatomic ring mobility and the free rotation of the phenyl group (12). The BDZ receptor affinity of BDZs and ABDZs was evaluated both in the cerebellum and in the cortex (which contains primarily α1 receptors, but has most subtypes), as well as in cell lines expressing recombinant receptors of structure α1β3γ2, α2β3γ2, α3β3γ2, α5β3γ2, and α6β3γ2.

To better understand the in vivo activity of these compounds, their anticonvulsant effects were studied in DBA/2 mice. This strain is genetically susceptible to sound-induced seizures and has been considered an excellent animal model for the study of certain kinds of human epilepsy and for test-





Drug	R	R^1	R^2	Ar	Drug
Diazepam	Cl	CH_3	H	C_6H_5	ABDZ1
Camazepam	Cl	CH_3	$OCON(CH^3)$	C_6H_5	ABDZ2
Halazepam	Cl	CH_2CF_3	H	C_6H_5	ABDZ3
Pinazepam	Cl	CH2C≡CH	H	C_6H_5	AGDZ4
Prazepam	Cl	CH_2	H	C_6H_5	ABDZ5
Flunitrazepam	NO_2	CH_3	H	2-F-C_6H_4	ABDZ6

FIG. 1. Chemical structures of 1,4-benzodiazepine and 3H-azirino[1,2-*d*][1,4]benzodiazepine derivatives studied. Diazepam = 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one ; camazepam = 7-chloro-1,3-dihydro-3-(*N*,*N*-dimethylcarbamoyl)-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one; halazepam = 7-chloro-1,3-dihydro-5-phenyl-1-(2,2,2-trifluoroethyl)-2H-1,4-benzodiazepin-2-one; pinazepam = 7-chloro-1,3-dihydro-5-phenyl-1-(2-propynyl)-2H-1,4-benzodiazepin-2-one; prazepam = 7-chloro-1-cyclopropylmethyl-1,3-dihydro-5-phenyl-2H-1,4-benzodiazepin-2-one; flunitrazepam = 1,3-dihydro-5-(2′-fluorophenyl)-1-methyl-7-nitro-2H-1,4-benzodiazepin-2-one; ABDZ1 = 8-chloro-1,9b-dihydro-5-methyl-9b-phenyl-3H-azirino[1,2-*d*][1,4]benzodiazepin-4(5H)-one; ABDZ2 = 8-chloro-1,9b-dihydro-3-(*N*,*N*-dimethylcarbamoyl)-5-methyl-9b-phenyl-3H-azirino[1,2-*d*][1,4]benzodiazepin-4(5H)-one; ABDZ3 = 8-chloro-1,9b-dihydro-9b-phenyl-5-(2,2,2-trifluoro-ethyl)-3H-azirino[1,2-*d*][1,4]-benzodiazepin-4(5H)-one; ABDZ4 = 8-chloro-1,9b-dihydro-9b-phenyl-5-(2-propargyl)-3H-azirino[1,2-d][1,4]benzodiazepin-4(5H)-one; ABDZ5 = 8-chloro-5-cyclopropylmethyl-1,9b-dihydro-9b-phenyl-3H-azirino[1, 2-*d*][1,4]benzodiazepin-4(5H)-one; ABDZ6 = 1,9b-dihydro-9b-(2′-fluorophenyl)-5-methyl-8-nitro-3H-azirino[1,2- *d*][1,4]benzodiazepin-4(5H)-one.

ing new anticonvulsant drugs (9,43). Swiss mice were used to evaluate the anticonvulsant properties of BDZs and ABDZs against seizures induced by pentylenetetrazole. In addition, we addressed the question of whether the pharmacological effects of some of these derivatives were modified by flumazenil, a "neutral" BDZ receptor antagonist, and we investigated their ability to antagonize seizures induced by two β-carbolines: methyl-β-carboline-3-carboxylate (β-CCM) and methyl-6,6-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM).

MATERIALS AND METHODS

Testing of Anticonvulsant Activity in DBA/2 Mice

DBA/2 mice [6–12 g, 22–26 days old; purchased from Charles River (Calco, Como, Italy)] were exposed to auditory stimulation 45 min after intraperitoneal (IP) administration of vehicle or drugs (n = 10 animals for each dose and n = 40–50 mice for each compound). Procedures involving animals and their care were conducted in conformity with national and international law and policies. For systemic injections, all BDZs were given IP (0.1 ml/10 g of body weight of the mouse) as a freshly prepared solution in 50% dimethyl sulfoxide (DMSO) and 50% sterile saline (0.9% NaCl). Flumazenil was prepared and used at a dose level that did not affect audiogenic seizure response in DBA/2 mice (15). Individual mice were placed under a hemispheric Perspex dome (diameter 58 cm) and were allowed 60 s for habituation, during which time locomotor activity was assessed. Auditory stimulation (12–16 kHz, 109 dB) was applied for 60 s or until tonic extension occurred. Seizure response was graded on the following scale: 0 = no response, 1 = wild running, 2 = clonus, 3 = tonus, 4 = respiratory arrest (14). 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. Behavioural changes were observed during the period between drug administration and auditory testing. A second group of DBA/2 mice (10 for each group) was used to determine the time course of the anticonvulsant effects of prazepam, oxazepam, and ABDZ5. For these tests, animals were pretreated at a dose of 1 μmol/kg from 15 min to 6 h before exposure to auditory stimulation.

β-Carboline-induced Seizures in DBA/2 Mice

Seizures were induced in adult DBA/2 mice (6–8 weeks old) by IP injection of DMCM (1.2–2 mg/kg) or β-CCM (0.5–2 mg/kg) dissolved in a minimal amount (<5% of final volume) of glacial acetic acid and brought to volume with saline (n = 20–30 per group of pooled controls). The mice were observed for 30 min for the incidence of clonic seizures. To determine their anticonvulsant effects, diazepam (1 mg/kg), ABDZ4 (4.2 mg/kg), and ABDZ5 (1.6 mg/kg) were used to pretreat the animals 15 min before administration of DMCM or β-CCM (n = 10 animals for each dose and n = 40–50 mice for each compound).

Anticonvulsant Properties Against Pentylenetetrazole-induced Seizures in Swiss Mice

Male Swiss mice [20–26 g, 48–56 days old; purchased from Charles River (Calco, Como, Italy)] were pretreated with vehicle or drugs 45 min before subcutaneous (SC) administration of pentylenetetrazole (n = 10 animals for each dose and n = 40–50 mice for each compound). The convulsive dose 97 (CD_{97}) of pentylenetetrazole (85 mg/kg) was 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 indicated that the test substance had the ability to elevate the pentylenetetrazole seizure threshold.

Effects on Motor Movements

Groups of male Swiss mice [20–26 g, 48–54 days old; purchased from Charles River (Calco, Como, Italy)] were trained to do coordinated motor movements continuously for 2 min on a rotarod 3 cm in diameter rotating at 8 rpm (U. Basile, Comerio, Varese, Italy). Impairment of coordinated motor movements was defined as inhability of the mice to remain on the rotarod for a 2-min test period (21). Groups of 40–50 mice (n = 10 animals for each dose) were pretreated with the compounds studied. The ability of the mice to remain on the rotarod was tested 45 min after administration of various 1,4-benzodiazepine and ABDZ derivatives.

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 were rapidly dissected on ice. Brain regions 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 1,000 × 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 8,000 × 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 volumes 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 [^{3}H]flumazenil (specific activity 72.4 Ci/mmol) in a final volume of 1 ml of 50 mM Tris-HCl, 120 mM NaCl, and 5 mM KCl, pH 7.4. All BDZs were dissolved in DMSO to a final concentration of 1%. Incubations were carried out for 60 min at 4°C in triplicate, and nonspecific binding was measured in the presence of 10 μM diazepam. Reactions were stopped by the addition of 5 ml of ice-cold Tris-HCl followed by rapid filtration through Whatman GF/C glass fiber filters (Whatman Inc., Clifton, NJ, USA) and two additional washes. The radioactivity trapped on the filters was counted by liquid scintillation spectrometry after the addition of 8 ml of Filter Count (Packard). The experiments were run in triplicate with eight different concentrations of competing ligand; results are expressed as IC_{50} ± SD values.

Effects on Radioligand Binding in Stable Cell Lines

Stable cell lines were generated and radioligand binding studies carried out essentially as described by Hadingham et al. (25). Briefly, stable cell lines expressing α1β3γ2, α2β3γ2, α3β3γ2, α5β3γ2, and α6β3γ2 were grown as previously described (25). Cells were harvested by scraping and were washed twice by centrifugation at 1,000 × 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 various 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 analysis of variance followed by post hoc Dunnett's *t*-test (rectal temperatures). The percent 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 percent response. ED_{50} values (with 95% confidence limits) for each compound and each phase of seizure response were estimated by the method of Litchfield and Wilcoxon (29); relative anticonvulsant activities were determined by comparison of respective ED_{50} values. The dose that induced 50% of mice to fall from the rotarod [the TD_{50} value (with 95% confidence limits)] for each compound was estimated by the method of Litchfield and Wilcoxon (29). The relative activities of the drugs were determined by comparison of TD_{50} values. For the binding experiments, ID_{50} values for [^{3}H]flumazenil displacement were determined by a nonlinear curve-fitting program based on LIGAND (32).

Drugs

The sources of the drugs used were as follows: diazepam [molecular weight (MW) 284.76] was purchased from Sigma (St. Louis, MO, USA); camazepam (MW 371.83), pinazepam (MW 308.77), prazepam (MW 324.81), flunitrazepam (MW 313.31), and oxazepam (MW 286.7) were extracted with chloroform from the corresponding drugs; halazepam (MW 351.74) was 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); and ABDZ derivatives were synthesized as previously described (12). The ABDZs have the following MWs: ABDZ1, 367.67; ABDZ2, 454.75; ABDZ3, 435.66; ABDZ4, 391.69; ABDZ5, 407.73; and ABDZ6, 396.21. Flumazenil (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-*a*] [1,4]benzodiazepine-3-carboxylate; MW 303.3) was obtained from Hoffman-La Roche (Basel, Switzerland) and prepared as previously described (15). [^{3}H]Flumazenil was obtained from New England Nuclear (Boston, MA, USA).

RESULTS

Anticonvulsant Activity of Classical 1,4-Benzodiazepines

As shown in Table 1, classical 1,4-benzodiazepines were able to protect DBA/2 mice against the clonic and tonic phases of the audiogenic seizure response. In particular, audiogenic seizures were significantly reduced 45 min after IP administration of diazepam (0.33, 0.66, and 1.0 μmol/kg), camazepam (6.6, 10, and 33 μmol/kg), halazepam (3.3 and 10 μmol/kg), pinazepam (1.0 μmol/kg), prazepam (3.3 and 10 μmol/kg), and flunitrazepam (0.33, 0.66, and 1.0 μmol/kg). The wild running phase was significantly reduced after IP administration of all BDZs at the highest doses tested (Table 1). A significant fall in body temperature and sedative effects were observed after the highest doses of all BDZ derivatives studied (data not shown). The relative ED_{50} values (with 95% confidence limits) are reported in Table 1. Following IP administration of prazepam (1 μmol/kg), the maximum protection was observed at 2–3 h, with a subsequent return to control seizure response at 6 h, whereas following IP administration of oxazepam (1 μmol/kg), the maximum protection was observed at 1–2 h, with a subsequent return to control seizure response at 4 h (Fig. 2).

TABLE 1

DOSES USED AND ED_{50} VALUES FOR VARIOUS 1,4-BENZODIAZEPINE AND AZIRINO[1,2-*d*][1,4]BENZODIAZEPINE DERIVATIVES AGAINST THE CLONIC AND TONIC PHASES OF AUDIOGENIC SEIZURES

Drug	Range of Doses (μmol/kg)	Clonic Phase (μmol/kg)	Tonic Phase (μmol/kg)
Diazepam	0.1–1	0.28 (0.20–0.39)	0.24 (0.15–0.39)
Camazepam	0.33–33	4.12 (1.78–9.55)	3.24 (1.24–8.45)
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)
ABDZ1	0.33–33.3	3.27 (1.08–9.93)	1.98 (0.81–4.83)
ABDZ2	0.33–33.3	56.18 (19.85–159.04)	38.55 (17.22–86.30)
ABDZ3	0.33–10	3.75 (1.82–7.71)	3.61 (1.50–8.67)
ABDZ4	0.1–10	1.18 (0.60–2.30)	0.71 (0.43–1.18)
ABDZ5	0.033–3.3	0.44 (0.21–0.94)	0.30 (0.15–0.62)
ABDZ6	0.33–33.3	11.75 (5.07–27.22)	8.12 (4.55–14.18)
Flumazenil + ABDZ4		4.30 (2.41–7.70)*	2.62 (1.84–3.73)*
Flumazenil + ABDZ5		3.57 (2.56–4.98)*	1.73 (1.18–2.54)*

ED_{50} values (with 95% confidence limits) were calculated according to the method of Litchfield and Wilcoxon (29). *Significant differences between ED_{50} values for group treated with flumazenil (8.24 μmol/kg) + ABDZ4 derivative and group treated with ABDZ4 alone or for group treated with flumazenil (8.24 μmol/kg) + ABDZ5 derivative and group treated with ABDZ5 alone, $p < 0.01$.

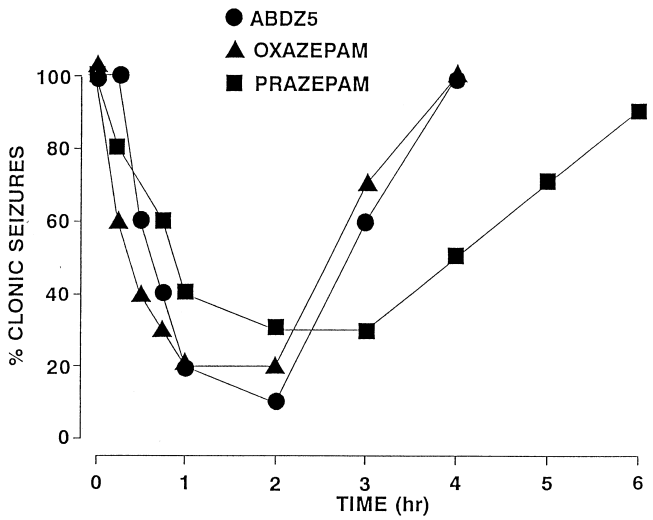


FIG. 2. Anticonvulsant effects of (●) ABDZ5, (▲) oxazepam, and (■) prazepam against audiogenic seizures in DBA/2 mice. The ordinate shows the % clonic seizures, and the abscissa shows the time in hours after IP administration. Ten animals were used for the determination of each point.

Anticonvulsant Activity of ABDZs

As shown in Table 1, ABDZs were able to protect DBA/2 mice against the clonic and tonic phases of the audiogenic seizure response. In particular, audiogenic seizures were significantly reduced 45 min after IP administration of ABDZ1 (3.3, 10, and 33 μmol/kg), ABDZ2 (66 μmol/kg), ABDZ3 (10 μmol/kg), ABDZ4 (1.0, 3.3, and 10 μmol/kg), ABDZ5 (1.0 and 3.3 μmol/kg), and ABDZ6 (33 μmol/kg). The wild running phase was significantly reduced after IP administration of ABDZ1, ABDZ3, ABDZ4, ABDZ5, and ABDZ6, but was not significantly suppressed by ABDZ2. A significant fall in body temperature was observed after the highest doses of ABDZ1, ABDZ4, ABDZ5, and ABDZ6 (data not shown). The relative ED_{50} values (with 95% confidence limits) are reported in Table 1. Following IP administration of ABDZ5 at 1 μmol/kg, maximum protection was observed at 2 h, with a subsequent return to control seizure response at 4 h (Fig. 2).

Treatment with Flumazenil

To ascertain the possible involvement of BDZ receptors in the antiseizure activity of ABDZs, the most active compounds (ABDZ4 and ABDZ5) were administered concomitantly with flumazenil. In particular, the anticonvulsant effects of the ABDZ4 derivative (0.33, 1.0, 3.3, and 10.0 μmol/kg) were reduced by treatment with flumazenil (8.24 μmol/kg IP) 15 min after administration of ABDZ4. 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 with the corresponding groups receiving ABDZ4 alone. For the different phases of audiogenic seizures, the ED_{50} values for ABDZ4 in combination with flumazenil were significantly increased over the values for ABDZ4 alone (to 3.64 and 3.69 times values for ABDZ4 alone for clonic and tonic phases, respectively; Table 1). Administration of flumazenil (8.24 μmol/kg IP) also was able to suppress the antiseizure effects of ABDZ5 (0.1, 0.33, 1.0, and 3.3 μmol/kg). For the different phases of seizures, ED_{50} values for ABDZ5 in combination with flumazenil were significantly increased over values for ABDZ5 alone (to 8.11 and 5.77 times values for ABDZ5 alone for clonic and tonic phases, respectively; 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 the two ABDZ derivatives in DBA/2 mice (data not shown).

Anticonvulsant Properties Against Pentylenetetrazole-induced Seizures

Table 2 shows the ED_{50} values (with 95% confidence limits) of those compounds that were active against clonic seizures induced by SC administration of pentylenetetrazole in Swiss mice. In particular, the clonic seizures induced by pentylenetetrazole were significantly reduced 45 min after IP injection of diazepam (0.66 and 1.0 μmol/kg), camazepam (6.6, 10,

TABLE 2

ED_{50} VALUES AGAINST PENTYLENETETRAZOLE-INDUCED SEIZURES AND TD_{50} VALUES IN THE ROTAROD TEST FOR VARIOUS 1,4-BENZODIAZEPINE AND AZIRINO[1,2-*d*][1,4]BENZODIAZEPINE DERIVATIVES

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
Camazepam	5.77 (3.56–9.35)	52.6 (37.4–74)	9.1
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
ABDZ1	4.58 (3.02–6.94)	31.5 (19.6–50.6)	6.9
ABDZ2	78.61 (46.7–132.4)	>100	—
ABDZ3	5.88 (47.2–7.32)	27.3 (17.7–34.8)	4.6
ABDZ4	2.06 (1.23–3.45)	23.1 (14.9–35.6)	11.2
ABDZ5	0.56 (0.40–0.78)	12.1 (9.1–16.1)	21.6
ABDZ6	18.6 (13.4–25.8)	>100	—

ED_{50} and TD_{50} values (with 95% confidence limits) were calculated according to the method of Litchfield and Wilcoxon (29). TD_{50}/ED_{50}, the therapeutic index, represents the ratio between TD_{50} and ED_{50}.

TABLE 3

ANTAGONISM OF THE CLONIC CONVULSANT ACTION OF THE β-CARBOLINES β-CCM AND DMCM IN ADULT DBA/2 MICE BY DIAZEPAM AND SOME AZIRINO[1,2-*d*][1,4]BENZODIAZEPINE DERIVATIVES

	ED_{50} (mg/kg)		Potency Ratio	
Pretreatment (30 min before)	β-CCM	DMCM	β-CCM	DMCM
Vehicle	0.76 (0.47–1.13)	1.31 (1.18–1.45)	—	—
Diazepam (1 mg/kg)	1.56 (1.28–1.90)	2.71 (2.53–2.91)	2.1*	2.1*
ABDZ4 (4.2 mg/kg)	3.48 (2.43–4.98)	2.12 (1.70–2.65)	4.6*	1.6
ABDZ5 (1.6 mg/kg)	3.3 (2.31–4.80)	2.29 (1.75–2.98)	4.4*	1.7

ED_{50} values (with 95% confidence limits) were calculated according to Litchfield and Wilcoxon (29). Potency ratios are the ratios of ED_{50}s for drug and vehicle. *Significant differences vs. respective control groups, $p < 0.01$.

and 33 μmol/kg), halazepam (3.3, 6.6, and 10 μmol/kg), pinazepam (1.0 μmol/kg), prazepam (3.3 and 10 μmol/kg), and flunitrazepam (0.33, 0.66, and 1.0 μmol/kg). In addition, pretreatment 45 min before pentylenetetrazole administration with ABDZ1 (6.6, 10, and 33 μmol/kg), ABDZ2 (100 μmol/kg), ABDZ3 (10 μmol/kg), ABDZ4 (3.3 and 10 μmol/kg), ABDZ5 (1.0 and 3.3 pmol/kg), and ABDZ6 (33 μmol/kg) was able to significantly reduce the incidence of clonic seizures induced by pentylenetetrazole. Diazepam was at least 1.7-fold more potent than ABDZ5, and flunitrazepam was 1.9 times more potent than ABDZ5 and much more potent than the remaining active ABDZ derivatives. In addition, ABDZ5 and ABDZ4 were more potent than prazepam, halazepam, and camazepam.

Anticonvulsant Activity Against β-Carboline-induced Seizures

Following vehicle pretreatment (30 min before), β-CCM (0.5, 1, 1.5, and 2 mg/kg IP) induced clonic seizures in DBA/2 mice in a dose-dependent manner. In particular, the lowest dose of β-CCM (0.5 mg/kg IP) elicited clonic convulsions in 30% of the mice. β-CCM 1 mg/kg IP elicited clonic convulsions in 58% of the mice, β-CCM 1.5 mg/kg IP induced clonic seizures in 80% of the mice, and β-CCM 2 mg/kg produced clonus in 93% of the DBA/2 mice. The ED_{50} value for β-CCM-induced clonic convulsions was 0.76 mg/kg IP (Table 3). After vehicle pretreatment (30 min before), DMCM (1, 1.2, 1.5, and 1.8 mg/kg IP) produced clonic seizures in DBA/2 mice in a dose-dependent manner. In particular, the lowest dose of DMCM (1 mg/kg IP) induced clonus in 10% of the mice. DMCM 1.2 mg/kg IP elicited clonic seizures in 33% of the mice, DMCM 1.5 mg/kg IP caused clonic convulsions in 73% of the mice, and DMCM 1.8 mg/kg IP produced clonus in 100% of animals. The ED_{50} value for DMCM-induced clonic convulsions was 1.31 mg/kg IP (Table 3). A pretreatment 30 min before with diazepam (1 mg/kg IP), ABDZ4 (4.2 mg/kg IP), and ABDZ5 (1.6 mg/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, whereas ABDZ4 and ABDZ5 provided better anticonvulsant protection against β-CCM-induced seizures than against DMCM-induced seizures (Table 3).

TABLE 4

POTENCY OF VARIOUS 1,4-BENZODIAZEPINE AND AZIRINO[1,2-*d*][1,4]BENZODIAZEPINE DERIVATIVES AS INHIBITORS OF SPECIFIC [^{3}H]FLUMAZENIL BINDING TO MEMBRANES FROM CEREBELLUM OR CORTEX

	IC_{50} (nM)	
Drug	Cerebellum	Cortex
Diazepam	20 ± 3	42 ± 5
Camazepam	300 ± 15	1,000 ± 48
Halazepam	62 ± 5	919 ± 34
Pinazepam	28 ± 4	1,550 ± 45
Prazepam	80 ± 6	2,520 ± 38
Flunitrazepam	5 ± 2	7 ± 2
ABDZ1	322 ± 26	583 ± 48
ABDZ2	2,930 ± 150	>10,000
ABDZ3	>10,000	>10,000
ABDZ4	>10,000	>10,000
ABDZ5	360 ± 26	722 ± 29
ABDZ6	274 ± 19	177 ± 19

The IC_{50} ± SD values were estimated with a nonlinear curve program based on LIGAND (32).

Effects on Motor Movements

Table 2 shows the TD_{50} values (with 95% confidence limits) obtained 45 min after IP administration of various 1,4-benzodiazepine and ABDZ derivatives. In general terms, the 1,4-benzodiazepines appeared more potent than the related ABDZ derivatives in affecting the rotarod test, with the following rank order of potency: pinazepam > flunitrazepam > diazepam > ABDZ5 > prazepam > halazepam > ABDZ4 > ABDZ3 > ABDZ1 > camazepam > ABDZ2 = ABDZ6.

Relative Potency of Various 1,4-Benzodiazepine and ABDZ Derivatives as Inhibitors of Specific [^{3}H]Flumazenil Binding to Membranes from Cerebellum or Cortex

The IC_{50}s of various 1,4-benzodiazepine and ABDZ derivatives are reported in Table 4. The order of potency was the following: cerebellum: flunitrazepam > diazepam = pinazepam > halazepam > prazepam > ABDZ6 = camazepam = ABDZ1 = ABDZ5 > ABDZ2; ABDZ3 and ABDZ4 were inactive; cortex: flunitrazepam > diazepam > ABDZ6 > ABDZ1 > ABDZ5 > halazepam = camazepam > pinazepam > prazepam; ABDZ2, ABDZ3, and ABDZ4 were inactive. In general

TABLE 5

POTENCY OF VARIOUS 1,4-BENZODIAZEPINE AND AZIRINO[1,2-*d*][1,4] BENZODIAZEPINE DERIVATIVES 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
Camazepam	1.06 ± 0.13	1.28 ± 0.31	2.51 ± 0.98	0.75 ± 0.02	>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
ABDZ2	>10,000	>10,000	>10,000	>10,000	>10,000
ABDZ3	>10,000	>10,000	>10,000	>10,000	>10,000
ABDZ4	>10,000	>10,000	>10,000	>10,000	>10,000
ABDZ5	>10,000	>10,000	>10,000	>10,000	>10,000

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

terms, the BDZs were more potent than the related ABDZs in the cerebellum but not in the cortex.

Effects of Various 1,4-Benzodiazepine and ABDZ Derivatives on Radioligand Binding in Stable Cell Lines

None of ABDZ derivatives tested showed a particular subtype specificity (Table 5).

DISCUSSION

Several previous studies (3,4,10,11,16,17,27,33,48) have indicated that the systemic administration of BDZs to experimental animals produces anticonvulsant activity, and the present results confirm these effects. All BDZs studied demonstrated marked anticonvulsant activity in DBA/2 mice (Table 1), even if the ABDZs were generally less potent as anticonvulsants than the parent 1,4-benzodiazepines; only derivative ABDZ5, the most active compound of our new series, was more active than the related 1,4-benzodiazepine (prazepam). The binding studies indicate that, in both tissues studied, the inhibition of [^{3}H]flumazenil binding occurred in the micromolar range, but in the cortex the derivatives examined generally exhibited a higher IC_{50}. These results suggest a different interaction with the BDZ receptor subtypes, which could be interpreted in terms of selectivity for BDZ1 vs. BDZ2 receptors. In addition, the pretreatment of mice with some ABDZ derivatives provides better anticonvulsant protection against β-CCM-induced seizures than against DMCM-induced seizures, further supporting a preferential action on the BDZ receptor subtype BDZ1, analogous to the action of quazepam (10).

Interestingly, ABDZ3 and ABDZ4, which were active as anticonvulsants and impaired motor activity, failed to displace [^{3}H]flumazenil from its binding sites. Thus, the ABDZ derivatives, although appearing to be stable molecules, could behave as prodrugs by releasing metabolites active at BDZ receptors after biotransformation. This hypothesis was confirmed by the ability of flumazenil to increase the ED_{50} values of ABDZ4 and ABDZ5 (Table 1) vs. audiogenic seizures under identical experimental conditions. This behaviour suggests that the action of ABDZ derivatives or their metabolites is principally mediated by BDZ receptor sites. Furthermore, the time course of ABDZ5, the parent BDZ prazepam, and its metabolite oxazepam was studied: the graph (Fig. 2) shows a similar trend, suggesting that in vivo ABDZ5 may be converted into oxazepam as final metabolite.

The anticonvulsant activity of some ABDZs was also examined against the seizures induced by pentylenetetrazole in Swiss mice; the antiseizure activity was similar to that observed in DBA/2 mice, and we observed a similar degree of anticonvulsant potency (Table 2).

As expected, although ABDZs are more lipophilic than BDZs because of the presence of the CCl_2 group in the former derivatives (12), this characteristic generally does not induce major anticonvulsant activity and produces major impairment of locomotor performance for ABDZ1 and ABDZ3 in comparison to related BDZs (Table 2). In addition, compounds possessing very similar ED_{50} values, such as flunitrazepam, diazepam, and ABDZ5, have different degrees of lipophilicity (12), suggesting the importance of other parameters.

The lower efficacy of the ABDZs with respect to BDZ derivatives could be due to the spatial position of the phenyl group, which in the tricyclic compounds (ABDZs) cannot assume an orientation perpendicular to the major plane of the molecule owing to the steric hindrance of the azirine nucleus.

In addition, we may consider that the different anticonvulsant potencies of 1,4-benzodiazepine and ABDZ derivatives could be attributable to differences in transport by diffusion through the blood–brain barrier, which could provide easier access to the central nervous system for the more active compounds or their metabolites. This hypothesis was confirmed when we studied the time course of prazepam, its metabolite oxazepam, and ABDZ5 (Fig. 2). The anticonvulsant activity of these compounds was evident at dose levels that did not affect sedation, ataxia, and in some cases body temperature. However, sedation and ataxia were observed but not measured after the highest doses of ABDZs employed. Further experiments concerning efficacy, absorption, and metabolism of ABDZs will be carried out to better clarify their in vivo pharmacological effects.

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