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Short Communication

Characterization of the enantiomeric 3-substituted-1,4-benzodiazepin-2-ones binding to the GABA–BDZ–chloride ionophore receptor complex

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Introduction

Gamma aminobutyric acid (GABA) is an important and abundant inhibitory neurotransmitter in the mammalian central nervous system (CNS). Its interaction with the $GABA_A$ receptor subtypes opens anion channels in the membrane through which chloride ions pass down their electrochemical gradient, thus stabilizing the resting level of membrane potential. $GABA_A$ receptors, associated with the anion channel, are part of a protein complex containing distinct but interacting recognition sites for convulsants [such as picrotoxin and *t*-butylbicyclo-phosphorothionate (TBPS)] and depressants [such as benzodiazepines (BDZs) and barbiturates] [1]. Therefore, the macromolecular make-up and interactions of the BDZ binding sites with the GABA receptor and chloride channel have to be accounted for in models of BDZ receptor function [2–4].

Several papers report the structure activity relationships as well as the influence of the stereochemistry of BDZs on their *in vitro* binding to the CNS receptor [5–11]. However, to the best of our knowledge, nothing is known about BDZ receptor function depending on the enantioselective binding of chiral BDZs to their receptor.

In the present paper the interaction to the CNS receptor of a series of chiral 3-substituted-1,4-benzodiazepin-2-ones, resolved as pure enantiomers by preparative chiral HPLC, was studied. The study of the interaction of the BDZ receptor with the GABA receptor and chloride channel was utilized to profile the *in vitro* differences between enantiomeric BDZs as agonists, antagonists, and inverse agonists. Quantifiable differences in the coupling of the BDZ receptors to GABA receptors can be determined by measuring the GABA-shift in the affinities of BDZs to displace ^{3}H-flunitrazepam [2, 11–13]. Thus, the influence of the stereochemistry on the pharmacological profile of the BDZ receptor ligands has been investigated as GABA-ratio determination. Further, the study of possible interaction between BDZ receptor and the chloride channel has been performed by the use of ^{35}S-TBPS (a selective label of chloride anion recognition site). Thus the effect exerted by chiral BDZs on the chloride channel regulation was evaluated by the modulation of ^{35}S-TBPS binding.

Experimental

Materials

^{3}H-flunitrazepam (specific activity = 83 Ci

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mmol^{-1}) and ^{35}S-TBPS (specific activity = 84.6 Ci mmol^{-1}) were obtained from DuPont de Nemours (New England Nuclear Division, Germany). Other chemicals were reagent grade and from commercial supplies. BDZs were kindly provided, as racemates, by Prof. W.H. Pirkle, School of Chemical Science, University of Illinois at Urbana–Champaign, IL, USA.

Chromatographic resolution

The preparative separations (up to 5 mg each injection) were carried out using a Jasco liquid chromatograph 887-PU coupled with a Jasco multi-340 multi channel detector. The HPLC system was operated at room temperature and two Pirkle ionic columns (25 × 0.4 cm i.d., CSP I and 25 × 2 cm i.d., CSP II) were used as chiral stationary phases. These columns, i.e. (*R*)-*N*-(3,5-dinitrobenzoyl)-phenylglycine ionically bonded to a 5 μm γ-aminopropyl silanized silica, were prepared *in situ* following the procedure reported in the literature [14]. The 25 × 0.4 cm i.d. silica-NH_2 column was from Merck (Darmstadt, Germany) and the 25 × 2 cm i.d. column was from Spherisorb (Queensferry, Clwyd, UK).

Hexane–2-propanol mixtures were used as mobile phases, at a flow rate of 1.1–1.5 ml min^{-1} or at 9 ml min^{-1} for CSP I or CSP II, respectively. HPLC grade solvents were used and they were filtered and degassed before use.

The chromatographic retentions of the solutes were followed at 254 nm and reported as the capacity factors (k's) where k' is defined as $(t_{BDZ} - t_o)/t_o$ (t_{BDZ} = retention in seconds of the BDZ enantiomer; t_o = retention in seconds of a non retained solute). The stereochemical selectivity (α) where $\alpha = k'_2/k'_1$, was also calculated (k'_2 and k'_1 are the capacity factors of the second and the first eluted enantiomer, respectively). Circular dichroism spectra were carried out with a Jasco J-600 spectropolarimeter. Absorption spectra were obtained using a Perkin–Elmer Lambda 9 spectrophotometer.

Membrane preparation

Membrane preparation was obtained essentially as previously described [15]. In brief: after homogenization and differential centrifugation, the membrane fraction was frozen overnight. Then it was thawed at room temperature, suspended in 25 vol of 50 mM Tris–citrate buffer, pH 7.5 and centrifuged. The washing step was repeated four times and the fraction was frozen again. After thawing, the membranes were resuspended in 25 vol of Tris buffer and washed twice.

The estimation of proteins was based on the method of Lowry *et al.* [16] after membrane solubilization with 0.75 N NaOH. Bovine serum albumin was utilized as a standard.

Binding assays

Binding studies were performed by using a filtration technique and ^{3}H-flunitrazepam and ^{35}S-TBPS as ligands as previously described [17, 18].

^{3}H-flunitrazepam binding studies. The membrane suspension (0.5 mg of proteins) was incubated in triplicate together with approximately 0.4 nM ^{3}H-flunitrazepam and various concentrations of the displacers for 45 min at 0°C in 500 μl of Tris buffer. After incubation the samples were diluted with 5 ml of assay buffer and immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B) and then washed with 5 ml of the same buffer. The filter disks were then placed in polypropylene scintillation vials together with 8 ml of Ready Protein Beckman scintillation cocktail; the radioactivity of the filters was determined by a Beckman LS 1800 scintillometer. Nonspecific binding was determined by parallel experiments containing diazepam (10 μM) and accounted for less than 10% of total binding.

^{35}S-TBPS binding studies. The membrane suspension was incubated together with 2 nM ^{35}S-TBPS and various concentrations of the tested compounds for 90 min at 25°C in 500 μl (final volume) of Tris buffer containing 200 mM KBr and 0.1 mM EDTA. After incubation the samples were diluted with 5 ml of Tris buffer and immediately filtered under reduced pressure through glass fiber filter disks (Whatman GF/B) and then rinsed twice with 5 ml Tris buffer. The filter disks were then placed in polypropylene scintillation vials together with 8 ml of Ready Protein Beckman scintillation cocktail; the radioactivity of the filters was determined by a Beckman LS 1800 scintillometer. Specific binding was determined in the presence of 600 μM picrotoxinin in Tris buffer.

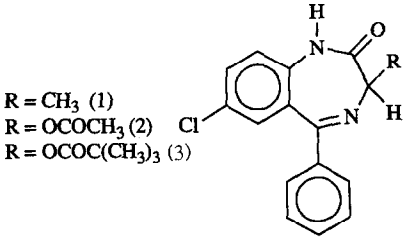
The BDZ derivatives were dissolved in

methanol-buffer (methanol concentration <2%) and the same mixture was present in blank experiments.

The concentrations of the investigated compounds that inhibit specific ^{3}H-flunitrazepam binding and ^{35}S-TBPS binding by 50% (IC_{50}) were determined by log-probit analysis with six concentrations of the displacers, each performed in triplicate. The inhibition constants of the unlabeled ligands (K_i) were derived according to the equation of Cheng and Prusoff [$K_i = IC_{50}/(1 + [L]/K_d)$], [19]. The ligand dissociation constant (K_d) of ^{3}H-flunitrazepam was 1.8 nM.

Results and Discussion

CSP I and CSP II were efficient in the resolution of **1**, **2** and **3** and 1–2 mg of the two enantiomers were recovered for each compound (10 runs using CSP I and one run using CSP II). The resolution of racemic **1** was obtained using CSP II ($k'_1 = 2.30$, $\alpha = 1.20$, eluent = hexane–2-propanol, 80:20, v/v, flow rate = 9 ml min^{-1}). The resolution of **2** was performed using CSP II ($k'_1 = 6.34$, $\alpha = 1.12$, eluent = hexane–2-propanol, 80:20, v/v, flow rate = 9 ml min^{-1}) but no pure enantiomers (enantiomeric excess, e.e. <98%) were obtained. A complete resolution of the two fractions was obtained using CSP I ($k'_1 = 6.55$, $\alpha = 1.29$, eluent = hexane–2-propanol, 80:20, v/v, flow rate = 1.5 ml min^{-1}). Compound **3** was resolved on CSP I, using hexane–2-propanol, 90:10, v/v, as mobile phase, at a flow rate of 1.2 ml min^{-1} ($k'_1 = 2.62$, $\alpha = 1.48$). The fractions of each compound were analysed for their e.e. using the same CSPs. Stock solutions in ethanol were prepared for the binding assays. Absorption and circular dichroism spectra of these solutions were recorded immediately before their use, in order to check concentrations and e.e. of the samples. This procedure is essential to ensure the reliability of receptor binding data.



The ability of enantiomeric derivatives to displace specific ^{3}H-flunitrazepam binding was studied in membranes prepared from bovine cerebral cortex with a radioligand concentration of 0.4 nM. The concentrations of the compounds able to give 50% inhibition of ^{3}H-flunitrazepam binding (IC_{50}) were determined from log-probit plots using six concentrations of the compounds.

Significant differences were observed in the values of K_i for the pure enantiomers (Table 1). For the tested compounds the racemic and *S*-enantiomeric form gave IC_{50} values according to other described benzodiazepines; in particular, the *S*-enantiomer was always more active than the *R*-form in displacing ^{3}H-flunitrazepam from bovine brain membranes, in agreement with literature data [5–10].

The enantioselectivity in the binding of BDZs to CNS receptor has been explained on the basis of the selectivity of the receptor binding site for one of the two possible conformations of the seven-membered ring of the drug [8]. These two conformations, defined M and P [20], interconvert in solution [21] if the BDZ has no substituent at C(3). On the contrary, only one conformation is largely prevalent for C(3) substituted BDZs. The prevailing conformation is that with the C(3) substituent in pseudoequatorial position (8). Thus, in practice, only the M conformation exists for the *S*-enantiomer [22] that is the conformational isomer which presents the higher affinity to the receptor binding site. Recently this hypothesis has been strongly supported by studying the 7-chloro-1,3-dihydro-1-(1,1-dimethylethyl)-5-phenyl-2H-1,4-benzodiazepin-2-one [23]. This BDZ needs a quite higher energy to interconvert between the M and P conformations, with respect to the

Table 1
Inhibition of ^{3}H-flunitrazepam binding and GABA ratio

Compound		K_i (nM)*	GABA ratio†
	(*S*)	53 ± 2	1.63
1	(*R*,*S*)	73 ± 5	1.50
	(*R*)	5760 ± 300	1.57
	(*S*)	109 ± 10	1.94
2	(*R*,*S*)	380 ± 50	1.79
	(*R*)	834 ± 70	1.34
	(*S*)	296 ± 20	1.50
3	(*R*,*S*)	371 ± 50	1.72
	(*R*)	>5000	—

* K_i values are means ±SEM of three determinations carried out in triplicate.
† IC_{50} (compound)/IC_{50} (compound + 50 μM GABA).

structural analogue diazepam, because of the presence of a bulky group at N(1). So, even if any asymmetric centre is not present, the two stereoisomers of this BDZ were isolated and the M-type conformer showed higher affinity to the receptor binding site [23].

Moreover, since the ability of BDZs to influence GABA neurotransmission is thought to occur via an allosteric link between BDZ and GABA receptors, we studied the ability of GABA to modulate the affinities of the enantiomeric BDZ ligands to their receptor to predict the pharmacological potency and efficacy of the compounds [2, 11–13, 24–26]. Using an exhaustively washed membrane preparation we evaluated the GABA ratio values as an *in vitro* indicator of the agonist, inverse agonist, or antagonist properties of the compounds. This GABA-shift is an index of the magnitude and quality of the cooperativity between the BDZ and GABA sites. Indeed the ligands can be roughly divided into three overlapping groups according to whether GABA enhances (GABA ratio >1, 'agonists'), leaves unaffected (GABA ratio = 1, 'antagonists'), or reduces (GABA ratio <1, 'inverse agonists') the affinity to the receptor [13, 27, 28]. The results, shown in Table 1, indicate that the examined compounds behave as agonists.

Specific chloride ionophore associated binding sites have been identified using the potent cage-convulsant ^{35}S-TBPS [29]. The differential modulation of ^{35}S-TBPS binding by the occupancy of BDZ receptors by its various ligands suggests another approach for distinguishing BDZ receptor agonists, antagonists and inverse agonists *in vitro* [2, 24, 30–33]. In fact, in the presence of micromolar concentrations of GABA, BDZ receptor agonists inhibit ^{35}S-TBPS binding, BDZ receptor antagonists have no marked effect whereas inverse BDZ receptor agonists enhance ^{35}S-TBPS binding.

In order to evaluate if stereoselectivity affected the modulation of chloride ionophore, we investigated the effects of racemic and enantiomeric BDZs on the ^{35}S-TBPS binding in the presence of GABA. Racemic and *S*-enantiomers of the tested BDZs were able to inhibit ^{35}S-TBPS binding in a dose dependent manner in the presence of 1 μM GABA while (*R*) enantiomers were less effective in modulating ^{35}S-TBPS binding (Figs 1–3). Table 2 indicates the concentrations of the studied

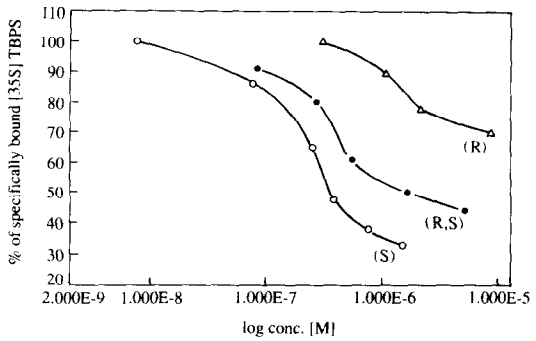


Figure 1
Modulation of ^{35}S-TBPS binding by (*S*)-**1** (○); (*R*,*S*)-**1** (●); and (*R*)-**1** (△) in the presence of 1 μM GABA.

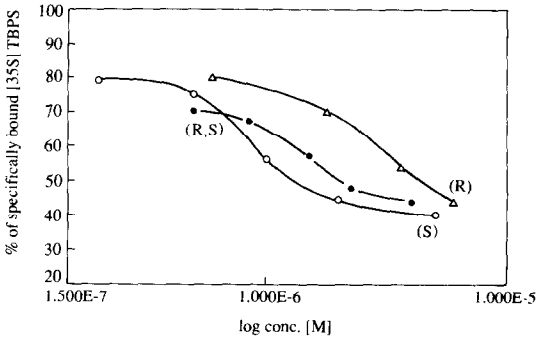


Figure 2
Modulation of ^{35}S-TBPS binding by (*S*)-**2** (○); (*R*,*S*)-**2** (●); and (*R*)-**2** (△) in the presence of 1 μM GABA.

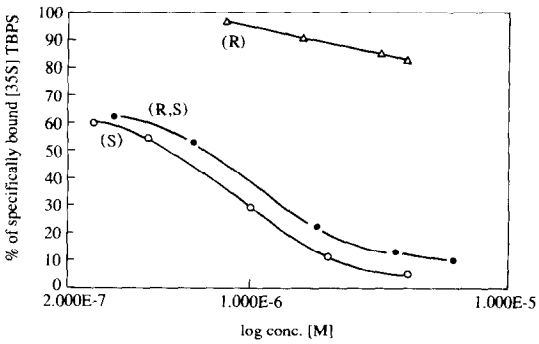


Figure 3
Modulation of ^{35}S-TBPS binding by (*S*)-**3** (○); (*R*,*S*)-**3** (●); and (*R*)-**3** (△) in the presence of 1 μM GABA.

compounds able to inhibit 50% of ^{35}S-TBPS binding. In conclusion, the observed higher activity of the *S*-antipodes shows that chirality has a strong influence on the benzodiazepine receptor and on the ability of the ligand to modulate ^{35}S-TBPS binding.

Table 2
Effects of chiral benzodiazepines on ^{35}S-TBPS binding

Compound		IC_{50} (nM)*
	(*S*)	395 ± 40
1	(*R*,*S*)	1742 ± 200
	(*R*)	>5000
	(*S*)	1460 ± 120
2	(*R*,*S*)	2164 ± 180
	(*R*)	3950 ± 350
	(*S*)	445 ± 50
3	(*R*,*S*)	664 ± 50
	(*R*)	>5000

*Concentrations necessary for 50% inhibition (IC_{50}) are means ±SEM of three determinations.

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